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POLYPLOIDNÍ KOMPLEX *ALLIUM OLERACEUM* L. V EVROPĚ

POLYPLOID COMPLEX OF *ALLIUM OLERACEUM* L. IN EUROPE

DOKTORSKÁ DISERTAČNÍ PRÁCE
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Abstrakt: Polyploidie je přírodní proces, hrající významnou úlohu v evoluci rostlin, většina z nich ve své minulosti pravděpodobně prodělala genovou duplikaci následovanou procesem diploidizace. S využitím metody průtokové cytometrie významně stoupla možnost studia polyploidie, struktury polyploidních komplexů i cytotypové variability jednotlivých rostlinných druhů.

Jako modelový organizmus pro zkoumání polyploidie byl zvolen česnek planý (*Allium oleraceum* L). Jedná se o polyploidní komplex, u něhož byly na počátku výzkumu známé čtyři ploidní úrovně: triploidní (2n=3x=24), tetraploidní (2n=4x=32), pentaploidní (2n=5x=40) a hexaploidní (2n=6x=48). Sběr vzorků probíhal na třech odlišných prostorových škálách – na úrovni populací, na úrovni přibližného středu rozšíření druhu (Česká Republika, Slovensko) a na úrovni Evropy. Celkem bylo studováno 620 populací a metodou průtokové cytometrie analyzováno 7354 rostlin.

Byly potvrzeny všechny doposud známé ploidní úrovně, nově byly zjištěny heptaploidní populace (2n=7x=56) a jedenkrát byla nalezena populace oktoploidní (2n=8x=64). Na úrovni celého areálu druhu jsou dominantním cytotypem pentaploidi následovány tetraploidy a hexaploidy. Triploidní a heptaploidní cytotypy jsou extrémně vzácné. Významně převažují populace pouze s jedním cytotypem, daleko menší množství je populací se dvěma a se třemi cytotypy. Populace s více jak třemi cytotypy nebyly nalezeny. U jednotlivých ploidních úrovní byla zjištěna ekologická i prostorová diferenciace. Nejmarkantnější rozdíl mezi populacemi různých ploidních úrovní je ve stupni ovlivnění habitatů člověkem, se vrůstajícím stupněm ploidie narůstá i stupeň ruderalizace stanovišť. Ploidní úrovně se rovněž liší v klimatických charakteristikách jako je průměrná roční teplota a průměrné roční množství srážek. Výše uvedená fakta mohou být důkazy pro tzv. adaptivní model diferenciace mezi jednotlivými ploidními úrovněmi. Vzhledem k relativně četné existenci smíšených populací je však třeba brát v úvahu i další faktory ovlivňující výskyt jednotlivých ploidií. Vliv na současné rozšíření má patrně vysoký stupeň asexuální reprodukce a omezená schopnost šíření na větší vzdálenosti, odlišné využívání krajiny v minulosti a v neposlední řadě opakovaný vznik polyploidů v různých částech areálu.

U vybraných jedinců všech ploidních úrovní byl změřen absolutní obsah DNA. Monoploidní obsah DNA signifikantně klesal se vzrůstající ploidní úrovní (kromě heptaploidů, jejichž monoploidní obsah DNA se nelišil od hexaploidů). U tetra- a hexaploidního cytotypu byla zjištěna variabilita ve velikosti jaderného genomu, která byla těsně spjata se zeměpisnou šířkou a délkou.

Klíčová slova: *Allium oleraceum*, polyploidie, ploidní úroveň, cytotyp
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Abstract: Polyploidy is a natural process that play a major role in the evolution of majority groups of plants. Most plants have probably undergone past polyploidization followed by diploidization. With use of flow cytometry techniques strongly increase the possibilities for studying polyploid, polyploid complexes or cytotype variation of single species.

We choose field garlic (*Allium oleraceum* L.) as a model plant for study polyploidy. *Allium oleraceum* is polyploid complex comprised four documented ploidy levels: triploid ($2n=3x=24$), tetraploid ($2n=4x=32$), pentaploid ($2n=5x=40$) and hexaploid ($2n=6x=48$). Research run on three different spatial scale: on the level of the population, on the level of the approximate center of the distribution (Czech Republic, Slovak Republic) and on whole natural area distribution (Europe). We sampled 620 populations and by flowcytometry analyzed 7354 plants.

We confirmed all of the known ploidy levels. In addition, newly were found heptaploids ($2n=7x=56$) and once were detected octoploids ($2n=8x=64$). On the level of whole distribution area dominated pentaploids, followed by tetraploids and hexaploids. Triploids and heptaploids were extremely rare. Significantly predominated single cytotype population, much more less were found two and free cytotype per population. Never were found population with more then three cytotypes.

Single cytotypes differ ecologically as spatially. Research revealed considerable variation in niche breath and optimum of cytotypes. Main differentiation among the cytotypes was related to the level of anthropic pressure. The ruderal character of cytotypes increase with a ploidy level. Cytotypes differ also in climatic characteristic like annual mean temperature or annual precipitation. These results could be evidence for adaptive differences among ploidy levels, which may influence their distribution pattern. Local coexistence of cytotypes may support the prevalence of asexual reproduction connected with limited dispersal, equilibrium disrupting processes and repeated origin of the polyploids (unreduced gametes).

Selected individuals for single ploidy level were subjected genome size measurement. The monoploid DNA amount (1Cx) was significantly dependent on ploidy level and genome downsizing between two successive cytotypes was significant, except for nonsignificant differences between hexa- and heptaploids. In tetraploids and pentaploids we found intra-ploidy variation in 2C DNA, these variation were tightly connected with longitude and latitude.

Key-words: *Allium oleraceum*, polyploidy, ploidy level, cytotype
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Prohlášení

Prohlašuji, že jsem tuto práci vypracovala osobně spolu s uvedenými spoluautory za použití citované literatury.

Vymezení podílu jednotlivých spoluautorů

1. Vědecký článek: Complex distribution patterns, ecology and coexistence of ploidy levels of *Allium oleraceum* (Alliaceae) in the Czech Republic. – Annals of Botany.

   M. Duchoslav a L. Šafářová realizovali sběr dat a jejich statistické zpracování. F. Krahulec pomohl se sběrem části populací. L. Šafářová provedla laboratorní analýzy. Na tvorbě textu se podíleli všichni uvedení autoři.

2. Vědecký článek: Cytotype distribution in mixed populations of polyploid *Allium oleraceum* measured at a microgeographic scale. – Preslia.

   L. Šafářová a M. Duchoslav provedli sběr dat. L. Šafářová provedla laboratorní analýzy a statistické výpočty, oba autoři se podíleli na tvorbě textu.

3. Vědecký článek: *Allium oleraceum* in Slovakia: cytotype distribution and ecology – Preslia.

   Všichni autoři se podíleli na sběru dat. L. Šafářová a M. Jandová provedly laboratorní analýzy, M. Duchoslav provedl statistickou analýzu dat, F. Krahulec vytvořil první verzi textu, která byla L. Šafářovou a M. Duchoslavem dopracována.

4. Manuskript: Large cytotype diversity, contrasting distributional patterns and genome size variations in polyploid geophyte *Allium oleraceum* (Alliaceae) on European scale.

   L. Šafářová a M. Duchoslav provedli sběr větší části populací, L. Šafářová provedla laboratorní analýzy, větší část statistické analýzy dat a oba autoři se podíleli na tvorbě textu.

Jednotliví spoluautoři byli seznámeni s výše uvedeným textem a vymezením podílu na jednotlivých článcích. Se zněním prohlášení souhlasí.

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Obsah

1. Úvod

1.1 Polyploidie

Polyploidie, stav kdy v jádře buňky jsou více než dvě identické chromosomové sady, je považována za jeden z klíčových procesů v evoluci rostlin (Grant 1981, Levin 1983, Thompson & Lumaret 1992, Ramsey & Schemske 1998, Wendel 2000). U živočichů má zmnožení chromozomů převážně letální nebo defektní následky, přesto i v živočišné říši není polyploidie vzácností (Mable 2004a).

První ranné odhady zastoupení polyploidů byly založeny na základě počtů chromozómů. Stebbins (1950) hovoří u krytosemenných rostlin o 35% polyploidů, Grant (1981) považuje za polyploidní rostliny s haploidním počtem chromozomů větším než 14 a dle známých chromozómových čísel považoval 43% dvouděložných a 58% jednoděložných za polyploidní. Goldblatt (1980) stanovil počet chromozomů pro polyploida vyšší než n = 9 (10) a odhadl, že v rámci jednoděložných existuje 70 – 80 % polyploidů. Masterson (1994) zvolil jiné kritérium než počet chromozomů. Studoval existenci polyploidie u fosilních rostlin a porovnával velikosti svěracích buněk průduchů s dosud žijícími druhy. Protože velikost svěracích buněk je vetší u polyploidů než u diploidů, odhadl množství polyploidů mezi fosilními rostlinami a stanovil, že téměř 70 % krytosemenných prodělalo ve svém vývoji polyploidizaci. Novější vědecké práce (Simillion 2002, Blanc et al. 2003) zjistily, že i rostlina s n = 5 (*Arabidopsis thaliana*) prodělala v minulosti až tři genomové duplikace. Je tedy možné, že téměř všechny rostliny prodělaly ve svém více či méně vzdáleném vývoji polyploidizaci.

K znásobení počtu chromozomových sad dochází často opakovaně, v různých částech areálu a v různých populacích diploidních nebo polyploidních předků (Soltis & Soltis 1995, Soltis & Soltis 2000). Celý systém vzniku a vývoje polyploidů je otevřený, nezřídka dochází ke genovému toku mezi rodiči a polyploidními potomky, různé genotypy vzniklé z nezávislých případů polyploidizace poskytují příležitost pro rekombinaci a vznik nových genotypů (Soltis & Soltis 1995).

Polyploidizace je spojena s reorganizací jádra. Změny mohou zahrnovat chromozomové přestavby, změny genové exprese („gene silencing"), divergenci duplikovaných genů a vývoj směrem k diploidizaci (Soltis & Soltis 1999, Cifuentes et al. 2009, Gaeta & Pires 2009). S přestavbami uvnitř jádra souvisí změna celkové velikosti jaderného genomu. Předpoklad, že polyploidní potomci budou obsahovat lineární násobek obsahu DNA svých rodičů, se ukázal být platný pouze v některých případech, například u nově vzniklých polyploidů. Nejčastěji dochází se stoupajícím stupněm polyploidie k poklesu průměrného množství DNA (2C DNA dělená stupněm ploidie) (Leitch & Bennet 2004, Šmarda & Bureš 2006, Kubátová et al. 2008, Zonneveld 2010a).

Téměř na všech úrovních biologické organizace lze nalézt vliv znásobení počtu chromozomů. Ve srovnání s rodičovskými druhy existuje u polyploidů díky duplikaci alel vyšší genetická diverzita, potažmo i značná fenotypová variabilita a v neposlední řadě vyšší stupeň heterozygozyty lépe vyvažující chromozomové mutace a následky inbrední deprese (Kubátová et al. 2008). Násobení počtu chromozomů má vliv na způsob reprodukce, stoupá význam vegetativního rozmnožování, samoopylení a apomixie. Ontogenetické procesy se zpomalují, často dochází k posunutí doby kvetení (Fowler & Levin 1984, van Dijk and Bijlsma 1994).

Nové genové kombinace umožňují polyploidům jinou a často širší odpověď vůči životnímu prostředí (Otto & Whitton 2000, Soltis et al. 2003). Nově vzniklí polyploidní jedinci se tak mohou úspěšně šířit do nových typů prostředí, což může vést až ke geografické diferenciaci jednotlivých ploidních úrovní jak na lokální tak na velké prostorové škále. Tento proces je označován jako tzv. adaptivní model. Ekologická a geografická diferenciace byla zjištěna u řady taxonů, z nověji studovaných např. u *Senecio*

carniolicus (Sonnleitner et al. 2010) nebo *Actinidia chinensis* (Li et al. 2010). Rovněž u řady invazně se šířících druhů je právě polyploidie jedním z možných faktorů ovlivňující úspěšnost pronikání rostlin do nového prostředí, jak bylo zjištěno např. u rodu *Spartina* (Ainouche et al. 2004), *Tragopogon* (Soltis et al. 2004), *Reynoutria* (Mandák et al. 2003), *Senecio* (Schlaepfer et al. 2008) nebo *Centaurea* (Treier et al. 2009).

Prostorová diferenciace a separace jednotlivých cytotypů může mít i jiné než adaptivní vysvětlení. Neadaptivní teorie – model vyloučení minoritního cytotypu (Levin 1975, Fowler & Levin 1984, Ramsey & Schemske 2000, Husband 2004, Köhler et al. 2010) – je založena na frekvenci závislé selekci proti nově vzniklým cytotypům v populaci, kdy minoritní cytotyp dosahuje nízké zdatnosti, protože má malou možnost se křížit s dalšími polyploidními jedinci. Tato teorie má však významné omezení – předpokládá pohlavní způsob rozmnožování, přestože polyploidie je často spojena právě s asexuální reprodukcí (Stebbins 1971), která pomáhá překonat nevýhodu „malých populací".

Velké množství studií se zabývá srovnáváním diploidních rodičů a jejich tetraploidních potomků (např. Gelber-Girard et al. 1996, Ramsey & Schemske 1998, Hardy et al. 2000, Levin 2002, Doyle et al. 2004, Rosenbaumová 2004, Španiel et al. 2008). Zejména v poslední době vzrostl počet prací studujících polyploidní komplexy vyšších ploidních stupňů (Keeler 1990, Burton & Husband 1999, Suda 2002, Baack, 2004, 2005, Pečinka et al. 2006, Suda et al. 2004, 2007, Mandáková & Münzbergová 2006, Halverson et al. 2008, Mráz et al. 2008, Kolář et al. 2009, Duchoslav et al. 2010, Sonnleitner et al. 2010).

1.2 Velikost genomu

Obsah jaderné DNA patří k důležitým znakům všech živých organizmů. Lze ho považovat za relativně stabilní konstantu v rámci dostatečně velké vzájemně se křížící populace jedinců. Při rozdělení populace však může docházet a často dochází k diferenciaci velikosti jaderného obsahu DNA (Greilhuber 1998). Přesto je intraspecifická variabilita důležitou taxonomickou charakteristikou, pomůckou při stanovení taxonomické koncepce (Trávníček et al. 2009), nástrojem při odhalování „drobných" druhů (Greilhuber 1998, Lysák 2000) a potažmo i nástrojem sloužícím ochraně biodiverzity.

Variabilita velikosti jaderné DNA v rostlinné říši je obrovská. Doposud známý rozsah 1C DNA u krytosemenných rostlin sahá od 0,063 pg (*Genlisea margaretae*) (Greilhuber et al. 2006) až k 125 pg (*Frilillaria assyriaca*) (Bennett & Leitch 1995), respektive 133 pg (*Trillium* x *hagae*) (Zonneveld 2010b). I velmi příbuzné druhy se liší množstvím repetitivních nekódujících sekvencí a tedy i velikostí svého genomu (Bennetzen et al. 2005, Grover & Wendel 2010). Variabilita velikosti genomu v rámci krytosemenných rostlin však není rozložena rovnoměrně. Zatímco ve všech skupinách lze nalézt rostliny s malými genomy, rostliny s velkými genomy se vyskytují zejména v rámci jednoděložných rostlin (Leitch et al. 2010). Variabilita nijak nekoreluje se stupněm vývoje ani komplexitou organizmu, tento jev je v literatuře označován jako „C-value paradox" nebo „C-value enigma" (Greilhuber 2010).

Zásadní pro zkoumání polyploidie, polyploidních komplexů a velikosti jaderné DNA se ukázala být vhodná metoda výzkumu a přístrojové vybavení. Nejdříve užívané biochemické metody nepřinášely ani zdaleka uspokojivé výsledky. Významný posun přinesla Feulgenova mikrospektrofotometrie. Avšak skutečným zlomem byla průtoková cytometrie, která velmi rychle a relativně přesně dokáže stanovit stupeň ploidie a obsah jaderné DNA u velkého množství vzorků (Doležel et al. 2007). Standardně jsou jádra pro stanovení obsahu DNA izolována z čerstvé rostlinné tkáně ve vhodném prostředí – pufru zabraňujícímu degradaci DNA, potlačující účinky cytosolických inhibitorů a umožňující barvení DNA (Doležel et al. 1991, Doležel & Bartoš 2005, Loureiro et al. 2006). Poté je

vzorek s neznámým obsahem DNA měřen společně se standardem – rostlinou se známým obsahem DNA. Tato metoda, tj. metoda vnitřního standardu, je považována za relativně nejpřesnější způsob zjištění velikosti jaderné DNA. Ale ať již jakkoli elegantní, i průtoková cytometrie skrývá řadu úskalí – například zjištěná variabilita velikosti DNA (Frediani et al. 1994, Singh et al. 1996) nemusí být vždy projevem skutečné diverzity, ale jak dokázal Greilhuber (1998, 2005) artefaktem vzniklým špatným metodologickým přístupem, působením buněčných inhibitorů ovlivňujících vazbu barviva na DNA nebo nevhodným nastavením přístroje. Ke znehodnocení výsledků může dojít i použitím standardu s nedostatečně přesně stanoveným obsahem DNA (Doležel & Greilhuber 2010).

Vnitrodruhová variabilita velikosti jaderného genomu je prezentována současnými studiemi u řady druhů rostlin (např. Suda et al. 2003, Bureš et al. 2004, Šmarda & Bureš 2006, Gasmanová et al. 2007, Slovák et al. 2009, Cires et al. 2010). Ke změnám velikosti jaderné DNA dochází zejména poklesem či znásobením množství repetitivních a mobilních sekvencí (Walbot & Cullis 1985). Odlišná velikost jaderné DNA u různých populací téhož druhu a s ní spojená reorganizace na úrovni genomu může být chápána jako schopnost rostlin odpovídat na odlišné podmínky prostředí (Ceccarelli et al. 2011). U *Linum usitatissimum* (Schneeberger & Cullis 1991), *Festuca arundinacea* (Ceccarelli et al. 1997) nebo *Dasypyrum villosum* (Caceres et al. 1998) je změna velikosti genomu spojována s odlišnými podmínkami prostředí, například s teplotou, živinami v půdě, dostatkem vody nebo nadmořskou výškou.

Efekt změny množství DNA v jádře se může projevovat na několika úrovních. Nukleotypický efekt (Bennett 1971, 1987) charakterizuje změny na úrovni fenotypu. Meagher et al. (2005) popsal rozdíly ve velikosti květů u *Silene latifolia* v závislosti na velikosti jaderné DNA, naopak Gupta et Reeds (1975) žádný efekt vnitrodruhové variability velikosti DNA u druhů rodu *Lolium* nepozorovali. Vnitrodruhovou variabilitu množství jaderné DNA nelze tedy automaticky spojovat se změnou na úrovní fenotypu (Murray 2005). Kromě nukleotypového efektu může mít velikost genomu dopad v oblasti evoluce a ekologie polyploidů (Knight 2005).

1.3 *Allium oleraceum* L. – česnek planý

Allium oleraceum L. bylo vybráno jako modelový organizmus pro studium polyploidie. Taxonomicky je řazeno do sekce Codonoprasum (Krahulec & Duchoslav 2010) a společně s dalšími blízce příbuznými druhy do skupiny *Allium paniculatum*. Celý rod Allium zahrnuje přibližně 750 druhů rozdělených do patnácti podrodů s těžištěm rozšíření ve Starém světě (Stearn 1992, Friesen et al. 2006). Polyploidie se vyskytuje napříč celým rodem (Ohri et al. 1998). Známí jsou polyploidi od triploidního po oktoploidní stupeň, nejčastější ploidní úrovní je tetraploidní, dále triploidní, hexaploidní a pentaploidní, heptaploidi a oktoploidi se vyskytují velmi vzácně. (Gohl & Kouhl 1973 sec. Fialová 1996, Goldblatt P & Johnson 2008). Základní chromozómové číslo v rodu se pohybuje od x = 7 po x = 11 (Májovský et al. 1987, Tzanoudakis & Vosa 1988). V Evropě se vyskytuje 110 druhů rodu (Stearn 1980) z toho v České republice 13 druhů původních a 14 pěstovaných (Krahulec 2002).

Allium oleraceum L. je polyploidní komplex zahrnující čtyři publikované ploidní úrovně: triploidní 2n = 24; doposud nalezena pouze v Rusku (Vachtina 1984) a Maďarsku (Krahulcová 2003), tetraploidní 2n = 32, pentaploidní 2n = 40 a hexaploidní 2n = 48; známé z celého areálu druhu. Původem polyploidního komplexu (tehdy byl znám pouze tetraploidní cytotyp) se jako první zabýval Levan (1937), který na základě pokusného křížení navrhl autopolyploidní vznik a jako rodiče označil diploidní *Allium paniculatum*. Při podrobnějším studiu jeho pokusů však vyšlo najevo, že použité "diploidní populace *Allium paniculatum*" patrně reprezentovaly dva příbuzné druhy, mj. *Allium paniculatum*

s *Allium podolicum*. Je tedy pravděpodobné, že *Allium oleraceum* je alopolyploidního původu (Krahulec & Duchoslav 2010).

Allium oleraceum má euro-suboceanický-(submediteránní) areál (Meusel et al. 1965). Vyskytuje se v širokém spektru biotopů od přírodních – lužní lesy, dubohabřiny, stepi, skalnaté svahy a skály, až po člověkem silně ovlivněné – okraje polí, silniční meze, ruderální remízky, porosty akátu (Duchoslav 2001a, b, Hæggström & Åström 2005, Karpavičienė 2007). Je řazen mezi geofyty – vytrvalé byliny s obnovovacími orgány pod povrchem půdy (Raunkier 1934), v případě česneku planého je to cibule obalena blanitou šupinou. Lodyha nese 1–4 ploché polooblé žebernaté listy a u fertilních jedinců dosahuje výšky až 100 cm.

Plodné rostliny tvoří lichookolík oboupohlavných květů s pacibulkami v květenství (někdy pouze pacibulky bez květů). Na bázi lichookolíku vyrůstá toulec, který u druhu *Allium oleraceum* vytrvává i v době květu (Krahulec 2002). Reprodukce probíhá převážně vegetativně, ale i prostřednictvím generativně vzniklých semen. Vegetativní propagule jsou dvojího typu; pacibulky v lichookolíku květenství a dceřiné cibule, které se vyvíjí na bázi mateřské rostliny. Během roku vyprodukuje rostlina průměrně 1-2 zralá neabortovaná semena, okolo 40 pacibulek a 1-2 dceřiné cibule (Duchoslav 2000, Karpavičienė 2002, Ohryzek 2007). Jandová (2010) prokázala velkou cytotypovou variabilitu na úrovni semen a semenáčků. U tetraploidních populací byly v potomstvu na úrovni semen zjištěny cytotypy 2n = 3x, 4x, 5x, 6x, 7x, u pentaploidních rostlin 2n = 4x, 5x, 6x, 7x, 8x a u hexaploidních rostlin 2n = 5x, 6x, 7x, 8x. Tato variabilita již byla daleko nižší na úrovni semenáčků a v průběhu ontogenetického vývoje zemřely vysoké (7x, 8x) a nízké (3x) ploidní úrovně.

Jednotlivé ploidní úrovně *Allium oleraceum* jsou si morfologicky velmi podobné, doposud studované české populace se dají rozlišit na základě znaků: produkce pacibulek (4x produkují nejvíce pacibulek), produkce květů (6x vykazují nejnižší produkci květů) a dále na základě kombinace znaků: papilnatost listu, velikost průduchů, velikost okvětních lístků, šířka a délka listu a délka toulce (Ohryzek 2007). Experimentálně bylo zjištěno, že jednotlivé ploidní úrovně se mezi sebou liší v klíčivosti semen a procentu vypučených pacibulek (Ohryzek 2007). Nejlepší klíčivosti dosahují překvapivě hexaploidní semena, nejnižší tetraploidní. U pacibulek nejlépe pučí propagule pentaploidů, naopak nejhůře hexaploidní pacibulky.

Fenologicky se jednotlivé cytotypy lišily dle teoretických předpokladů (Jírová 2007). Na úrovni květu i jedinců vykvétaly nejdříve tetraploidi a nejpozději hexaploidi. Pentaploidi dokvetly nejdříve, následovány tetraploidy a hexaploidy. Při pokusném opylování byla prokázána autoinkompatibilita u triploidů, tetraploidů i pentaploidů. Naopak hexaploidi byly schopny rozmnožovat se autogamně, což podporuje teorii o prolomení autoinkompatibility u vyšších ploidních stupňů (Levin 1983, Mable 2004b). Další experimenty s křížením mezi cytotypy bohužel nebyly úspěšné, většina květů brzo po opylení abortovala (Jírová 2007).

Vnitrodruhové variabilitě v rámci polyploidního komplexu *Allium oleraceum* nebyla doposud věnována žádná pozornost. Známé jsou pouze údaje pro obsah jaderné DNA u pentaploidního cytotypu. Velikost genomu *A. oleraceum* pro pentaploida uvádí ve své práci Labani & Elkington (1987) 2C DNA = 52,78 pg a Baranyi & Greilhuber (1999) 2C DNA = 60,37 pg. Obě práce použily pro stanovení jaderného obsahu DNA metodu Feulgenovy denzitometrie, jako standard byla použita cibule - *Allium cepa*.

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2. Cíle práce

Jak bylo naznačeno v úvodu, *Allium oleraceum* představuje složitý polyploidní komplex vyskytující se na území Evropy, k porozumění chování jednotlivých cytoypů byly stanoveny následující cíle a oblasti výzkumu:

1. Cytogeografie polyploidního komplexu *Allium oleraceum* ve střední Evropě
   - Podrobné zhodnocení rozšíření a frekvence cytotypů na několika prostorových škálách na území ČR a SR
   - Zhodnocení vzniku, existence, složení a rozšíření cytotypově smíšených populací

2. Cytoekologie polyploidního komplexu *Allium oleraceum* ve střední Evropě
   - Zhodnocení ekologické amplitudy jednotlivých cytotypů
   - Zhodnocení významu ekologické diferenciace mezi cytotypy pro současný pattern rozšíření na různých prostorových škálách

3. Studium variability velikosti genomu polyploidního komplexu *Allium oleraceum*
   - Stanovení velikosti genomu jednotlivých ploidních úrovní
   - Zhodnocení míry intraspecifické variability ve velikosti genomu (mezipopulační a vnitropopulační) jednotlivých cytotypů
   - Interpretace zjištěného pattern ve vztahu k faktorům prostředí

4. Studium cytogeografie a cytoekologie polyploidního komplexu *Allium oleraceum* na škále celé Evropy
   - Zhodnocení rozšíření a ekologie jednotlivých cytotypů, porovnání s údaji získanými na menším území střední Evropy
   - Interpretace zjištěného pattern s ohledem na variabilitu ve velikosti genomu: vznik a šíření jednotlivých cytotypů.

5. Vytvoření vhodných metodických postupů pro měření česneku planého (*Allium oleraceum*) na průtokovém cytometru.

3. Původní vědecké práce

3.1 Complex distribution patterns, ecology and coexistence of ploidy levels of *Allium oleraceum* (Alliaceae) in the Czech Republic.

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Complex distribution patterns, ecology and coexistence of ploidy levels of *Allium oleraceum* (Alliaceae) in the Czech Republic

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• *Background and Aims* Despite extensive study of polyploidy, its origin, and ecogeographical differences between polyploids and their diploid progenitors, few studies have addressed ploidy-level structure and patterns of ecogeographical differentiation at various spatial scales using detailed sampling procedures. The pattern of coexistence of polyploids in the geophyte *Allium oleraceum* at the landscape and locality scale and their ecology were studied.
• *Methods* Flow cytometry and root-tip squashes were used to identify the ploidy level of 4347 plants from 325 populations sampled from the Czech Republic using a stratified random sampling procedure. Ecological differentiation among ploidy levels was tested by comparing sets of environmental variables recorded at each locality.
• *Key Results* Across the entire sampling area, pentaploids ($2n = 5x = 40$) predominated, while hexaploids ($2n = 6x = 48$) and tetraploids ($2n = 4x = 32$) were less frequent. The distribution of tetra- and hexaploids was partially sympatric (in the eastern part) to parapatric (in the western part of the Czech Republic) whereas pentaploids were sympatric with other cytotypes. Plants of different ploidy levels were found to be ecologically differentiated and the ruderal character of cytotypes increased in the direction $4x \rightarrow 5x \rightarrow 6x$ with the largest realized niche differences between tetra- and hexaploids. Most populations contained only one ploidy level (77 %), 22 % had two (all possible combinations) and 1 % were composed of three ploidy levels. The majority of $4x + 5x$ and $5x + 6x$ mixed populations occurred in sympatry with uniform populations of the participating cytotypes in sites with ecologically heterogeneous or marginal environment, suggesting secondary contact between cytotypes. Some mixed $4x + 6x$ populations dominated by tetraploids being sympatric and intermixed with uniform $4x$ populations might represent primary zones of cytotype contact. Almost no mixed accessions were observed on the fine spatial scale in mixed populations.
• *Conclusions* The results provide evidence for adaptive differences among ploidy levels, which may contribute to their complex distribution pattern. The prevalence of asexual reproduction, limited dispersal and equilibrium-disrupting processes may support local coexistence of cytotypes.

Key words: *Allium oleraceum*, contact zones, Czech Republic, ecological differentiation, distribution, DNA ploidy level, ploidy mixture, polyploidy, spatial scales.

INTRODUCTION

Polyploidy is a highly dynamic process that plays a major role in the evolution and speciation of angiosperms, and a significant role in the evolutionary history of other eukaryotes (Grant, 1981; Thompson and Lumaret, 1992; Wendel, 2000; Soltis *et al.*, 2003). Previous studies have estimated the proportion of polyploids within angiosperms at about 50 % (Müntzing, 1936; Grant, 1981). Later studies suggested that at least 70 % of angiosperms are of polyploid origin (Goldblatt, 1980; Masterson, 1994). Recent genomic studies suggest that perhaps all eukaryotes possess genomes with gene redundancy, much of which is the result of past genome duplication. Most plants have probably undergone polyploidization followed by diploidization through genomic rearrangements, gene silencing and gene divergence (Wendel, 2000; Soltis *et al.*, 2003). Molecular data support the recurrent origin of many polyploids (e.g. Soltis and Soltis, 1993, 1999, 2000; Segraves *et al.*, 1999; Soltis *et al.*, 2003; Guo *et al.*, 2005). Different genotypes resulting from

independent polyploidization events come into contact and afford the opportunity for recombination and production of new genotypes. Recurrent formation and gene flow between progenitors and polyploids enrich the gene pool of polyploids (Soltis and Soltis, 1995).

One of the prerequisites for polyploid research is knowledge of the geographical distribution of cytotypes. Distribution data can offer insight into the extent of reproductive isolation between cytotypes and the mechanisms responsible for their spatial separation (Baack, 2004; Suda *et al.*, 2007a, b; Kao, 2008). Such data may serve as a foundation for answering questions about polyploid origins and exploring the history of contemporary distribution patterns using molecular techniques (van Dijk and Bakx-Schotman, 1997; Segraves *et al.*, 1999).

Two scenarios have been proposed to explain differences in patterns of cytotype distribution. According to the adaptive evolutionary scenario, novel genetic combinations can produce novel characters that endow polyploids with new

responses to environmental conditions (Levin, 1983, 2002; Soltis and Soltis, 1993; Otto and Whitton, 2000; Soltis et al., 2003). Consequently, polyploid populations often expand to wider or different ranges of habitats relative to those of their progenitors (e.g. Rothera and Davy, 1986; Bayer and Stebbins, 1987; Lumaret et al., 1987; Jay et al., 1991; Petit and Thompson, 1999; Petit et al., 1999; Levin, 2002; Johnson et al., 2003; Soltis et al., 2003). Ecological sorting along environmental gradients usually results in spatial separation of polyploids and their ancestors, a phenomenon observed in many plant species (Stebbins, 1950; Lewis, 1980). Such spatial relationships between cytotypes may be of several types, ranging from sympatry to parapatry or even allopatry (Levin, 2002). Accordingly, the coexistence of sympatric cytotypes requires niche differentiation and highly localized spatial patterns of habitat differentiation between cytotypes (Thompson and Lumaret, 1992). In the case of parapatry, contact zones (= ecotones) are maintained by divergent selection pressures along some gradient, often with selection against parental types in non-native environments (Barton and Hewitt, 1985; Fritsche and Kaltz, 2000).

Although ecological sorting along abiotic and biotic environmental gradients has been considered as the main mechanism underlying spatial separation between polyploids and their diploid ancestors and between cytotypes within polyploid complexes (Endler, 1977; Levin, 2002), alternative, environmentally independent explanations ('non-adaptive scenarios') also exist. Spatial separation between cytotypes may be directed by frequency-dependent mating success that results from low fitness of hybrids formed from between-cytotype matings and which gradually leads to the elimination of the minority cytotype from the population ('minority cytotype exclusion model'; Levin, 1975; Fowler and Levin, 1984; Ramsey and Schemske, 1998). As a consequence, this model predicts that most populations should be cytologically uniform and that cases of multiple coexisting cytotypes represent transient situations following frequent generation or immigration of an alternative cytotype (Kao, 2007). Although this process was originally considered for primary hybrid zones (*sensu* Petit et al., 1999), an analogous process can occur across zones of secondary contacts between cytotypes when dispersal leads to mixed cytotype populations (Dorken and Pannell, 2007).

Differences in present-day distribution among cytotypes may also reflect history, i.e. the place of origin or past environmental heterogeneity. Widespread cytotypes may have been superior colonizers of areas which became available upon amelioration of the climate after the last ice age (Pleistocene) or due to human activities such as deforestation and agricultural use (Levin, 1983; Stebbins, 1985; Gornall and Wentworth, 1993; Xie-Kui et al., 2008). Alternatively, such distribution patterns may be explained non-adaptively through the position of past cytotype refuges relative to the sites which became available for colonization by single cytotypes (van Dijk et al., 1992; van Dijk and Bakx-Schotman, 1997; Štěpánková, 2001; Mandáková and Münzbergová, 2006).

The increasing number of diploid–polyploid contact zones studied, accelerated by the introduction of flow cytometric techniques, allowing more samples to be analysed than previously possible by classical chromosome counting, has revealed that mixed cytotype populations are much more frequent than anticipated (e.g. Keeler, 1990, 1998; Burton and Husband, 1999; Suda, 2002; Suda et al., 2004, 2007a; Halverson et al., 2008; Kao, 2008). This influx of data is challenging and altering our knowledge of the establishment, persistence and distribution of polyploids (Kron et al., 2007; Suda et al., 2007b). More recent models investigating the role of several mechanisms that increase the probability of polyploids evading minority cytotype exclusion have shown (Felber, 1991; Rodriguez, 1996; Felber and Bever, 1997) that when diploids produce relatively high frequencies of unreduced gametes or when cytotypes differ in fitness, fecundity, longevity and level of self-compatibility (but see Mable, 2004), polyploids can become established and maintained in the populations. Husband (2004) showed in computer simulations evaluating the effect of triploids on autotetraploid evolution in *Chamerion angustifolium* that partially fit triploids can increase the likelihood of diploid–tetraploid coexistence, and in some cases they can facilitate tetraploid fixation. In addition, the evolution of assortative mating – attained by a variety of factors such as divergence in flowering time or differences in pollinators (Fowler and Levin, 1984; van Dijk and Bijlsma, 1994; Segraves and Thompson, 1999), iteroparity, parthenogenesis (Bierzychudek, 1985; Yamauchi et al., 2004; Kao, 2007, 2008) or short-distance pollen and seed dispersal (Li et al., 2004; Baack, 2005) – might suffice to allow coexistence of cytotypes.

Numerous studies have examined ploidy-level structure and patterns of ecogeographical differentiation at various spatial scales in established polyploid complexes (Chmielewski and Semple, 1983; Stutz and Sanderson, 1983; Rothera and Davy, 1986; Lumaret et al., 1987; Keeler, 1990; Brochmann and Elven, 1992; Hroudová and Zákravský, 1993; Burton and Husband, 1999; McArthur and Sanderson, 1999; Hardy et al., 2000; Suda, 2002; Weiss et al., 2002; Johnson et al., 2003; Stuessy et al., 2004; Baack, 2004, 2005; Suda et al., 2004, 2007a; Mandáková and Münzbergová, 2006; Halverson et al., 2008; Kao, 2008; Mráz et al., 2008; Španiel et al., 2008; Xie-Kui et al., 2008; Kolář et al., 2009), but many of these studies were based on rough measures of the environment and small population samples. In consequence, ecogeographical patterns observed vary widely when multiple data are compared (Mable, 2003). There is a need for a quantitative approach (Johnson et al., 2003; Halverson et al., 2008), and this may be particularly important for detecting less obvious cases of habitat differentiations, as in ploidy variation within species (Lewis, 1980).

Allium oleraceum (Alliaceae), a bulbous geophyte distributed in most of Europe (Stearn, 1980; Hultén and Fries, 1986), is a polyploid complex comprising four documented ploidy levels ranging from triploids to hexaploids ($2n = 24, 32, 40, 48$; see Table 1). Low generative reproduction is a common feature of all the ploidy levels, but there is significant asexual reproduction by means of daughter bulbs and vegetative bulbils within the inflorescence (Duchoslav, 2000; Karpavičienė, 2002; Åström and Hæggström, 2004). A review of published *A. oleraceum* chromosome counts across Europe shows (Table 1) that, except for four instances, papers report only a single ploidy level per population with tetraploids and pentaploids being the most frequently reported

TABLE 1. *Summary of the ploidy levels found in* Allium oleraceum *in Europe according to the literature and present records*

Country	References	Ploidy level
Austria	Geitler and Tschermak-Woess (1946), Tschermak-Woess (1947), Wittmann (1984), Speta (1984), Wetschnig (1992), Baranyi and Greilhuber (1999), Dobeš and Vitek (2000)	4x, 5x, 6x
Belorussia	Parfenov (1980) sec. Agapova (1990)	4x
Czech Republic	Měsíček and Jarolímová (1992)*, Fialová (1996)*, present study*	4x, 5x, 6x
Finland	Arohonka (1982), Halkka (1985), Åström and Hæggström (2004)*	4x, 5x
France	Jauzein and Tison (1999)	4x
Germany	Speta (1984), Åström and Hæggström (2004)	4x, 5x
Great Britain	Vosa (1976)	4x
Hungary	Krahulcová (2003)	3x
Italy	Gadella and Kliphuis (1970) sec. Moore (1973), Capineri *et al.* (1978), Baranyi and Greilhuber (1999)	4x, 5x
Lithuania	Vakhtina and Kudrjassova (1985), Karpavicienė (2007)*	4x, 5x
Macedonia	Šopova (1972)	4x
Norway	Laane and Lie (1985)	4x, 5x
Poland	Pogan *et al.* (1986), Joachimiak in Pogan *et al.* (1990)	4x
Russia (European part)	Vakhtina (1984), Vakhtina and Kudrjassova (1985)	3x, 5x
Slovakia	Váchová and Feráková (1978), Májovský and Murín (1987), Murín *et al.* (1999)	4x
Spain	Fernandez Casas *et al.* (1980), Pastor (1982)	4x, 5x, 6x
Sweden	Wittmann (1984), Lövkvist and Hultgard (1999), Åström and Hæggström (2004)	4x, 5x
Switzerland	Baranyi and Greilhuber (1999)	5x
Ukraine	Kish (2001)	5x

* Occurrence of cytotype-mixed populations was reported in the source.

cytotypes in Europe. Fialová (1996) and Karpavicienė (2007), using few population samples, observed the occasional co-occurrence of penta- and hexaploids and tetra- and penta-ploids in the Czech Republic and Lithuania, respectively. The occurrence of three of four known ploidy levels and evidence for the unusual co-occurrence of penta- and hexaploids in the Czech Republic make this area very suitable for exploring patterns of ecogeographical distribution and the frequency of coexistence of different cytotypes. Understanding the factors responsible for the distribution of the cytotypes in this region may offer excellent opportunities to gain deeper knowledge of the ecological and evolutionary significance of chromosome number variation within this polyploid complex. There are two additional reasons for choosing this species as a model. First, *A. oleraceum* is relatively common throughout the whole of central Europe, so studies can be based on extensive sampling, and distribution patterns can therefore be insensitive to random fluctuations (see also results on another common species, *Pilosella officinarum* – Mráz *et al.*, 2008). Secondly, the limited possibility of seed reproduction and, for that reason, low dispersion ability over larger distances, is another advantage of this species, as it conserves distribution patterns.

Here a large sampling study of *A. oleraceum* populations and their environment at two spatial scales in the Czech Republic (central Europe) was performed. The following questions were addressed. (1) What are the frequencies and distribution patterns of plants of different ploidy levels at the landscape and local scales? (2) Are there any ecological differences between ploidy levels allowing for interpretation of the observed distribution pattern as a result of environmentally dependent selection? (3) Do populations with cytotype mixtures exist? And if so, (4) can their composition and ecogeographical distribution allow the inference of their mode of origin (primary or secondary hybrid zones or contact zones without inter-cytotype gene flow)? (5) Are such mixed populations dominated by a single cytotype, which might suggest the 'minority cytotype exclusion' effect?

MATERIALS AND METHODS

Study species

Allium oleraceum L. is a bulbous geophyte occurring in most of Europe (Meusel *et al.*, 1965). It is distributed throughout western, central and eastern Europe and in southern Scandinavia. In the Czech Republic, the species is quite common and its distribution is concentrated between 300 and 500 m a.s.l. (Duchoslav, 2001*a*). It grows in a wide range of natural and human-influenced habitats, ranging from rocky grounds and dry grasslands through field margins and road ditches to scrub and deciduous forests (Duchoslav, 2001*a*, *b*, 2009; Karpavičienė, 2004; Hæggström and Åström, 2005).

The plant has one to four leaves. They are linear to filiform, fistular in the lower part and sheathing the bottom half of the scape. The terminal bulb in non-flowering plants and the major offset bulb in flowering plants replace the parent bulb at the end of the growing season. The plants rarely form non-dormant daughter bulbs. Sexually mature plants produce a lax umbel with a few hermaphroditic protandrous flowers (0–20) and many bulbils (10–60) at the top of the scape. Each flower can produce up to six seeds (Stearn, 1980), but in practice seed production varies greatly and seedling establishment is low (Duchoslav, 2000; Karpavičienė, 2002; Åström and Hæggström, 2004).

The origin of the *A. oleraceum* polyploid complex is still unclear. Levan (1938) considered *A. oleraceum* to be an autopolyploid form of diploid *Allium paniculatum* that arose by somatic doubling of the chromosomes, although he did not rule out fusion of unreduced gametes as an alternative. An autopolyploid origin of the species was proposed by Pastor (1982) and Fialová (1996). Vosa (1976), using the C-banding technique, stated that tetraploid plants of *A. oleraceum* are of allopolyploid origin. Careful analysis of Levan's paper shows that the 'synthetic' *A. oleraceum* obtained therein by crossing plants from two distant diploid populations [one being '*A. paniculatum*' from the botanical garden in Cluj-Napoca (Romania) and the other *A. podolicum* from the botanical garden in Stockholm] may actually be an interspecific hybrid formed by unreduced gametes. It is not clear whether the Romanian plant was a true specimen of *A. paniculatum* or another member of this

group – there are at least two other native species in Romania, namely *A. fuscum* and *A. fussii* (Brullo *et al.*, 1996). Also, the second parent is not certain, and the name suggests a Ukrainian origin. Russian and Ukrainian authors (Omelchuk-Myakushko, 1979; Dobrotchaeva *et al.*, 1999; Seregin, 2005) distinguish three species within this group (*A. paniculatum*, *A. podolicum* and *A. praescissum*) occurring in Ukraine.

Study area and sampling procedure

The present research was carried out in the Czech Republic (78 865 km^2), which is covered by a heterogeneous cultural landscape of arable fields, broadleaved and coniferous forests, and human settlements. Its western part (the Bohemian massif) has a Palaeogene relief of rolling plains, hills and plateaus surrounded by the densely forested Hercynian mountains. The younger areas comprise river canyons and areas with Tertiary volcanism. Mostly acidic Variscan regions were later covered by Permo-Carboniferous and Mesozoic sediments. Base-rich bedrocks are concentrated in the lower altitudes. The eastern part of the Czech Republic is the flat northernmost projection of the Pannonnian basin and is surrounded by the western slopes of the Carpathian mountains. Relief here is of Tertiary age. The bedrock is more diverse than in the west, being mostly of Mesozoic and Tertiary origin. Calcium-rich substrates occur from the lowlands to the mountains. The vegetation cover has a more fine-grained distribution in comparison with the Bohemian massif (Ložek, 1988).

Samples of *A. oleraceum* were collected throughout the Czech Republic during early spring from 2001 to 2004. A stratified random sampling procedure was used to sample populations and subsamples within populations. The Czech Republic was divided according to a road atlas (Hlaváček, 2000) into 144 quadrats, each with an area of approx. $6 \cdot 0 \times 9 \cdot 3$ km. Within each quadrat, we randomly searched for two populations and within each sampled population all plants in at least five randomly placed subsamples were sampled, each with an area of approx. 30×30 cm. To take into account habitat diversity and variation in population density (Duchoslav, 2001*a*), additional rules concerning the sampling procedures were defined. (1) In each quadrat one population from a natural habitat and one from an human-impacted one (see 'Habitat and population characteristics' below for explanations) were sampled (if available). (2) The minimum distance between two populations from the same type of habitat was specified as 10 km. (3) The minimum distance between sampled subsamples was (if available) specified as 1 m. The standard sampling procedure (i.e. number of populations per quadrat and number of subsamples per population) was modified in some cases to reflect population size, population density at the landscape level and habitat variation within quadrats (number of sampled populations per quadrat: mean 2·27, s.d. 1·15, minimum 1, maximum 8). Samples were transported to and planted in the garden of the Palacký University in Olomouc, Czech Republic. In total, 325 populations and 4481 plants of *A. oleraceum* were collected in the field (number of sampled plants per population: mean 13, s.d. 4, minimum 3, and maximum 32). The early spring was chosen as the sampling time because at that time of year even

populations consisting of non-flowering plants are easily recognizable in the field. Later in the year, when other plants grow as well, it is difficult to find *A. oleraceum*, and vegetative plants usually disappear during June (Duchoslav, 2009).

Habitat and population characteristics

Initially, the habitat of each population was investigated in the field. The following set of primary variables (see Appendix for a survey) was recorded at each locality. (1) Habitat type was assessed in the field according to EUNIS habitat classification (Davies *et al.*, 2004). Because of the low frequency of some habitats in the sample, they were translated here into one of seven common habitat types (rock; steppe; mesic & wet grassland; semi-natural forest; ruderal scrub; planted *Robinia pseudacacia* forest; arable field & field margins). Correspondence between this and EUNIS habitat classifications is explained in the Appendix. (2) Light conditions were assessed in the field according to the visually estimated proportion of full sunlight reaching the ground during late spring (1 = strong shade, 2 = half-shade, 3 = low shade, 4 = full insolation). (3) Populations were classified into two categories according to their distance to the nearest arable field ('Presence of arable land'; 0 = distance to the nearest field > 20 m, 1 = distance to the nearest field ≤ 20 m). (4) Populations were classified into two categories according to the degree of anthropogenic impact ['Habitat naturalness'; 0 = vegetation strongly influenced or created by humans, typically with higher proportions of ruderal or alien species ('human-impacted'), 1 = natural and semi-natural vegetation without strong anthropogenic influence ('natural')]. Examples of 'human-impacted' vegetation represent natural forests with ruderal or alien species, woody vegetation outside forest, intensively managed or disturbed grasslands, etc.

A secondary data set of geographical characteristics of the sites was obtained from tourist maps and from a digitized database of climatic parameters as follows: (1) altitude was estimated using 1 : 50 000 tourist maps (SHOCart, Inc., Zádveřice, Czech Republic), and (2) each locality was classified into one of three categories according to prevailing climatic conditions ('Climatic region'; C = cold region, SW = slightly warm region, W = warm region; Quitt, 1971).

Soil samples (topsoil, 5–10 cm) were taken from the sites during field sampling. Soil samples were passed through a 2-mm sieve. Soil pH was measured in water suspension potentiometrically. The oxidizable carbon concentration (C) was determined by oxidation with potassium dichromate in sulphuric acid. Oxidative mixture redundancy was determined via volumetry with Mohr's salt (Zbíral, 1995). Organic nitrogen concentrations (N) were determined after mineralization with sulphuric acid, conversion to ammonium ions and subsequent distillation with water vapour (Zbíral, 1997) on a Kjeltec System Instrument (TECATOR; FOSS, Inc., Hillerød, Denmark). Phosphorus pentoxide concentrations (PO_4^{3-}) were determined after extraction in Mehlich II solution (Mehlich, 1978) using a DR 2000 spectrophotometer. Determination of metallic cation (Ca, Mg, K) concentrations in soil samples were made after extraction in Mehlich II solution using an AVANTA atomic absorption spectrometer.

The size of each population was assessed visually on an ordinal scale (less than 50, 51–500, more than 500 individuals) and population area was estimated in square metres.

The spatial pattern of individuals within each population ('Morphological pattern') was described according to the prevailing type observed *in situ* (i.e. either separate individuals or clumping).

Chromosome counts

Chromosome counts were obtained from somatic mitotic cells in root-tip cuttings of pot-cultivated plants. Plants for chromosome counts were raised in a greenhouse. After 2 weeks, the root tips were excised and pre-treated with a 0·5 % solution of colchicine at room temperature for 3 h, fixed in a cold mixture of ethanol and acetic acid (3 : 1) overnight and then stored at 4 °C in 70 % ethanol until use. The fixed root tips were hydrolysed in cold 5 M HCl, stained with Feulgen and squashed in 45 % acetic acid (Lillie, 1951). Chromosomes were counted using an Olympus CX-31 light microscope.

Estimation of DNA ploidy levels

DNA ploidy levels (Suda *et al.*, 2006) were measured from most of the surviving plants ($n = 4347$, i.e. 97 % of all sampled plants) using flow cytometry. Leaf tissue of analysed *Allium* plant(s) with an appropriate volume of the internal reference standard (*Triticum aestivum* 'Saxana') were chopped with a new razor blade in a Petri dish containing 1 mL LB01 buffer (Doležel *et al.*, 1989). The suspension was filtered through a 42-μm nylon mesh and the samples were stained with DAPI (final concentration 2 μg mL^{-1}). The relative fluorescence intensity of stained nuclei was analysed using a Partec PAS instrument (Partec GmbH, Münster, Germany) equipped with an HBO-100 mercury arc lamp. Histograms of fluorescence intensity were registered over 512 channels. In each sample, 1000–2000 nuclei of both the standard and the test plant G1 peaks were analysed. The gain of the instrument was adjusted so that the G1 peak of wheat was approximately on channel 50. The ploidy level of each sample was determined by the position of its G1 peak relative to the G1 peak of an internal standard. Known tetra-, penta- and hexaploid plants with known chromosome counts were used for the specification of internal standard-sample position. The ratios between the positions of sample and internal reference standard peaks were 2·4–2·6, 2·8–3·0 and 3·3–3·4 for tetraploids, pentaploids and hexaploids, respectively. DAPI staining yielded histograms with coefficients of variance (CV) below 5 % for both the standard and the sample in the majority of DNA-ploidy measurements (mean standard CV = 4·48 %, s.d. = 0·94; mean sample CV = 4·67 %, s.d. = 0·82). In total, 99·9 % of the surviving plants were successfully analysed by flow cytometry. Chromosome numbers for samples that could not be analysed by flow cytometry were ascertained cytologically.

Statistical analyses

Univariate statistical analyses of variation in the environmental and population parameters of ploidy levels were performed using NCSS 2001 software (Hintze, 2001). Two data sets were prepared from the original data matrix. The first set included only cytotype-uniform populations ($n = 250$) while the second data set comprised all (uniform and mixed) populations ($n = 325$). The first data set was used for an analysis of morphological patterns without accounting for the effects of different ploidy levels in mixed populations. The second data set was used in the rest of the analyses. In the case of mixed populations and univariate analyses, the environmental data were duplicated for each respective ploidy level. Either a paired *t*-test or an *F*-test (randomized blocks; ANOVA) evaluated whether one ploidy level was consistently dominant in the mixed cytotype sites. Contingency tables were used for the analyses of qualitative environmental variables; ANOVA and the Kruskal–Wallis test were used for the analyses of quantitative and ordinal data, respectively (Zar, 1996). The Bonferroni correction of α for multiple tests (Gotelli and Ellison, 2004) was applied in the case of environmental variables.

Environmental variables were subsequently subjected to multivariate data analysis. Due to different types of descriptors (nominal, ordinal, quantitative), the primary data matrix was replaced by a secondary data matrix with the Gower general coefficient of similarity for combined data (Legendre and Legendre, 1998) using MVSP 3·12 software (Kovach Computing Service; http://www.kovcomp.co.uk/). The secondary matrix was subjected to principal coordinate analysis (PCoA; Legendre and Legendre, 1998) in MVSP 3·12. PCoA results were then subjected to constrained PCoA (db-RDA; Legendre and Anderson, 1999) where the independent X matrix contained ploidy-level identifiers and the dependent Y matrix consisted of the principal coordinates. Calculations were done in the program CANOCO 4·5 (ter Braak and Šmilauer, 2002) according to Lepš and Šmilauer (2003). First, differences in habitat conditions among different cytotypes were tested with a Monte Carlo permutation test (999 permutations). Fuzzy coding of independent variables was used to accommodate the existence of mixed populations and variable representation of respective ploidy levels within mixed populations. Differences in habitat conditions between pairs of ploidy levels were tested by partial db-RDA with respective pairs as explanatory variables and the third ploidy level as a covariable. Secondly, differences in habitat conditions were tested among groups of populations classified by ploidy-level composition with a Monte Carlo permutation test (999 permutations). In total, six groups were established, which included uniform ($4x$, $5x$, $6x$) and mixed ($4x + 5x$, $4x + 6x$, $5x + 6x$) populations. Groups of populations with mixed $4x + 5x + 6x$ cytotypes were excluded from the analysis due to the small number of these populations. Because the overall test was significant (see Table 6), we next tested for differences in habitat conditions between *a priori* selected pairs of groups on a reduced set of data matrices consisting of only populations of the respective groups. The Bonferroni correction of α (at $\alpha = 0.05$) for multiple tests was applied.

The niche breadth of each ploidy level was expressed by the Gower general coefficient of dissimilarity ($1 - G$, where G is the Gower similarity coefficient). The mean and its 95 % bootstrap confidence interval (from 200 bootstrap samples) were calculated for all dissimilarity coefficients among populations in the presence of the respective ploidy level.

TABLE 2. *Ploidy-level composition of 325 populations of* Allium oleraceum *from the Czech Republic*

Number of ploidy levels per population			Populations containing one ploidy level			Populations containing two ploidy levels		
1	2	3	$4x$	$5x$	$6x$	$4x + 5x$	$4x + 6x$	$5x + 6x$
0·77	0·22	0·01	0·14	0·57	0·29	0·21	0·08	0·71

Populations were grouped and relative frequencies calculated according to the number of ploidy levels per population (one, two or three). Populations were further subdivided according to specific cytotypes present.
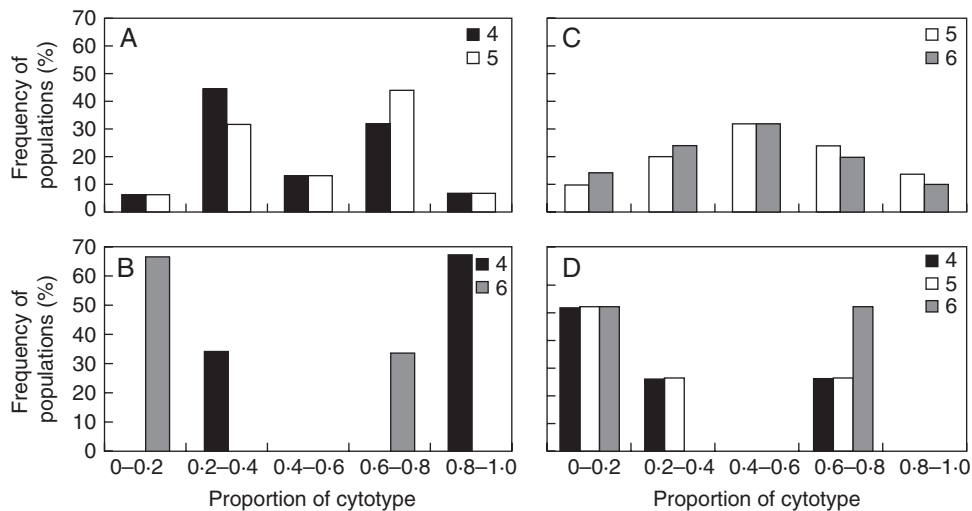

FIG. 1. Ploidy-level frequencies in *Allium oleraceum* mixed populations of tetra- and pentaploids (A), tetra- and hexaploids (B), penta- and hexaploids (C), and tetra-, penta- and hexaploids (D) in the Czech Republic.

RESULTS

Cytotype composition in the Czech Republic

Chromosome numbers were estimated for two individuals from each of 16 populations (32 individuals in total), confirming the presence of tetra- ($2n = 4x = 32$), penta- ($2n = 5x = 40$) and hexaploid ($2n = 6x = 48$) cytotypes (see Supplementary data, available online). DNA tetraploid, pentaploid and hexaploid cytotypes were also observed within the study area. Neither other ploidy levels nor aneuploid counts were found. Of the 325 populations, tetraploids occurred in 18 %, pentaploids in 65 % and hexaploids in 41 %.

Within the area sampled, populations consisted of one, two or three ploidy levels (Table 2). Most of the populations (77 %) contained only one ploidy level and 22 % contained two. Populations that contained three ploidy levels were extremely rare (1 %). Among the populations consisting of a single ploidy level, 57 % contained pentaploids, 29 % contained hexaploids and 14 % contained tetraploids. Among the populations comprising two ploidy levels, 71 % contained pentaploids and hexaploids, while combinations of tetraploids and hexaploids had the lowest frequency (Table 2).

There was strong heterogeneity in the relative frequency of ploidy levels among populations containing cytotype mixtures (Fig. 1). Hence, no single ploidy level was consistently dominant in mixed populations containing various cytotype combinations (Table 3). Within $4x + 5x$ mixed populations, usually one ploidy level tended to be predominant, and the other

was in the minority. The distribution of tetraploids and hexaploids within mixed $4x + 6x$ populations showed an antimodal pattern with either tetraploids or hexaploids predominating. By contrast, the distributions of pentaploids and hexaploids within $5x + 6x$ mixed populations were similar, and the two ploidy levels were almost uniformly distributed with a tendency toward evenly mixed populations. The distribution of ploidy levels in mixed $4x + 5x + 6x$ populations was characterized by the weak dominance of some ploidy levels and rare occurrences of others (Fig. 1).

Small-scale spatial pattern of ploidy levels within mixed populations

The small-scale spatial pattern of ploidy levels was rather uniform, and ploidy levels formed mostly homogeneous stands at a fine spatial scale (30 × 30 cm). In total, only 5·0 % of subsamples within mixed populations contained two or more ploidy levels. Populations of tetraploids mixed with any other ploidy level showed a higher (but not significantly different: $\chi^2 = 0.61$, d.f. = 2, $P = 0.74$) proportion of mixed subsamples ($4x + 5x$: 3·1 %; $4x + 6x$: 3·2 %) than mixed populations of penta- and hexaploids (1·7 %).

Population parameters

Population size and area differed significantly with respect to the cytotype composition of populations (size: chi-square

TABLE 3. *Ploidy-level relative frequencies in mixed populations of* Allium oleraceum *in the Czech Republic*

Ploidy-level combination	n	Mean frequency of tetraploids $\pm$ s.e.	Mean frequency of pentaploids $\pm$ s.e.	Mean frequency of hexaploids $\pm$ s.e.	Paired t (F^*)	P
$4x + 5x$	15	$0{\cdot}46 \pm 0{\cdot}06$	$0{\cdot}54 \pm 0{\cdot}06$	–	$-0{\cdot}02$	$0{\cdot}98$
$4x + 6x$	6	$0{\cdot}70 \pm 0{\cdot}13$	–	$0{\cdot}30 \pm 0{\cdot}13$	$1{\cdot}03$	$0{\cdot}35$
$5x + 6x$	50	–	$0{\cdot}53 \pm 0{\cdot}03$	$0{\cdot}47 \pm 0{\cdot}03$	$1{\cdot}17$	$0{\cdot}25$
$4x + 5x + 6x$	4	$0{\cdot}32 \pm 0{\cdot}14$	$0{\cdot}28 \pm 0{\cdot}12$	$0{\cdot}40 \pm 0{\cdot}14$	$0{\cdot}01^*$	$0{\cdot}99$

The mean frequency of tetraploid, pentaploid and hexaploids is given for populations containing two ploidy levels and populations containing three ploidy levels from the entire sampling area.

Either a paired *t*-test on the number of 4*x* vs. 5*x*, 4*x* vs. 6*x* and 5*x* vs. 6*x* individuals or GLM ANOVA (*F*-test) on the number of 4*x* vs. 5*x* vs. 6*x* individuals evaluated whether one ploidy level was consistently dominant in the mixed sites.

test, $\chi^2 = 35{\cdot}00$, d.f. = 5, $P < 0{\cdot}001$; area: Kruskal–Wallis test, $\chi^2 = 17{\cdot}83$, d.f. = 5, $P = 0{\cdot}003$). Populations of uniform Ploidy level showed a tendency towards smaller population sizes and areas than mixed populations. Both parameters increased slightly with increasing population ploidy level (Fig. 2). An increasing tendency to form clumps of individuals was observed with increasing population ploidy level (4*x*: 34·3 %, 5*x*: 53·2 %, 6*x*: 71·2 %; chi-square test, $\chi^2 = 14{\cdot}00$, d.f. = 2, $P = 0{\cdot}001$).

Ploidy-level distribution in the Czech Republic

The distribution of particular ploidy levels in the Czech Republic is shown in Fig. 3. It is clear from this that ploidy levels differ in their distribution. Uniform pentaploid populations occur regularly throughout the entire study area. By contrast, uniform tetra- and hexaploid populations occur in narrower ranges and are partially sympatric in the eastern part but rather parapatric in the western part of the Czech Republic. Some small, single-cytotype areas were observed: for example, those of hexaploids in the western part and those of tetraploids in the eastern part of the Czech Republic. Except for mixed populations consisting of tetra- and hexaploids, the distribution of mixed populations coincides with areas of sympatric occurrence of uniform populations of the respective ploidy levels. Mixed populations of tetra- and hexaploids are mostly located in broad contact zones between uniform tetra- and hexaploid populations. Mixed populations of three ploidy levels occur rarely in areas of sympatric occurrence of all ploidy levels.

Ecological differentiation among ploidy levels – univariate analyses

No significant differences in relative frequencies of tetra-, penta- and hexaploids in different habitat types were found (Table 4), although tetraploids tended to occur more frequently in deciduous forests (oak–hornbeam and hardwood floodplain forests) and less frequently in field and field margins than plants of other ploidy levels. However, penta- and hexaploids were increasingly frequent in localities in close contact with arable land (Armitage test for trend in proportions, Z-value = 4·33, $P < 0{\cdot}001$) and in human-impacted vegetation (Z-value = 4·34, $P < 0{\cdot}001$; Table 4, Fig. 4). Whereas tetra-ploids were evenly distributed between natural and
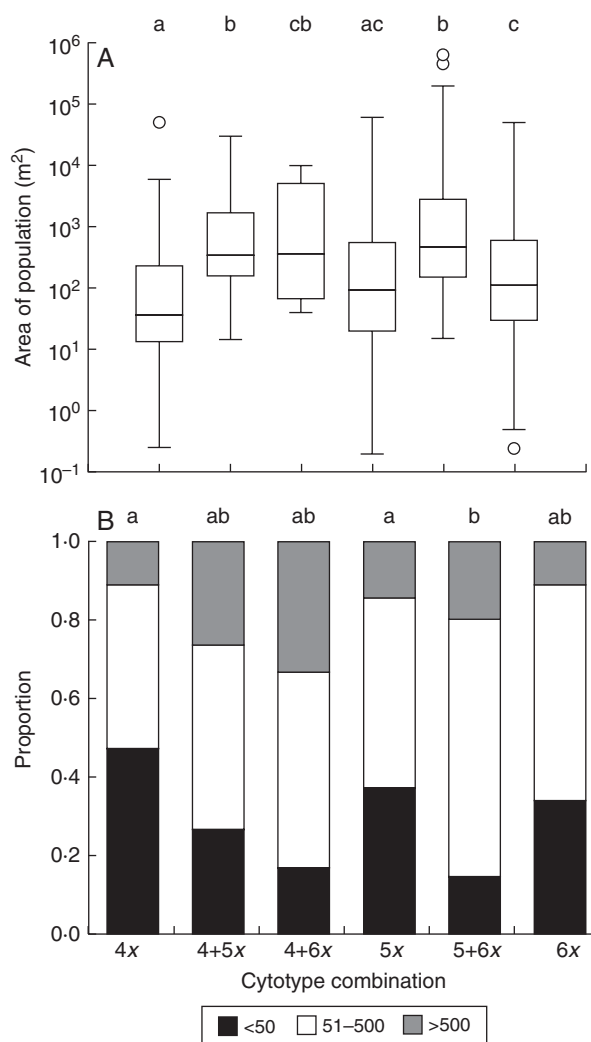

FIG. 2. Box plot of the area of the population (A) and frequency diagram of population size (B; <50, 51–500, >500 individuals, as indicated) in single- and mixed-ploidy-level populations of *Allium oleraceum* in the Czech Republic. Mixed populations of $4x + 5x + 6x$ cytotypes were excluded from the analyses due to small sample size. Note the log-scale of the y-axis in (A). Significant differences in medians between pairs of populations with different ploidy-level combinations (A; Dunn's test with $P < 0{\cdot}05$) and in frequencies of population size categories between pairs of populations with different ploidy-level combinations (Cross-tabulation; with $P < 0{\cdot}05$) are marked by different letters in rows above the diagrams.
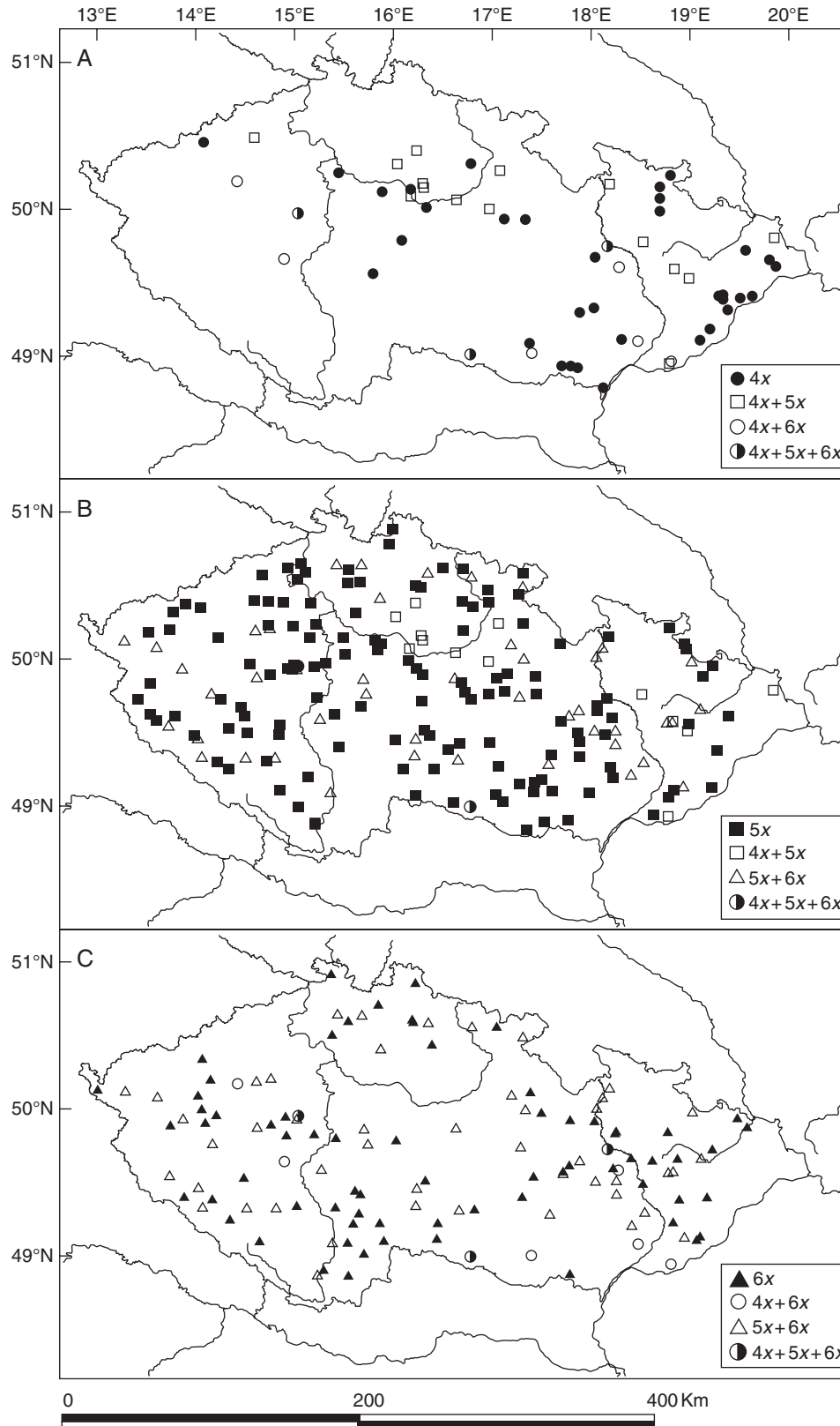
Fig. 3. Geographical distribution of uniform and mixed-ploidy-level populations of *Allium oleraceum* in the Czech Republic. (A) Uniform $4x$ and mixed $4x + 5x$, $4x + 6x$ and $4x + 5x + 6x$ populations; (B) uniform $5x$ and mixed $4x + 5x$, $5x + 6x$ and $4x + 5x + 6x$ populations; (C) uniform $6x$ and mixed $4x + 6x$, $5x + 6x$ and $4x + 5x + 6x$ populations.

Table 4. *Summary of the associations between ploidy levels and selected environmental variables in populations of* Allium oleraceum *in the Czech Republic*

Variable	Test	d.f.	Test statistics	P
Habitat type	CT	12	14·32	0·286
Presence of arable land	CT	2	18·96	≪**0·001**
Habitat naturalness	CT	2	20·39	≪**0·001**
Climatic region	CT	4	46·94	≪**0·001**
Altitude	KW	2	3·40	0·183
Light conditions	KW	2	1·47	0·478
Soil C*	ANOVA	2	2·12	0·122
Soil N	KW	2	6·68	0·035
Soil PO_4^{3-}	KW	2	12·48	**0·002**
Soil pH	KW	2	5·49	0·064
Soil Ca^{2+}	KW	2	8·43	0·014
Soil Mg^{2+}*	ANOVA	2	0·92	0·631
Soil K^+*	ANOVA	2	0·68	0·534

Differences were tested either by one-way ANOVA, Kruskal–Wallis test (KW) or contingency tables (CT).

*Data were log $(x + 1)$ transformed before analysis. P-values in bold are significant after Bonferroni correction ($P < 0.004$).

human-impacted vegetation, hexaploids mostly (80·4 %) occurred in human-impacted vegetation: field margins, road verges, in ruderal scrub, eutrophicated forests, etc. Tetra- and pentaploids occurred on soils with a higher content of nitrogen and calcium than hexaploids. Both ploidy levels also occurred on slightly less acidic soils than hexaploids, but the difference was not significant. By contrast, penta- and hexaploids occurred on soils with a higher content of phosphorus than tetraploids (Fig. 5). There was no difference in other soil properties (C, Mg^{2+}, K^+), light conditions and altitude among ploidy levels (Table 4).

Analysis of the cytotype frequencies in different climatic regions showed that tetraploids, in contrast to the other ploidy levels, were not concentrated in any climatic region. Pentaploids and hexaploids, however, were concentrated in areas with mesic climatic conditions (Table 4).

Ecological differentiation among ploidy levels – multivariate analyses

Although all three ploidy levels have overlapping ecological niches, overall and paired tests showed that the ploidy levels are ecologically differentiated, with the largest realized niche differences between tetra- and hexaploids (Tables 5 and 6; Fig. 6). In general, hexaploids occurred predominantly in human-impacted open habitats (usually at field margins and in fields) in mesic climatic conditions at higher altitudes, whereas tetraploids were confined to more natural habitats, both exposed and shaded along the whole altitudinal gradient. Pentaploids were confined to warm and mesic regions in the lower and middle altitudes, but were found in a wide range of habitats on soils with higher levels of minerals and higher soil pH. The niche breadth of tetra- and pentaploids was similar and higher than that of hexaploids [Gower dissimilarity coefficient: mean (95 % bootstrap confidence interval) – 4x: 0·361 (0·354–0·367); 5x: 0·359 (0·358–0·361); 6x: 0·320 (0·319–0·321)].
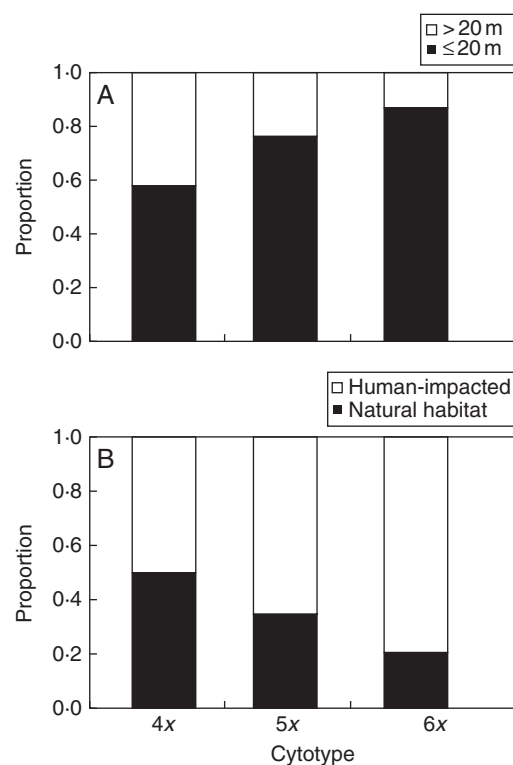

Fig. 4. (A) Relative frequencies of ploidy levels in relation to the distance of their populations from the nearest arable field ('Presence of arable land'; distance to the nearest field >20 m or ≤20 m, as indicated). (B) Relative frequencies of ploidy levels in relation to the degree of anthropogenic impact applied on their populations ('Habitat naturalness'; human-impacted or natural habitat, as indicated).

Significant differences in habitat conditions were found even among groups of populations classified according to ploidy-level composition (Table 5b). Pure 4x, 5x and 6x populations differed from each other with regard to environmental conditions. By contrast, the environments of mixed-ploidy-level populations did not differ from those of uniform populations of the respective cytotypes, pointing to the heterogeneity of the environments where cytotypes co-occur. This is in agreement with the finding that mixed ploidy populations occur more frequently (24·3 %) at sites with two or more adjoining habitats than uniform populations (16·9 %).

DISCUSSION

The results of this study indicate that pentaploids are the most common cytotype in the Czech Republic, and surprisingly, that hexaploids are more frequent than tetraploids there (Table 2). Other ploidy levels or aneuploid plants were not detected in this area. This is in good agreement with previous reports of chromosome numbers in the Czech Republic (Table 1), especially with the local study of Fialová (1996) who investigated 206 individuals from 24 *A. oleraceum* populations in the eastern part of the Czech Republic and found mostly penta-ploids and hexaploids, with only rare occurrences of tetra-ploids and no aneuploid plants. The absence of mature
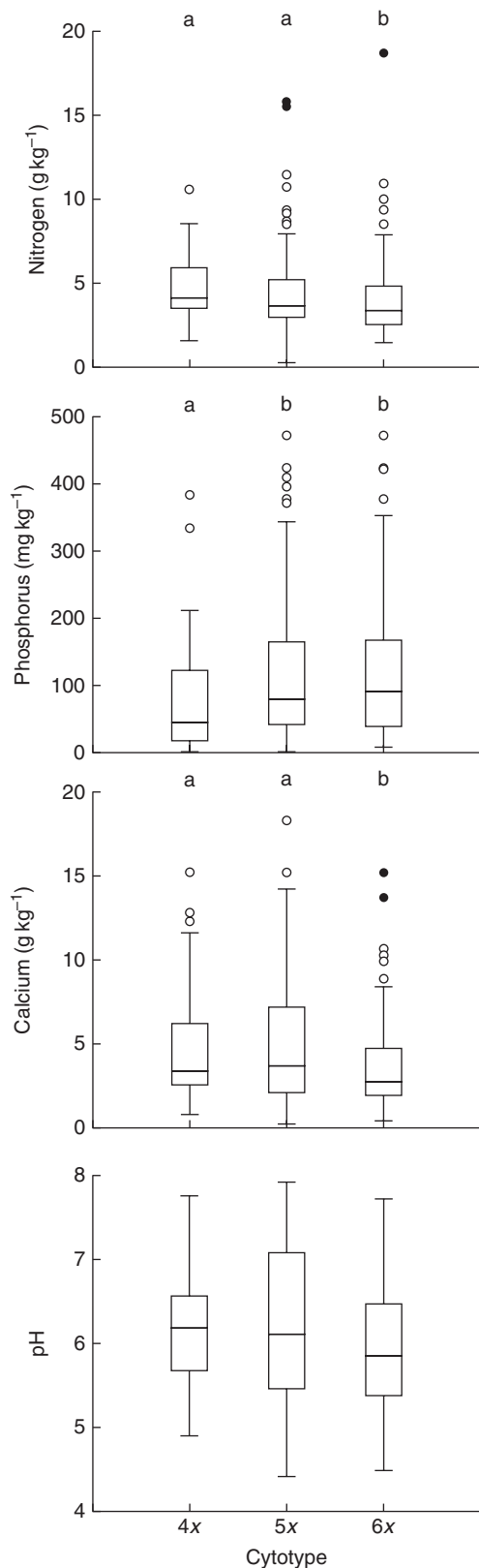
FIG. 5. Box plots of selected chemical soil properties at sites with occurrence of different ploidy levels of *Allium oleraceum*. Significant differences in medians between ploidy levels (Dunn's test; $P < 0.05$) are marked by different letters in rows above the box plots. For overall tests see Table 4.

TABLE 5. *Summary of the analyses using constrained coordinates analysis applied to environmental variables recorded in populations of* Allium oleraceum *in the Czech Republic: (a) effects of ploidy levels; (b) effects of groups of populations classified by observed ploidy-level combinations*

Model	Trace (1st axis)	F	P
(a) Ploidy levels			
Overall model	0·047	7·22	0·001
4x vs. 5x	0·033	5·70	**0·001**
4x vs. 6x	0·082	9·06	**0·001**
5x vs. 6x	0·016	3·99	**0·001**
(b) Groups			
Overall model	0·063	3·93	0·001
4x vs. 5x	0·030	5·18	**0·001**
4x vs. 6x	0·084	9·21	**0·001**
5x vs. 6x	0·016	4·00	**0·002**
4x vs. 4x + 5x	0·010	1·76	0·071
4x vs. 4x + 6x	0·018	1·96	0·039
5x vs. 4x + 5x	0·016	3·12	**0·003**
5x vs. 5x + 6x	0·007	1·75	0·065
6x vs. 4x + 6x	0·009	0·95	0·473
6x vs. 5x + 6x	0·008	1·98	0·033

All effects were tested by Monte Carlo permutation tests using 999 random permutations. See Methods for details.

P-values in bold (except for overall models) are significant after Bonferroni correction [$P < 0.017$ in the section (a)] or are significant at $P = 0.01$ in the section (b).

TABLE 6. *Survey of environmental variables that are best correlated with occurrence of ploidy levels in constrained principal coordinates analysis applied to environmental variables recorded in populations of* Allium oleraceum *in the Czech Republic*

4x (+) vs. 5x (−)	r	4x (+) vs. 6x (−)	r	5x (+) vs. 6x (−)	r
Forest	0·68	Habitat naturalness	0·65	Habitat naturalness	0·57
Habitat naturalness	0·65	Forest	0·64	Soil Ca^{2+}	0·54
Colder climate	−0·28	Light conditions	−0·55	Colder climate	−0·49
Light conditions	−0·42	Soil Ca^{2+}	0·26	pH	0·42
Arable field & field margin	−0·57	Arable field & field margin	−0·58	Arable field & field margin	−0·52
Presence of arable land	−0·66	Presence of arable land	−0·75	Presence of arable land	−0·67

Within each analysis, variables showing the highest positive or negative correlations with the first canonical axis are reported together with their sign and correlation coefficient. The sign of correlation coefficients corresponds to the position of the respective ploidy level along the first canonical axis within each analysis (i.e. 4x vs. 5x; 4x vs. 6x; 5x vs. 6x). For explanations of variables see Methods.

aneuploid plants, particularly within those populations containing pentaploids, does not mean that they could not be formed. Fialová (1996) observed a few aneuploid seedlings from 5x maternal plants. It is probable that aneuploid offspring are either non-viable or are eventually out-competed due to their reduced fitness.
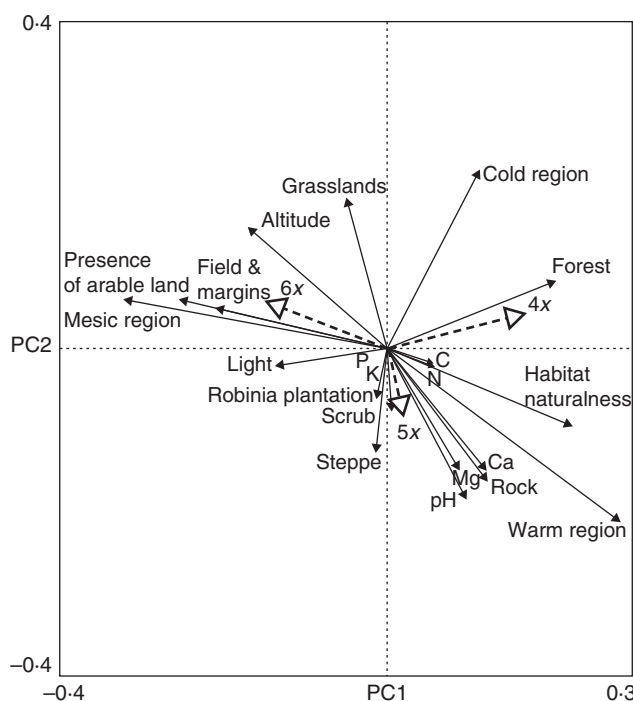
FIG. 6. The first and the second axis of the constrained principal coordinate analysis testing environmental differences among ploidy levels of *Allium oleraceum*. Vectors of the environmental variables were used as supplementary data to help interpret the ordination. See Methods for analysis settings.

Screening results also clearly indicate that ploidy-level distribution patterns are much more complex and finely grained than previously thought (see Table 1 and Fig. 3). Widespread sympatry of cytotypes of *A. oleraceum* that result in the contact zones, where at least two ploidy levels are intermixed, contrasts with the majority of diploid–polyploid contact zones studied. They are thought to have resulted from secondary contacts, the distribution of cytotypes is mostly parapatric, and only a few inter-cytotype hybrids are normally recorded (Soltis and Soltis, 1993; Petit *et al.*, 1999; Segraves *et al.*, 1999). The present study also found more mixed-ploidy-level *A. oleraceum* populations (23 %) than had previously been reported (8·3 %, Fialová, 1996; 12 %, Karpavičienė, 2007). In fact, co-occurrences of all possible combinations of 4*x*, 5*x* and 6*x* ploidy levels were observed, and among these 4*x* + 6*x* and 4*x* + 5*x* + 6*x* populations had not previously been identified in Europe.

High frequency of mixed-ploidy-level populations has been recently observed in several other plant species (Burton and Husband, 1999; Suda, 2002; Keeler, 2004; Suda *et al.*, 2007*a*; Halverson *et al.*, 2008; Kao, 2008). However, it is impossible to identify unambiguously the sole unifying mechanism that explains the existence of those populations. Likewise, the complex distribution patterns of the three ploidy levels of *A. oleraceum* observed do not permit an easy explanation. Several non-exclusive mechanisms that could explain the observed geographical patterns of the three cytotypes are discussed below.

Ecological differentiation among ploidy levels

Although their ecological preferences partially overlap, both univariate and multivariate analyses indicate that *A. oleraceum* of different ploidy levels show ecological differentiation. This difference in ecological requirements of the cytotypes is thus consistent with the generally accepted fact that polyploidization can produce novel characters which lead to niche shifts (reviewed by Ehrendorfer, 1980; Lewis, 1980; Levin, 1983, 2002; Soltis *et al.*, 2003), although we acknowledge that the differences observed may have emerged later via selection (Petit *et al.*, 1999). Higher ploidy levels also sometimes confer a wider ecological amplitude (e.g. Hancock and Bringhurst, 1981; Rothera and Davy, 1986; Thompson and Lumaret, 1992; Burton and Husband, 1999). Brochmann and Elven (1992) showed in a detailed study of three diploid and 13 polyploid (4*x*–16*x*) species of *Draba* that ecological amplitude, heterozygosity and biochemical diversity all increased significantly with increasing ploidy level. This is not the case in *A. oleraceum* here: the niche breadth of tetra- and pentaploids is similar, being higher than that of hexaploids. This is in agreement with the markedly lower biochemical diversity of hexaploids than either tetra- or pentaploids noted by Staňková (2005) in an electrophoretic enzyme study of 30 Czech populations of *A. oleraceum*. Similar observations, in which a broader ecological niche was found for lower ploidy levels, were recently reported from a diploid–hexaploid complex of *Senecio carniolicus* (Schönswetter *et al.*, 2007) and diploid–tetraploid complexes of *Centaurea stoebe* (Španiel *et al.*, 2008) and *Santolina pectinata* (Rivero-Guerra, 2008).

Ploidy-level distributions on regional and local scales: is the adaptive scenario the most plausible explanation?

Hypotheses concerning ecological diversification among cytotypes predict trends towards parapatry or allopatry of cytotypes at larger spatial scales if the fitness of cytotypes is a function of the environment, which itself changes with geographical scale (Engen *et al.*, 2002; Johnson *et al.*, 2003), or towards partial or complete sympatry but ecological isolation between cytotypes if the environmental factors are mosaic in structure (Thompson and Lumaret, 1992; Levin, 2002). The character of ecogeographical differentiation observed among the *A. oleraceum* ploidy levels is more probably consistent with the latter model because the most important environmental factors contributing to the ecological differentiation among the cytotypes (e.g. habitat naturalness; presence of arable land) have a rather mosaic pattern in the central European landscape. Furthermore, the coarse-grained spatial pattern of certain environmental factors that cause lower landscape heterogeneity in some Czech regions, especially at higher altitudes (Petřík and Wild, 2006), may also explain (1) the existence of a few small single-cytotype areas, especially those of hexaploids; and (2) the extremely rare occurrence of tetraploids in the large upland areas of central and western parts of the Czech Republic (Bohemia) in contrast to their common occurrence in climatically similar or even harsher upland areas in the eastern part of the Czech Republic (eastern Moravia), despite their broad ecological

niche (see Fig. 3). The contrasting distributions of tetraploids probably reflect differences in environmental conditions and historical processes that influence floristic composition in these regions. Rather acidic and mineral-poorer soils dominate in the Bohemian upland; a mosaic of mineral-poor and mineral-rich soils occur in eastern Moravia (Demek, 1987; Ložek, 1988). In fact, the distribution of tetraploids in Bohemia roughly corresponds to the occurrence of mineral-rich soil substrates at altitudes under approx. 350(400) m (Ložek, 1988; Sádlo, 2007), and this may also partly explain the absence of hexaploids from large areas of the Bohemian lowlands despite the vast expanses of arable land. Mráz et al. (2008) recorded similar cytotype distribution patterns in *Pilosella officinarum* in Bohemia, albeit with rare penta- and hexaploids confined to warm, low-elevation regions and common tetraploids prevailing in the whole of Bohemia.

At the population level, detected differences in ecological niches among cytotypes of *A. oleraceum* seem to support the observation of predominantly monotypic populations, i.e. a situation when plants invading a population of the other cytotype would do poorly in the unsuitable habitat (Baack, 2004). The present data demonstrate that a majority of $4x + 5x$ and $5x + 6x$ mixed populations do not show distinct geographical patterns, and are sympatric and intermixed with single-ploidy-level populations. The existence of such mixed populations could be best explained as a stochastic event based on mutually independent dispersal of cytotypes through the landscape followed either by: (1) their successful establishment and persistence at sites that represent less typical or marginal (unfavourable) environments for either one or both cytotypes but where they can both persist due to partial overlaps in realized ecological niches; or (2) local secondary contacts between cytotype-different, but uniform, populations at borders between different habitats. The present analyses support the idea that environmental conditions at mixed-population sites are marginal to or intermediate ('heterogeneous') between the environmental conditions of the respective uniform cytotype population sites. Similar patterns, i.e. the coexistence of cytotypes in sites possessing either sufficient environmental heterogeneity or ecologically marginal environment, were observed by Lumaret et al. (1987), Keeler (1990), and Husband and Schemske (1998) in mixed-cytotype populations of *Dactylis glomerata*, *Andropogon gerardii* and *Chamaerion angustifolium*, respectively.

The hypothesis of secondary contacts between ploidy levels is further supported by the larger population sizes and areas of mixed populations relative to the uniform populations (Fig. 2). The present results suggest that mixed-ploidy populations occur somewhat more frequently at sites with two or more adjoining habitats than do uniform populations, i.e. the larger area of mixed-cytotype populations also entails higher environmental heterogeneity (Legendre and Legendre, 1998; Koenig, 1999). As no single cytotype was strongly dominant in the majority of mixed $4x + 5x$ and $5x + 6x$ populations, which cytotype dominates at a site could therefore be due either to specific responses of the cytotypes to local environmental conditions or to random factors such as chance colonization (Kliber and Eckert, 2005; Kao, 2008). Cytotype uniformity of small populations can be explained through events such as colonization of a new site by a small number of individuals, probably representing a clone (founder

effect), or through processes such as habitat change when large populations are strongly reduced in size (bottleneck effect and/or drift).

By contrast, here some mixed $4x + 5x$ and $5x + 6x$ populations were observed at sites with homogeneous environment typical of one of the participating cytotypes. This suggests that niche differentiation is insufficient to explain the existence of these mixtures, although we cannot exclude that such differentiation occurs on a very fine spatial scale. Furthermore, ecological differentiation fails to explain the markedly contrasting cytotype frequencies in a majority of mixed populations of tetra- and hexaploids, because, in most of these populations, tetraploids predominate even if habitat conditions appear less suitable for them than for hexaploids on the basis of multivariate analysis.

We therefore consider ecological differentiation to be an important but not sole driving force behind the distribution patterns observed. Rather, our non-exclusive explanations take into account (1) the generation of cytotype mixtures as a result of the interploidy crosses or the emergence of a higher polyploid within a lower polyploid population, (2) predominant vegetative reproduction and localized dispersal that retard the effect of exclusion of emerging or invading cytotype and (3) human impact influencing distribution of cytotypes.

First, we suggest that *in situ de novo* production of hexaploids in some tetraploid populations seems to occur in *A. oleraceum* through the union of reduced and unreduced gametes, as evidenced by the fact that (1) some mixed $4x + 6x$ populations are intermixed with uniform $4x$ populations outside the hexaploid range, (2) the cytotype structure of the majority of $4x + 6x$ populations is characterized by strong dominance of tetraploids and rare occurrence of hexaploids, and (3) the tetra- and hexaploids have identical multilocus isozyme phenotypes within the two $4x + 6x$ populations (Staňková, 2005). However, the origin of hexaploid plants in tetraploid populations seems to be an extremely rare event given that no previous study on the cytology of *A. oleraceum* (see Table 1) reported mixed $4x + 6x$ populations, despite the frequent reports of tetraploid populations. In turn, the origin of $4x + 5x$ and $5x + 6x$ mixed populations is difficult to explain on the basis of a primary origin of a novel ploidy level (Ramsey and Schemske, 1998) because of the absence of one 'compatible' donor ploidy level, i.e. $4x$ in $5x + 6x$ or $6x$ in $4x + 5x$ mixed-ploidy populations. Indeed, we cannot exclude the possibility that our research simply failed to detect the minority ($4x$ or $6x$, respectively) cytotype, but this is unlikely because (1) the within-population screening was quite extensive, and, as stated above, (2) the 'missing' donor ploidy usually did not occur in the surroundings. Hybridization between the $4x$ and $6x$ plants would yield pentaploids, but only three mixed populations of tetra-, penta- and hexaploids were found. This suggests limited gene flow between $4x$ and $6x$ cytotypes due to extremely rare production of flowers in hexaploids (Ohryzek, 2007) and rather indicates secondary contacts between cytotypes.

Second, asexual reproduction via aerial bulbils and daughter bulbs strongly predominates over sexual reproduction in *A. oleraceum* (Duchoslav, 2000; Karpavičienė, 2002; Åström and Hæggström, 2004; Ohryzek, 2007). Hence, even a single plant of one cytotype emerging within or invading a uniform population of another cytotype has the potential to persist as it

can maintain itself and spread through asexual reproduction (Kao, 2007). This feature is analogous to apomictic seed formation, which allows plants of the other cytotype to produce their exact copies and thus to escape reproductive costs due to their minority status. Yamauchi *et al.* (2004) showed that if asexuality dominates over sexual reproduction, the outcome of mixed-ploidy occurrences are probably determined by direct competition between the cytotypes. We therefore suggest that existence of some mixed populations is a result of (recent) cytotype invading a uniform population of another cytotype and that eventually one cytotype suppress the other(s) in the ensuing period. Longevity of *A. oleraceum* individuals and their ability to propagate via daughter bulbs even under suboptimal ecological conditions hindering the production seeds and bulbils, for example in deep shade (Duchoslav, 2009), may retard competitive exclusion of one cytotype.

Additionally, direct competition among cytotypes can be reduced if cytotypes show local pollen/propagule dispersal leading to local spatial segregation of cytotypes (Li *et al.*, 2004; Baack, 2005). The present data show that *A. oleraceum* forms predominantly cytotype-homogeneous patchy stands within mixed populations and that local cytotype homogeneity tends to increase with ploidy level. This pattern may be explainable simply by limited dispersal (Hardy and Vekemans, 2001; Meirmans *et al.*, 2003). *A. oleraceum* is functionally similar to *A. vineale*, for which Ronsheim (1994) found that dispersion distances did not differ between bulbils and seeds and that most propagules fall within 30 cm of their mother plants. She also observed, however, that a very small number of seeds dispersed far from mother plants (> 1 m). This can, in the case of co-occurring cytotypes, result in a mixture with established patches of the other cytotype. Seed recruitment may, however, be inhibited by competition for safe sites from clonal bulbils and daughter bulbs (Abrahamson, 1980; Eriksson, 1997; Kliber and Eckert, 2005). This would also decrease the probability of invasion by an 'alien' cytotype. Much scarcer seed production by higher ploidy levels (the average production of seeds increases from zero seeds per plant in hexaploids to one and two seeds per plant in penta- and tetraploids, respectively; M. Fialová and M. Duchoslav, pers. obs.) and prevailing vegetative reproduction could explain not only increased local cytotype homogeneity within mixed populations of higher ploidy levels but also the increased clumping of higher ploidy levels presented here. Local dispersal and clumping of *A. oleraceum* cytotypes can thus effectively separate the cytotypes and thereby decrease reproductive interference and inter-cytotype competition (Baack, 2005). Recently, Kolář *et al.* (2009) proposed that founder effects together with limited seed distribution capacity leading to clumping is a plausible explanation for the existence of some mixed-ploidy populations of *Knautia arvensis* agg. Undoubtedly, data regarding the fine-scale spatial and environmental distribution of cytotypes within mixed-ploidy-level populations is needed to assess the relative importance of various coexistence mechanisms.

Third, the present-day complex distribution patterns of ploidy levels and high proportion of mixed-ploidy populations may also reflect human impact (Balfourier *et al.*, 2000; Perný

et al., 2008), i.e. the weedy character of *A. oleraceum* (Duchoslav, 2001*a*). The spread of the species was indeed influenced by agricultural practices and transport of crops and hay. Its frequent present-day occurrence along roadsides and field margins is evidence of its past abundance in arable land before the implementation of subsoil ploughing and the use of selective herbicides (Håkansson, 1963; Willmans, 1985; Duchoslav, 2001*a*). In the face of these equilibrium-disrupting processes, the abilities of niche differentiation and competitive exclusion to limit co-occurrence of cytotypes may be weakened.

General pattern of ploidy-level distribution in Europe and hypothetical origins of polyploidy

The pattern of ploidy-level distribution observed by us in the Czech Republic contrasts with that seen on the European scale. Because published karyological data on *A. oleraceum* are based on chromosome counting in small numbers of plants, such an approach is able to detect the most frequent cytotypes but fails to detect rare ones (Burton and Husband, 1999; Halverson *et al.*, 2008). It can therefore evaluate frequencies and large-scale spatial patterns of detected cytotypes inaccurately. Hence, it is highly possible that the current understanding of cytotype distribution patterns in Europe is inaccurate.

The origin of *A. oleraceum* is still not fully understood. Polyploid *A. oleraceum* could be of autopolyploid and/or of allopolyploid origin. We consider Levan's (1938) experimental results, i.e. the creation of tetraploids in one step from diploids, to provide a highly probable explanation for the origin of *A. oleraceum*. The *A. paniculatum* group is an extremely complicated set of species of the section *Codonoprasum* that occurs from the westernmost parts of Macaronesia, northern Africa (de Wilde-Duyfjets, 1976) and the Iberian peninsula (Pastor and Valdes, 1983) through the whole Mediterranean area (Zahariady, 1975; Stearn, 1980; Jauzein and Tison, 1999, 2001; Brullo *et al.*, 1996, 2001) to Iran (Wendelbo, 1971) and south-western Siberia (Frizen, 1988). This group could represent the hypothetical parents. Furthermore, they are not only diploid, as were used by Levan, but also triploid, tetraploid and pentaploid. Thus, the high variation in ploidy levels of *A. oleraceum* from triploid to hexaploid might be the result of independent crosses between different members of this complex. At present, this hypothesis is supported by the occurrence of two types of hexaploids found by us: one type is represented by populations that differ from tetra- and pentaploids in their ecological requirements, and the second hexaploid type comprises rare individuals admixed in populations of tetraploid plants that probably originated from fusion of reduced and unreduced gametes.

No triploid plants were detected during our detailed screening within the Czech Republic, and only extremely rare records of triploids are known, all of which are from the northern edge of the ranges of the supposed diploid progenitors (Vakhtina, 1984; Krahulcová, 2003). Triploids probably result from the hybridization of reduced and unreduced gametes of diploid progenitors, but they may arise from pollinations between ancestor diploid and tetraploid *A. oleraceum* (Type I hybrids;

Petit *et al.*, 1999). Currently, it is not possible to differentiate between these two hypotheses.

Both tetra- and pentaploids are widely distributed and probably sympatric (Karpavičienė, 2007; present study) throughout Europe, even in northern areas that were covered by glaciers during the last glacial maximum (Huntley & Birks, 1983). By contrast, hexaploids are presently known just in the Czech Republic, Austria and Spain (Table 1). Pastor (1982) mentioned that higher cytotypes (5*x*, 6*x*) probably arose in tetraploid populations through the production of (un)reduced gametes and interploidy crosses, and this mode is partially (6*x*) supported by the present data. However, alternative origins of the higher cytotypes cannot yet be ruled out, including backcrosses of triploids or tetraploids with parental taxa and eventual polyploidization, and even polyphyletic crosses (see above). Apart from the origin of ploidy levels, the wide present-day distribution of tetra- and pentaploids probably reflects their superior colonization abilities, as evidenced by the breadth of their ecological niches, which were found to be greater than that of hexaploids. Alternatively, the common occurrence of hexaploids in the Czech Republic may represent evidence of a recent range expansion of a newly established hexaploid type in anthropogenic habitats.

Conclusions

A detailed investigation of the distribution of different ploidy levels of *A. oleraceum* showed a distribution pattern much more complex than could be deduced from published chromosome counts. Individual cytotypes differ in their ecological requirements at the regional scale and this contributes to the distribution patterns observed. Local cytotype coexistence is, however, widespread. It is therefore considered that high frequency of mixed-ploidy populations is a result of both the cumulative effects of various isolating mechanisms, including niche differentiation, localized dispersal and prevailingly asexual propagation, and equilibrium-disrupting processes, i.e. agricultural practices. Distributional data support the existence of both primary and secondary zones of cytotype contact.

The extensive distribution range of *A. oleraceum* over most parts of Europe, as compared with the narrower distribution of its supposed diploid (and perhaps also polyploid) progenitors (species of the *A. paniculatum* group) in southern Europe, is consistent with the idea that polyploids are more ecologically tolerant and therefore are able to colonize harsher environments than their diploid progenitor(s). We suggest the recurrent formation of particular cytotypes and consider their polyphyletic origin as highly probable. A logical extension of this research would be to determine whether the present results could be extrapolated outside central Europe. The growing number of chromosome counts reported from individual regions suggests an increasingly complex cytogeographical pattern.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and include a list of *Allium oleraceum* localities accompanied by brief descriptions of habitats, geographical coordinates and ploidy levels for data presented in this study.

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APPENDIX

Survey of environmental and population variables used in the study. Codes of habitats corresponding to the EUNIS habitat classification (Davies *et al.*, 2004), which are included within each habitat type used in the study, are given in parentheses.

Environmental/ population variable	Explanation [units]
Habitat type	rock (H2, H3); steppe (E1, E5·2); mesic & wet grassland (E2, E3); semi-natural forest (G1·2, G1·6, G1·7, G1·8, G1.A, G3·4, F3 p.p.); ruderal scrub (G5, FA, F3 p.p.); planted *Robinia pseudacacia* forest (G1.C); arable field & field margins (E5·1, H5·6, I, J4)
Presence of arable land	0 – distance to the nearest field >20 m, otherwise 1
Habitat naturalness	0 = human-impacted habitat, 1 = natural habitat
Light conditions	1 = strong shade, 2 = half-shade, 3 = low shade, 4 = full insolation
Chemical soil parameters	pH of water extract, PO_4^{3-} [mg kg^{-1}], Mg^{2+} [g kg^{-1}], K^+ [g kg^{-1}], Ca^{2+} [g kg^{-1}], N [g kg^{-1}], C [%]
Altitude	[m a. s. l.]
Climatic region	C = cold region, SW = slightly warm region, W = warm region
Population size	<50, 51–500, >500 individuals
Area of population	[m^2]
Morphological pattern	individuals, clusters

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

Šafářová L. & Duchoslav M.

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

Rozšíření ploidií v cytotypově smíšených populacích *Allium oleraceum*

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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 μg.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).
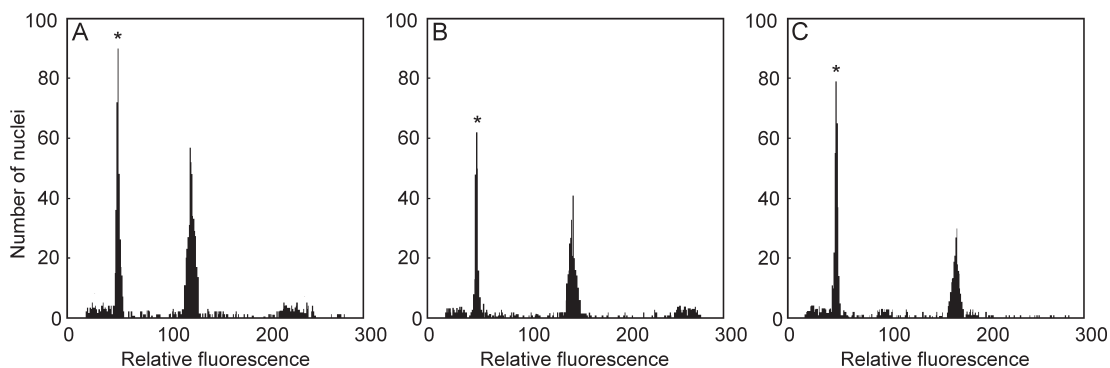

Fig. 1. – Fluorescence histograms of individual DNA ploidy levels of *Allium oleraceum*. Nuclei were simultaneously isolated from fresh leaf tissue of *Allium oleraceum* and an internal reference standard, *Triticum aestivum* 'Saxana', stained with DAPI and analyzed on a flow cytometer. A: tetraploid 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 ≤ 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	−0.616	**0.001**	**<0.001**	**<0.001**
	4, 5	27	63.0	37.0		scrub 74	forest 26		−0.817	**0.001**	**<0.001**	**0.032**
	4, 6	26	65.0		35.0	scrub 92	forest 8					
	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).
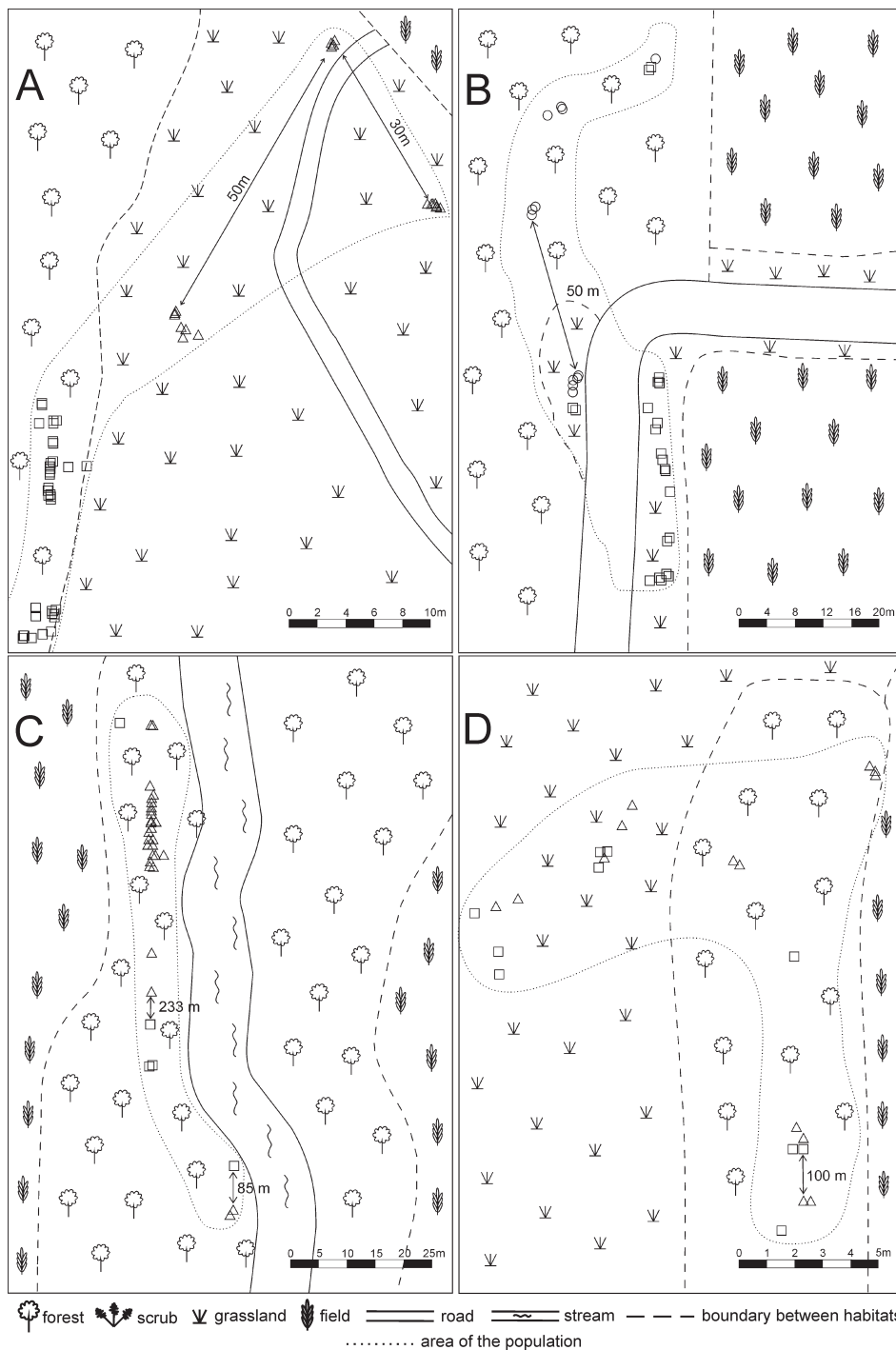
Fig. 2. – Examples of contrasting spatial structure of cytotypes at cytotype-mixed sites (A: site no. 18, B: site no. 3, C: site no. 13, D: site no. 14). Each plant sampled is represented by a symbol identifying its ploidy level (○= 4x, □ = 5x, △ = 6x). Population borders are demarcated by dotted lines 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.

Acknowledgements

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

3.3 *Allium oleraceum* in Slovakia: cytotype distribution and ecology.
Šafářová L., Duchoslav M., Jandová M. and Krahulec F.
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Allium oleraceum in Slovakia: cytotype distribution and ecology

Allium oleraceum na Slovensku: rozšíření cytotypů a jejich ekologie

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Šafářová L., Duchoslav M., Jandová M. & Krahulec F. (2011): *Allium oleraceum* in Slovakia: cytotype distribution and ecology. – Preslia 83: 513–527.

The spatial distribution of cytotypes can provide valuable insights into the evolution of polyploid complexes. Previously, only tetraploid *Allium oleraceum* was reported from Slovakia. Analysing 863 individuals from 93 populations from Slovakia revealed an extensive variation in the DNA ploidy levels of *Allium oleraceum* (3x, 4x, 5x and 6x). Of the main cytotypes, the penta- and tetraploids had strongly overlapping distributions, although the pentaploids exhibited a tendency to occur more frequently in the southern and the tetraploids had a tendency to occur in the northern regions of Slovakia. A triploid cytotype was found in one population in the southern part of Slovakia, which is the third locality worldwide for this cytotype. The hexaploid cytotype was rare and sparsely occurred in western and southern Slovakia. Sixteen per cent of the populations sampled consisted of more than one ploidy level; the most common was a combination of penta- and tetraploids. The cytotypes differed with respect to altitude; the tetraploids were found significantly more frequently at higher altitudes than the penta- and hexaploids. When compared with reanalysed altitudinal distribution data from the Czech Republic divided into two geographic areas (Carpathian and Herzynian) the pattern found in the Carpathian part of the Czech Republic was similar to that in Slovakia, with tetraploids at the higher altitudes. The distribution in the Herzynian part (Bohemian Massif) was just the opposite: the tetraploids were more often found at lower altitudes than the penta- and hexaploids. Both tetra- and pentaploid cytotypes occurred in a wide and similar spectrum of habitats, while hexaploids were limited to human-influenced habitats. A local-scale distribution of cytotypes analysed in detail in the Slovak Karst area, showed surprising differences in the distribution of cytotypes on particular karst plains, which can be related to different land uses. Concerning the contrasting altitudinal differentiation of tetraploids in the regions compared, the results suggest that at least two different types of tetraploids occur in Central Europe. The apparent cytotype diversity in the surrounding Slovak Karst area may suggest the existence of a primary contact zone.

K e y w o r d s: *Allium*, Czech Republic, distribution, flow cytometry, habitat differentiation, polyploidy, spatial scales, vertical distribution

Introduction

The use of flow cytometry techniques in plant ecology has strongly changed the potential for studying certain aspects of plant populations (Kron et al. 2007, Hülber et al. 2009, Kubešová et al. 2010, Loureiro et al. 2010, Suda & Pyšek 2010). As a consequence, the number of plant populations that are studied with respect to the variation in their chromosome/genome copy number has increased dramatically. Entirely new distributions of

cytotypes are recorded for many species, such as *Oxycoccus* (Suda 2002), *Empetrum* (Suda et al. 2004), *Elytrigia* (Mahelka et al. 2005), *Senecio carniolicus* (Schönswetter et al. 2007, Suda et al. 2007, Sonnleitner et al. 2010) and *Pilosella officinarum* (Mráz et al. 2008) in Central Europe and *Cardamine* (Marhold et al. 2010) in Eastern Asia. This information may be briefly summarized, as follows: the scale of cytotype variation has strongly decreased, the existing pattern is finer than was expected on the basis of chromosome counting and many species are now known to be variable in smaller geographic areas than previously.

One such species, with many cytotypes, is *Allium oleraceum* L., for which four cytotypes (2n = 3x = 24, 2n = 4x = 32, 2n = 5x = 40 and 2n = 6x = 48) are known from Central Europe (e.g. Měsíček & Jarolímová 1992, Krahulcová 2003). Detailed research has shown a complex pattern, both in the distribution and ecological preferences of particular (2n = 4x, 5x and 6x) cytotypes in the Czech Republic (Duchoslav et al. 2010, Šafářová & Duchoslav 2010). In neighbouring Slovakia, surprisingly, only tetraploid plants (2n = 32) are currently reported (Májovský & Murín 1987, Murín et al. 1999, Marhold et al. 2007). Therefore, we started to collect data from Slovakia to compare with the complex pattern found in the Czech Republic within the framework of a project on mapping the cytotype distribution in Europe. The data were not collected in such a systematic way as in the Czech Republic, but the intention was to collect data from a large spectrum of habitats, and, especially, from regions that were previously unexplored. Furthermore, we tried to carry out detailed sampling in some regions of southern Slovakia, where we anticipated the occurrence of cytotypes not previously reported from Slovakia. The questions we addressed were: Is *Allium oleraceum* represented in the area of Slovakia only as the tetraploid cytotype? If not, is there any clear pattern in the distribution of cytotypes, and is there any difference in the ecological preferences of the cytotypes?

Materials and methods

Species studied

Allium oleraceum is a member of sect. *Codonoprasum*, which also includes other species occurring in Central Europe, viz. *Allium flavum*, *A. carinatum*, *A. cirrhosum* and *A. paniculatum* (Stearn 1980, Krahulec & Duchoslav 2010). *Allium oleraceum* is closely related to the other species (e.g. *A. paniculatum*, *A. fuscum*, *A. fusii*, *A. pallens* and *A. podolicum*) in the *Allium paniculatum* group; *A. oleraceum* differs from the other species in this group by the presence of bulbils in its inflorescences. *Allium oleraceum* is most likely a hybrid between sexual members of the *A. paniculatum* group. Levan (1938) reports the production of an *A. oleraceum* that originated from the experimental hybridization between two distinct populations of *A. paniculatum*, most likely, *A. podolicum* and another species from Romania (three different species occur there, *A. paniculatum*, *A. fuscum* and *A. fussii*, cf. Brullo et al. 1996, Ciocarlân 2009). *Allium paniculatum* rarely occurs at several localities in the southern part of Slovakia (Somogyi 1999). *Allium oleraceum* occurs in a broad spectrum of habitats, from natural ones, such as rocks and forests, to anthropic ones, such as the margins of arable land (Duchoslav 2001a, b). This species produces little seed and propagates mainly vegetatively through daughter bulbs and, especially, the bulbils in the inflorescences.

Collection of plants and environmental variables

To characterize the large-scale pattern of distribution, an effort was made to cover the entire area of Slovakia. The area of Slovak Karst, consisting of a complex of huge karst plains and plateaus and their surroundings, was selected for the analysis of the distribution pattern of cytotypes at a local scale. Plants were collected from a wide spectrum of habitats to cover the large ecological variation of the species. Considering the existence of populations with several known cytotypes in the Czech Republic and the local propagation by bulbils and bulblets, we regularly collected several individuals that did not grow in close vicinity to each other, but covered the entire population. In total, we collected 1002 plants from 93 populations. Each population sample consisted of 2–53 plants (an average of 10.8 plants per sample). Sample size was variable and occasionally low because some of the populations sampled were composed of just one or two individuals or clusters of plants. A list of all of the populations included in the analysis, with additional data, is given in Electronic Appendix 1. The plants were transplanted into pots in the experimental garden of the Department of Botany, Palacký University at Olomouc and used later for flow cytometry measurements. Several ecological variables, identical with those used by Duchoslav et al. (2010), were recorded for each site sampled: (i) habitat type was assessed in the field according to the EUNIS habitat classification (Davies et al. 2004). Because of a low frequency of some habitats in the data set, we translated them into one of seven common habitat types (rock, dry grassland, mesic and wet grassland, (semi)natural forest, scrub, planted *Robinia pseudacacia* forest, and arable field and field margins). Correspondence between this and the EUNIS habitat classification is explained in Duchoslav et al. (2010); (ii) populations were classified into two categories, according to the degree of anthropic influence (human-influenced, vegetation strongly influenced or created by man, typically with a high proportion of ruderal or alien species of "habitat naturalness", and natural, natural and seminatural vegetation without strong anthropic influence; examples of human-influenced vegetation represent forests with ruderal or alien species, eutrophicated woody vegetation outside forests, and eutrophicated, intensively managed or disturbed grassland); and (iii) the altitude was recorded via GPS instrumentation on-site or later using the coordinates and the Google Earth application (Google Inc.). The altitude of published localities is that of the centre of a village or of a hill.

The best time to collect this species is April and the first half of May, provided students are able to correctly determine sterile plants. Later in the year, it is impossible to collect sterile individuals in shaded habitats, such as shrubs and forests, as they mostly finish growing in May (Duchoslav 2009). Flowering plants reported in summer are those growing in open, sunny habitats not shaded ones. Thus, the summer distribution does not reflect the full range of habitats occupied by this species.

Ploidy level

Several cultivated plants died before analysis so only two plants per population were analysed in some cases. DNA ploidy level (Suda et al. 2006) and chromosome number were determined using the procedures described in detail by Duchoslav et al. (2010). Briefly, DNA ploidy levels were determined by flow cytometry using the method of internal standardization. The nuclei of the standard and the sample were isolated, stained and analyzed together (Doležel 1991). *Triticum aestivum* cv. 'Saxana' was used as an internal standard

and calibrated against plant reference standard *Hordeum vulgare* with 2C DNA 10.43 pg (Doležel et al. 1998). The relative fluorescence intensity of propidium iodide (PI) stained nuclei was analysed using a Partec PAS instrument (Partec GmbH, Münster, Germany) equipped with an argon ion laser (535 nm). Histograms of fluorescence intensity were registered over 512 channels. In each sample, at least 2000 nuclei were analyzed. The ploidy level of each sample was determined by the position of its G_0/G_1 peak relative to the G_0/G_1 peak of the internal standard. Plants for which the number of chromosomes had been counted were used for the specification of internal standard-sample position. The fluorescence ratios between the positions of sample and internal reference standard peaks were 1.18–1.29, 1.40–1.69, 1.74–1.98, 2.02–2.23 for 3x–6x cytotype, respectively. PI staining yielded histograms with coefficients of variance (CV) of both standard and sample below 5% for the majority of the DNA-ploidy measurements (mean CV of standard was 4.22% ± 0.02, mean CV of samples were 4.04% ± 0.06, 4.10% ± 0.04, 4.19% ± 0.03, 4.32% ± 0.03 SE, for 3x–6x cytotype, respectively).

Data analyses

Because they were only observed at a single site, the triploids were excluded from the statistical analyses of habitat types and altitude. In the maps, we also include all of the published data provided in Table 1. These data were not included in the ecological comparisons (except for altitude) because there is insufficient information in the original sources.

Descriptive statistics and statistical tests were done using Statistica 9.0 software (Statsoft Inc.). Generalized linear models with multinomial distribution of dependent variable (habitat types) and logit-link function were used for the analyses of habitat differentiation among cytotypes. Breadth of ecological niche was expressed by Shannon diversity index H using $\log_e$ in the equation (Magurran 2004) based on frequencies of cytotypes in seven habitat types. Because Shapiro-Wilk normality test revealed non-normality of altitude even after various transformations, non-parametric Kruskal-Wallis test followed by multiple comparison Dunn's test were used in this analysis (Zar 1996).

Table 1. – Previously published data on the occurrence of *Allium oleraceum* cytotypes in Slovakia. In all of the original sources, only tetraploids (2n = 32) are reported.

Locality	Latitude (N)	Longitude (E)	Altitude (m)	Source
Strážovské vrchy Mts, Trenčianske Teplice village	48°54'43"	18°09'44"	267	Váchová & Feráková in Löve (1978)
Malá Fatra Mts, Párnica village	49°11'36"	19°11'31"	452	Murín et al. (1999)
Východoslovenská nížina lowlands, Komárany village	48°55'58"	21°38'58"	150	Murín et al. (1999)
Malé Karpaty Mts, Chľmec hill	48°11'43"	17°07'08"	325	Murín et al. (1999)
Chočské and Prosečianske vrchy Mts, Podbiel village, Biela skala	49°18'46"	19°28'53"	664	Murín et al. (1999)
Ipeľsko-rimavská brázda furrow, Šurice village	48°13'56"	19°54'54"	223	Murín et al. (1999)
Malé Karpaty Mts, Devínská Kobyla hill	48°11'41"	16°58'54"	287	Murín et al. (1999)
Brezovské kopce Hills, Bradlo hill	48°40'52"	17°34'00"	509	Murín et al. (1999)

Results

Cytotype distribution and ecology in Slovakia

We analysed 863 individuals (86% of the total sampled) from 93 populations in Slovakia using flow cytometry and an average of 9.3 plants per population (range 2–53). Four ploidy levels were recorded in Slovakia. Of the individuals analysed, 0.3% were triploid, 27.8% tetraploid, 66.3% pentaploid and 5.6% hexaploid. The triploid cytotype was found in one population (1.1%) and the tetraploids in 34 populations (36.6%). The most common cytotype was the pentaploid, being found in 68 populations (73.1%); the hexaploid cytotype was rare, as it was found in only seven populations (7.5%). Note that the sum of the percentages is higher than 100 because there are mixed populations. Sixteen per cent of the populations sampled by us consisted of more than one ploidy level, the most common being a combination of penta- and tetraploids. The detailed statistics, including the previously published data for 8 populations (Table 1), are shown in Table 2.

Table 2. – The cytotype composition of 101 populations of *Allium oleraceum* from Slovakia, including previously published data.

Cytotype composition	Count	Percent	Mean frequency of cytotype in mixed population (%)		
			4x	5x	6x
3x	1	1.0	–	–	–
4x	31	30.7	–	–	–
5x	53	52.4	–	–	–
6x	1	1.0	–	–	–
4x+5x	9	8.9	42.2	57.8	–
5x+6x	4	4.0	–	56.9	43.1
4x+5x+6x	2	2.0	19.4	38.9	41.7

The distribution of cytotypes is given in Fig. 1. The triploid cytotype was found in southern Slovakia. The pentaploid cytotype had a tendency to occur more frequently in the southern part of Slovakia, while the tetraploid had a tendency to occur in the northern parts. The hexaploid cytotype was sparsely found in western and southern Slovakia.

There was a clear relation between the distribution of cytotypes and altitude in Slovakia ($\chi^2 = 8.5$, P = 0.014; Fig. 2A), with tetraploids occurring more frequently at the higher altitudes and pentaploids and hexaploids occurring more frequently at lower altitudes. Regarding habitat type, there were weakly significant differences between the cytotypes ($\chi^2 = 21.2$, df = 12, P = 0.048; Fig. 3) due to tendency of hexaploids to occur more frequently in field margins than plants of other cytotypes. On the other hand, no differences were found between tetra- and pentaploids when the rare hexaploids are not included in the analysis ($\chi^2 = 8.6$, df = 6, P = 0.193). When only two habitats (i.e. habitat naturalness) were considered, the natural versus human-influenced, higher ploidy levels were increasingly found in the human-influenced habitats (4x: 37%, 5x: 56%, 6x: 100%; $\chi^2 = 14.0$, df = 2, P < 0.001). The Shannon index H for hexaploids (H = 1.17) was lower than that for tetra- (H = 1.83) and pentaploids (H = 1.86), suggesting a greater degree of habitat specialization of hexaploids than tetra- and pentaploids. The only locality with a triploid population was a relic site on an isolated volcanic hill with steppic vegetation, where it was found in fringe communities.
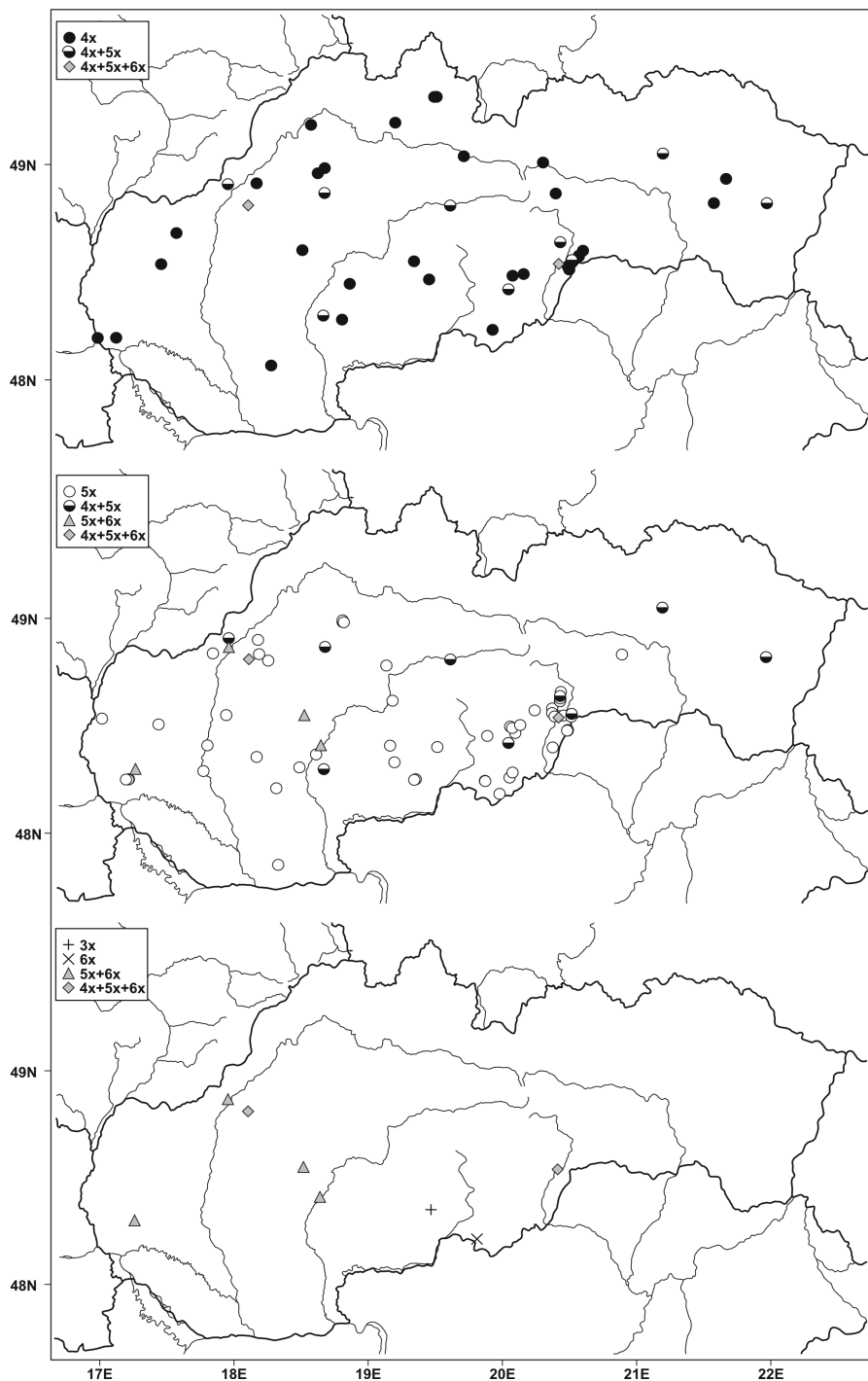
Fig. 1. – The distribution of cytotypes of *Allium oleraceum* in Slovakia, including previously published data. Cytotypes of uniform and mixed populations are distinguished by different symbols.
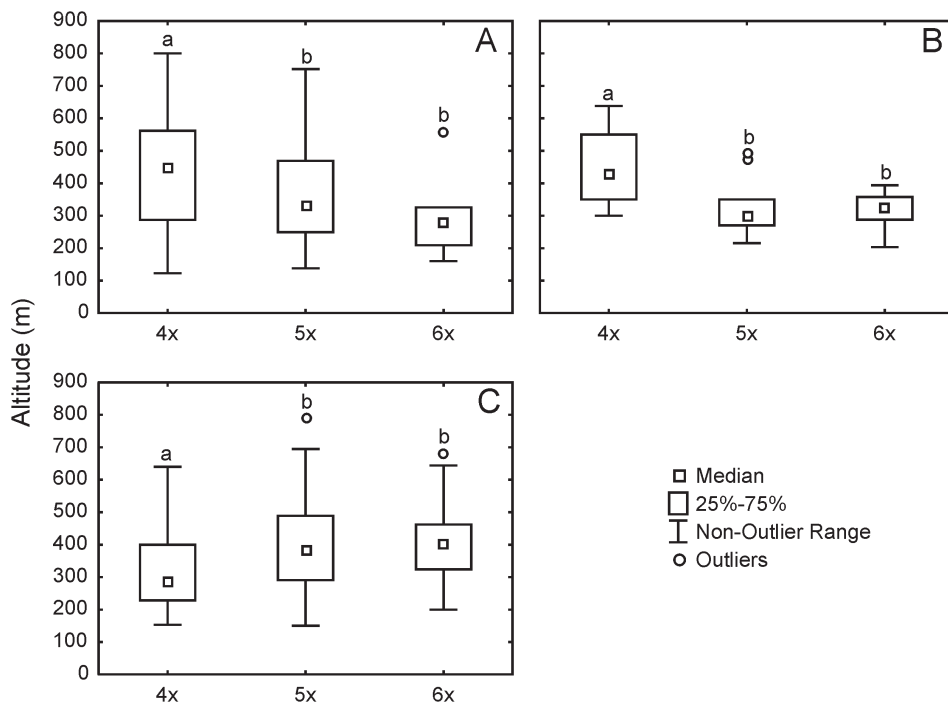
Fig. 2. – The altitudinal relationships of cytotypes in Slovakia (A) and in the Carpathian (B) and (C) Herzynian part of the Czech Republic (extracted from Duchoslav et al. 2010). Significant differences in the medians between ploidy levels (Dunn's test at P = 0.05) are indicated by different letters above the box plots, separately for each region.
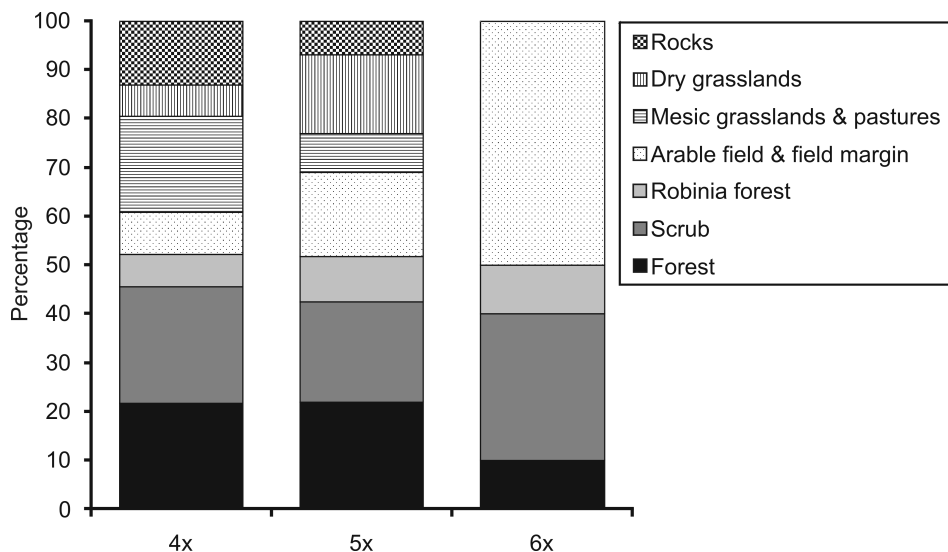
Fig. 3. – The distributions (%) of tetra- (n_{pop} = 34), penta- (n_{pop} = 68) and hexaploid (n_{pop} = 7) cytotypes of *Allium oleraceum* in common habitats in Slovakia. Triploids were not included in this analysis because they were found at only one locality.
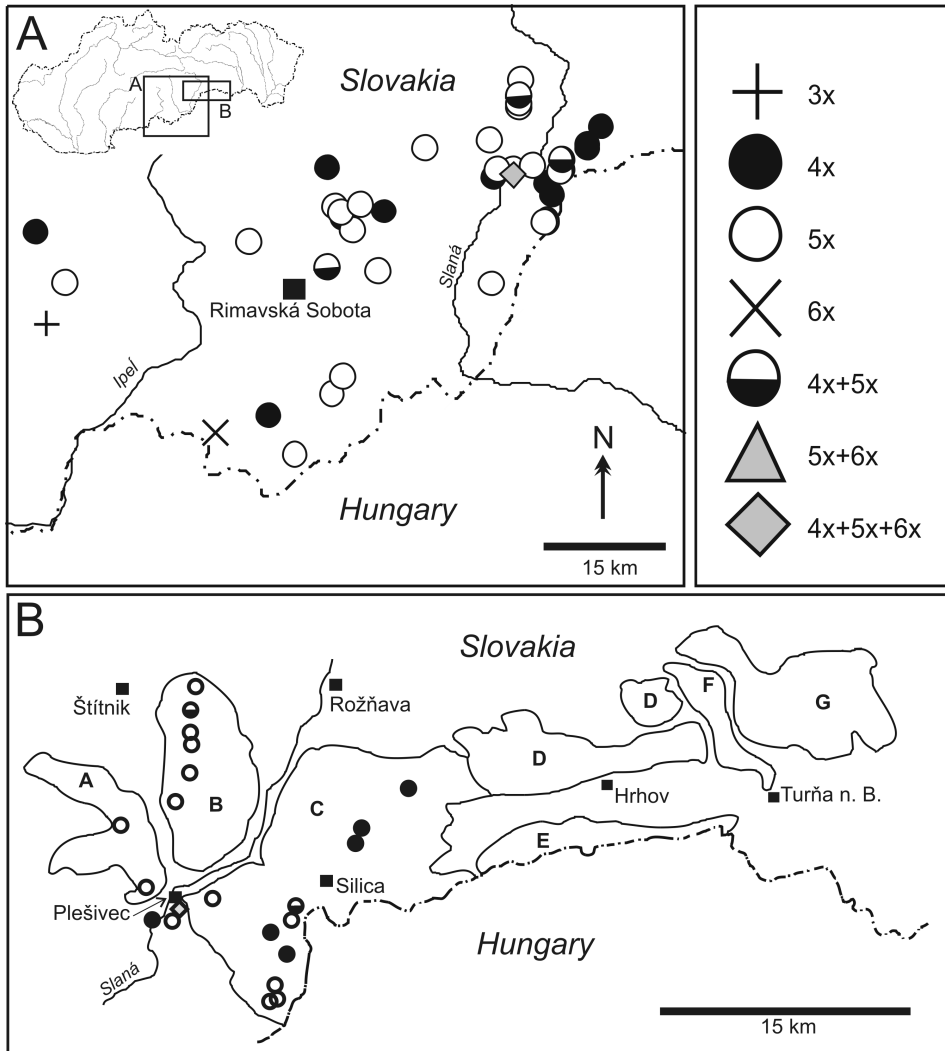
Fig. 4. – The distribution of *Allium oleraceum* cytotypes in Southern Slovakia in the surroundings of Rimavská Sobota (A) and the detailed distribution on the Slovak Karst (B). Particular karst plains are indicated, as follows: A – Koniarska planina; B – Plešivecká planina; C – Silická planina; D – Horný vrch; E – Dolný vrch; F – Zadielska planina; G – Jasovská planina.

Distribution on the Slovak Karst

After the discovery of a locality with three cytotypes ($2n = 4x+5x+6x$) at Plešivec (loc. no. 21), we paid special attention to the western part of the Slovak Karst and the surrounding areas of Southern Slovakia. The results are presented in Fig. 4 and in Electronic Appendix 1. The distribution on the different plains differed; *A. oleraceum* was found on three plains. On the Koniarska planina, *A. oleraceum* was rather rare and was represented by the

pentaploid cytotype. The Plešivecká planina was characterized by populations of the pentaploid cytotype, and only one of them was a mixture of pentaploids and tetraploids (loc. no. 62). The most complex pattern was found on the Silická planina, where the dominant cytotype was tetraploid, but in the central part, we also found a pentaploid population and a mixture of both. On the southwestern periphery of this plain, only pentaploids were found (loc. no. 44, 47, 59 and 60). We did not find *A. oleraceum* on the Horný vrch or Zadielská planina plains.

Discussion

Cytotype distribution

To date, the published data on *Allium oleraceum* from Slovakia includes only tetraploids; they are reported from eight different localities, with no special geographical preferences. Ironically, the most common pentaploid cytotype found by us in 73% of the populations is not previously recorded. This fact shows that even when a relatively high number of populations are included in studies they can still give misleading data for a region as there can be additional cytotypes where populations are mixed on a small spatial scale. The absence of data for rare triploid and hexaploid cytotypes is easily understandable. The preferential collection of tetraploids could be influenced by their more common occurrence in open seminatural stands including grasslands and rocks, locations where they regularly flower and can be more easily found than populations in forests and shrubs, which usually do not flower and almost all were collected by us in spring. In all other habitats, the pentaploid cytotype either dominated or was as common as the tetraploids (Fig. 3).

In contrast to the 23% of the populations consisting of a mixture of cytotypes reported by Duchoslav et al. (2010) for *A. oleraceum* in the Czech Republic, only 16% of the populations consisted of two or three cytotypes in Slovakia. This lower frequency of mixed populations may be partly due to the one third lower average number of plants per population analyzed (nine individuals per population) than in the Czech Republic ($\approx$ 13; Duchoslav et al. 2010). This necessarily increases the uncertainty in the estimates of the number of mixed ploidy populations, as rare cytotypes could easily have been missed (see Šafářová & Duchoslav 2010, Sonnleitner et al. 2010). However, when comparing frequencies of respective cytotypes within mixed populations, we observed an almost identical pattern to that found in the Czech Republic (Duchoslav et al. 2010), i.e. no single cytotype dominated within populations but cytotypes were usually in balanced proportions. This suggests, in accordance with previous studies (Duchoslav et al. 2010, Šafářová & Duchoslav 2010) that 'minority cytotype exclusion' (Levin 1975) has little effect within these populations.

The comparison of cytotype frequencies in Slovakia and the neighbouring Czech Republic (Duchoslav et al. 2010) revealed another significant difference. The pentaploid cytotype is the most common in both countries, more so in Slovakia than the Czech Republic. However, the second most common in the Czech Republic, the hexaploid cytotype, is rare in Slovakia and for this reason a greater proportion of the plants are tetraploid in Slovakia than in the Czech Republic. It appears that Slovakia is just on the eastern periphery of a continuous occurrence of hexaploids in Europe (L. Šafářová & M. Duchoslav, unpublished material), because the occurrence of hexaploids in the western part of Slovakia is tightly linked with their occurrence in the eastern part of the Czech Republic

and Austria (Dobeš & Vitek 2000). The isolated occurrence of hexaploids in the neighbourhood of the Slovak Karst may suggest a primary zone (sensu Petit et al. 1999) of hexaploid formation and, hence, different origins for these and the western hexaploids. However, the overall low frequency of hexaploids and the few plants sampled from each population might indicate there could be an undiscovered patchy distribution of hexaploids within southern Slovakia, which connect the eastern and western localities.

The tetraploids and pentaploids are broadly distributed in Europe; both ploidy levels occur sympatrically, for example, in the Baltic region (Finland, Lithuania and Sweden; Duchoslav et al. 2010: Table 1 and references therein). Therefore, their common occurrence in Slovakia is not exceptional. In contrast, only one triploid population was recorded in southern Slovakia, which is only the third locality worldwide and fills the gap between the previously reported triploids in northern Hungary (Krahulcová 2003) and the Ukraine (Vakhtina 1984). The previously recorded triploid populations and this new one are located at the northern limits of one of the supposed diploid progenitors, *A. paniculatum*, which was recently found in the area of the Drienčanský kras (Somogyi 1999, Kliment et al. 2000), and is also reported at Lillafüred, near Hamor (a few kilometres west of Miskolc) in Hungary, very close to the Slovak border (Rapaics 1917). However, the extremely rare records of triploids suggest that they do not serve as a bridge between diploids and tetraploids, and are only able to survive and form separate populations by producing bulbils within their inflorescences (M. Fialová & M. Duchoslav, unpublished material).

Cytotype mixed populations consisting of tetra- and pentaploids were sympatric and intermixed with single-cytotype 4x and 5x populations. Such a pattern is most likely a result of secondary contact between cytotypes and corroborates the results of a previous study (Duchoslav et al. 2010). On the other hand, mixtures of hexa- and pentaploids were more frequently recorded than pure hexaploid populations. However, because of the rarity of hexaploids in Slovakia this could be due to chance and thus no definitive conclusions on the origin of mixed populations can be proposed at present.

We found only two mixed populations with three cytotypes (4x+5x+6x) and therefore it would be premature to draw any conclusion based on this small sample. However, environmental conditions experienced by these two populations and Czech populations, which contain the same cytotype combination, are similar, i.e. scree slopes and rocky ground with outcrops of limestone or bedrock with traces of lime and fields in the close neighbourhood (see Duchoslav et al. 2010). Analysis of cytotype distribution in population no. 82 at a microgeographic scale showed that hexaploids occurred only under shrubs at field margins and tetra- and pentaploids in all microhabitats including rocky ground with steppic vegetation. It seems probable that strong habitat heterogeneity at a local scale combined with disturbance and calcium-rich bedrocks can increase the probability of the local co-occurence of different cytotypes. Whether these mixtures represent either primary or secondary contact zones is still an open question (see Šafářová & Duchoslav 2010).

Relationship with altitude

As mentioned above, the cytotypes differed in their relation to altitude, and this was significant for the material from Slovakia, whereas in the neighbouring Czech Republic, no significant relationship was found (Duchoslav et al. 2010). After the discovery of the relationship for Slovakia, we reanalysed the data from the Czech Republic. We divided the

data set according to the main geomorphological and biogeographical regions, the Carpathian and Herzynian portions (the Bohemian Massif). In the Carpathian part of the Czech Republic, we found the same relationship as in Slovakia (Fig. 2B). However, the situation in the Herzynian region was different; the tetraploids differed from the penta- and hexaploids as in the Carpathian region, but in a different way. The relationship was the opposite, with the tetraploids found at lower altitudes than the pentaploids and hexaploids (Fig. 2C). There are several possible explanations. For us, it seems probable that at least the tetraploids in the Carpathians were different from the tetraploids on the Bohemian Massif. These western tetraploids had an evident tendency to occur mainly at lower altitudes in forests (i.e., floodplain forests; see Duchoslav et al. 2010), while this was certainly not true for the Carpathian tetraploids. Comparing both regions, the differentiation within the tetraploids was greater than within the pentaploids and hexaploids.

This example clearly shows that correlations, which were not detected at one level (administrative unit, the Czech Republic) may be detected at another level (more natural units, the Carpathians and Bohemian Massif). The border between the Bohemian Massif and the Carpathians is known as an important biogeographical boundary for the distribution of species in Central Europe (Hendrych 1987), and it is the main reason for their biogeographical separation. Recently several studies have shown that the distribution of cytotypes of several species differ in these regions, for example, those of *Vicia cracca* (Trávníček et al. 2010) and *Pilosella officinarum* (Mráz et al. 2008). The present report and a previous one (Duchoslav et al. 2010) show this differentiation also for *Allium oleraceum* and the present report clearly shows that, for example, the altitudinal correlations were opposite in the two regions. These facts suggest that the differences found have deep roots in the history of both of these main geographical regions. The differences between the Bohemian Massif and the Carpathians are discussed by Mráz et al. (2008).

Ecology

With respect to habitat preferences, both the tetra- and pentaploid cytotypes occurred in a wide spectrum of habitats, while the hexaploids were limited to narrower spectrum of habitats, as in the Czech Republic (Duchoslav et al. 2010). However, in contrast to the data from the Czech Republic, no increase in the frequency of tetraploids in (semi)natural forest stands was observed in Slovakia. This finding partially supports the hypothesis that the tetraploids in the Carpathian and Herzynian regions have different origins (see above). However, a consistent pattern of an increasing frequency for the higher ploidy-number cytotypes in human-influenced vegetation both in the Czech Republic and Slovakia suggest that, regardless of the origin and regional differences in the composition of the vegetation, these cytotypes maintain their ecological strategies.

Some localities were rather extreme. In fact, near the village of Drienčany, we found *A. oleraceum* growing on the temporarily emerging bottom of a karst lake, within the community of *Agropyro-Rumicion* (loc. no. 31). This habitat was flooded for at least part of the growing season, yet the plants were strong and showed no signs of stress.

Slovak Karst

The surprising difference in the distribution of the cytotypes on particular karst plains in the area of the Slovak Karst can be related to the different land use in these regions

(Rozložník 1994), which is corroborating evidence for the scenario explaining the ploidy-level distribution of *A. oleraceum* in the Czech Republic (Duchoslav et al. 2010). The Koniarska and Plešivecká planina plains had no permanent settlements in the past; most areas of the Koniarska planina are wooded and the Plešivecká planina is a mixture of pastures and forests. Conversely, the Silická planina does have permanent settlements, namely, the villages of Silica and Silická Brezová. Human activities have created not only pastures, as on the Plešivecká planina, but also arable lands. We found each of the 4x and 5x cytotypes in the vicinity of the villages. Near Silica, cytotypes grew together in a broad-leaf forest (loc. no. 56), whereas in the vicinity of Silická Brezová, cytotypes were found in different habitats: tetraploids occurred at the edge of a forest (loc. no. 57) and between arable land (loc. no. 46) and the pentaploids in grassland, on a small hill, with steppic communities (loc. no. 58). Similarly, a complex pattern also occurred in the Drienčanský kras karst area, where tetraploids and pentaploids grow close together with no clear habitat preferences. Near the village of Hostišovce, both tetraploid and pentaploid cytotypes occurred in a rocky habitat (loc. no. 33 and 37).

Conclusions

Only tetraploid *A. oleraceum* are previously reported from Slovakia. This study, however, suggests that both the composition and pattern of distribution of *A. oleraceum* cytotypes are remarkably complex at various spatial scales with as many as four cytotypes detected (2n = 3x, 4x, 5x, 6x) in Slovakia. The results thus fit well into and broaden the results of detailed screening in the neigbouring Czech Republic (Duchoslav et al. 2010) with Slovakia representing the most cytotype-diverse region so far detected for *A. oleraceum*. From the methodological point of view, our study clearly shows that to correctly estimate (co-)distribution and frequency of cytotypes, (i) both intensive (many plants per site) and extensive (many sites) sampling (Halverson et al. 2008) of (ii) a wide range of habitats inhabited by the species are required. Contrasting the altitudinal distribution of tetraploids in the Czech Republic and Slovakia suggest that at least two different types of tetraploids occur in Central Europe. Both tetra- and pentaploid cytotypes showed similar and wide ecological amplitude in contrast to the hexaploids, which were limited to human– influenced habitats. The majority of mixed-ploidy populations were found in sympatry with cytotype-uniform populations of participating cytotypes suggesting rather secondary contact between cytotypes. The existence of mixtures containing tetra-, penta- and hexaploids and apparent local cytotype diversity in the Slovak Karst area may indicate primary contact zones. For detailed investigations on the evolutionary dynamics of populations with cytotype heterogeneity *Allium oleraceum* is a promising plant.

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

Acknowledgements

Souhrn

Studie se zabývá rozšířením a ekologií cytotypů *Allium oleraceum* na Slovensku. Doposud publikované práce uváděly z území Slovenska pouze tetraploidní cytotyp. Protože byla recentně zjištěna komplexní cytotypová variabilita (2n = 4x–6x) v sousední České republice, vyvstala otázka, zdali se skutečně vyskytuje na Slovensku jen tetraploidní *A. oleraceum* nebo je situace složitější. Vedle geografického rozšíření a stanovištních nároků cytotypů na území Slovenska jsme se dále zaměřili na podrobnější rozšíření cytotypů na lokální škále Slovenského krasu a blízkého okolí, kde jsme, na základě údajů o výskytu blízce příbuzného taxonu a jednoho z předpokládaných diploidních rodičů, *A. paniculatum*, poblíž studovaného území předpokládali komplexnější cytotypovou kompozici. Pomocí průtokové cytometrie byl analýzou 863 jedinců z 93 populací zjištěn výskyt čtyř cytotypů: tri- (2n = 24), tetra- (2n = 32), penta- (2n = 40) a hexaploidů (2n = 48). Nejčastěji (v 73 % studovaných populací) byl zaznamenán pentaploidní cytotyp, tetraploidní cytotyp byl zjištěn ve 37 % populací, hexaploidní cytotyp byl zjištěn pouze v 7 populacích (8 %) a triploidní v jedné populaci (1 %). Zjištěné údaje jsou zcela v rozporu s dosavadními znalostmi cytotypové variability studovaného taxonu na Slovensku a ukazují, že i relativně vyšší počet v minulosti studovaných populací (8 lokalit) může poskytnout nepřesné a zavádějící údaje. Šestnáct procent analyzovaných populací bylo cytotypově smíšených, byly zjištěny kombinace cytotypů 4x+5x, 5x+6x a 4x+5x+6x. Tetraploidní cytotyp byl četnější v severní zatímco pentaploidní cytotyp v jižní části Slovenska. Hexaploidní cytotyp se vyskytoval na západním a jižním Slovensku. Nález triploidní populace na kopci Veľký Lysec poblíž obce Luboreč (okres Lučenec) představuje teprve třetí známou lokalitu na světě, která leží, podobně jako další dvě známé lokality (severní Maďarsko, Ukrajina) na severní hranici areálu *Allium paniculatum*. Byly zjištěny statisticky signifikantní rozdíly ve vazbě cytotypů na biotopy, přičemž hexaploidní cytotyp vykazoval silnější vazbu na biotop pole a polní okraje (meze, příkopy atp.) než tomu bylo u tetra- a pentaploidních cytotypů. V případě, kdy byly lokality klasifikovány podle míry antropického tlaku, stoupala s rostoucí ploidií relativní frekvence výskytu populací na ruderalizovaných stanovištích. (Re)analýza výskytového rozšíření 4x–6x cytotypů v České republice, rozdělené do dvou geomorfologických/biogeografických celků (Karpaty a Hercynikum), a na Slovensku ukázala, že zatímco tetraploidní cytotyp je četnější ve vyšších nadmořských výškách než penta- a hexaploidní cytotypy jak na Slovensku, tak v karpatské části České republiky, opačný vztah byl nalezen v Hercyniku. Z výše uvedeného lze usuzovat, že se ve střední Evropě vyskytují přinejmenším dva typy tetraploidů. Rozšíření cytotypů na planinách v západní části Slovenského krasu bylo nenáhodné a je dáváno do souvislosti s rozdílným využíváním krajiny.

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3.4 Large cytotype diversity, contrasting distributional patterns and genome size variations in polyploid geophyte *Allium oleraceum* (Alliaceae) on European scale

Šafářová L. & Duchoslav M.

manuskript

Large cytotype diversity, contrasting distributional patterns and genome size variations in polyploid geophyte *Allium oleraceum* (Alliaceae) on European scale

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● *Background and Aims*   *Allium oleraceum* is polyploid complex consisting of several cytotypes. Detailed screening on local and regional scales in Central Europe revealed complex distribution pattern of cytotypes that was results of several interacting mechanisms. The pattern of coexistence of polyploids in the geophyte *Allium oleraceum* over continental scale together with variation in their genome size was studied with aims to elucidate evolutionary history of polyploidy in *A. oleraceum*.

● *Methods*   277 populations of *Allium oleraceum* were sampled over Europe and also all available data on ploidy level variation were exctracted from literature resulting in 765 populations. Root-tip squashes and flow cytometry were used to identify ploidy levels and to measure 2C DNA content. Ecological differentiation among ploidy levels was tested by comparing sets of environmental and climatic variables for each locality. Genome size variation was correlated with spatial, climatic and habitat variables.

● *Key Results*   Four ploidy levels were confirmed in Europe: pentaploids (2n = 40) and tetraploids (2n = 32) predominated over whole Europe, hexaploids (2n = 48) were limited to the (sout)western and Central Europe, triploids (2n = 24) occurred in the eastern part of Central Europe and in the eastern Europe. Moreover, two new cytotypes were discovered: rare heptaploids (2n = 56) in France and octoploids (2n = 64) in Spain. Plants of different ploidy levels were found to be ecologically differentiated, though cytotypes have overlapping ecological niches. Triploids prefer natural habitats in continental type of climate, while hexaploids and heptaploids occured human-impacted habitats in regions with mild climate. Tetra- and pentaploids showed wide ecological niche and occured in wide spectrum of climatic conditions. Mixed populations represented 15 % of total sample with commonly found mixtures 4x+5x and 5x+6x. Significant variation in genome size was revealed in *A. oleraceum*. Major part of variation within each of *3x-7x* cytotypes was attributable to interpopulation variation (range 4.9-18.3 %), while within-population variation was not significant. Monoploid DNA amount (1Cx) was significantly dependent on ploidy level, and genome downsizing were observed between all ploidy levels except for nonsignificant differences between hexa- and heptaploids. Significant variation and spatial structuring of genome size were observed in tetra- and pentaploids, where 2C DNA significantly increased with latitude and longitude suggesting gradient of continentality. On the contrary, 2C DNA content in hexaploids showed low variation and was only weakly correlated with geographical and climatic variables.

● *Conclusions*   Cytotypes of *A. oleraceum* showed complex distributional pattern on European scale with frequent occurence of mixed populations. Greater cytotype variation was found in the southern latitudes where the contact zones with presupposed progenitors are developed. In more northerly located regions without such contacts usually tetra- and pentaploids dominated. Differences in monoploid genome size among cytotypes in *A. oleraceum* and dominant east-west gradient in genome size in tetra- and pentaploids can be product of local adaptation along ecological gradients or rather may suggest existence of at least two independent lineages: eastern and western. Two contact zones between cytotypes are suggested: western Central Europe and eastern Central Europe. Polyphyletic origin of the species is also highly probable. Distributional data support the existence of both primary and secondary zones of cytotype contacts.

Key words: Alliaceae; chromosome numbers; flow cytometry; genome size; geophytes; polyploidy, spatial scales

Introduction

Polyploidy is a widesplead phenomenon among flowering plants (Wendel, 2000). Several lines of evidence suggest that at least 70% of angiosperm species have polyploid ancestry (Masterson, 1994; Blanc and Wolfe, 2004), and polyploidization is also believed to be a major speciation mechanism (Otto and Whitton, 2000; Rieseberg and Willis, 2007; Wood et al., 2009). Before becoming evolutionary successful, newly formed polyploids often have to overcome numerical inferiority, incompatible matings and competition with parents (Levin, 1975). It was believed in past that these mechanisms results in situations where cytotype mixtures are extremely rare and cases of localy coexisting cytotypes represent transient situations following frequent generation or, in the case of secondary contacts, immigrations of an alternative cytotype (Kao, 2007). Polyploidization has, hovewer, profound consequences for gene expression (Adams and Wendel, 2005), the physiological and ecological behaviour of plants (Levin, 1983, 2002; Lumaret, 1988; Soltis et al., 2003; Ramsey, 2011) as well as genetic diversity (Soltis and Soltis, 2000). Increased genetic buffering provided by having extra genome copies and changes in gene expression may affect the potential for novel adaptive responses to selection in polyploids (Levin 1983, 2002; Bretagnole and Thompson, 1996; Soltis and Soltis, 1999; Wendel, 2000; Otto and Whitton, 2000; Soltis et al., 2003). It has been evidenced many times that polyploids have a broader niche or they may differ in their niche optima from their diploid conspecifics (Thompson and Lumaret, 1992; Petit et al., 1999; Soltis and Soltis, 1995, 2000) with frequently reported increased ability to tolerate stressful conditions (Levin, 2002). As a results, various local and global spatial patterns of polyploid distibution ranging from less frequently reported sympatry of cytotypes with microhabitat differentiation through more common parapatry with cytotype-mixed populations over contact zones to the allopatry has been observed (Thompson and Lumaret, 1992; Levin, 2002). Due to their better adaptation to extreme climates, polyploids are also believed to have wider geographic distributions than their progenitors (Soltis and Soltis, 2000), especially in regions that were previously glaciated (Brochmann et al., 2004; Wu et al., 2010), but alternatively, widespread cytotypes could be more rapid and efficient colonizers (Levin, 2002; Brochmann et al., 2004). Polyploids are also better invaders in introduced ranges (Pandit et al., 2006; Treier et al., 2009). Same patterns may be, however, explained non-adaptively as results of random processes, e.g. founder effect (Kliber and Eckert, 2005).

With the implementation of flow cytometry, it is clear that mixed-ploidy populations are much more frequent than previously anticipated (e.g., Burton and Husband, 1999; Weiss et al., 2002; Stuessy et al., 2004; Suda et al., 2007a; Halverson et al., 2008; Kao, 2008, Marhold et al., 2010; Cires et al., 2010; Trávníček et al., 2010, 2011ab). Several models investigating the mechanisms of cytotype establishment and local coexistence showed that the evolution of assortative mating attained by a variety of factors such as divergence in flowering time or differences in pollinators (Fowler and Levin, 1984; van Dijk and Bijlsma, 1994), iteroparity, apomixis and greater vegetative reproduction (Bierzychudek, 1985; Yamauchi et al., 2004; Kao, 2007, 2008) or localized pollen and seed dispersal (Li et al., 2004; Baack, 2005) might suffice to allow coexistence of cytotypes. Nonetheless, we have still little empirical data exploring mechanisms shaping cytotype distributions and co-existence in details (Husband, 2004; Kao and Parker, 2010) and recent research (Trávníček et al., 2011a) suggests, in contrast to generally accepted theories, possibility of a long-term existence of ploidy mixtures when free inter-cytotype mating interactions results in sufficiently frequent polyploid origin and no apparent decrease in fitness of cytotypes occur.

Allium L. section *Conodoprasum* Reichenb. represents evolutionary young group of bulbous geophytes (Friesen *et al*., 2006) consisting of a set of diploid and polyploid species. These species occur from the westernmost part of Macaronesia, northern Africa and Iberian Peninsula through the whole Mediterranean area and Europe to the Iran and southwestern Siberia (Meusel *et al*., 1965; Stearn, 1980; Friesen, 1987; Jauzein and Tison, 2001; Brullo *et al*., 1996*ab*, 1997, 2001, 2003*ab*, 2008; Fraga, 2002; Bogdanovic *et al*. 2008, 2009; Aedo, 2010). Within this section, one of the most interesting and hitherto less investigated group is the *Allium paniculatum* complex, characterized by plants with ribbed and glabrous leaves with semicylindrical to flat outline, spathe valves with long appendage, and campanulate perigon with stamens included or just slightly exterted (Levan, 1937, 1938; Brullo *et al*., 2001, 2008). This complex is taxonomically extremely difficult (Stearn, 1981) and the latest monograph dealing with the complex and covering whole Europe was that one by Stearn (1980) in *Flora Europaea*. Within the complex, Stearn distinguished three widespread species (*A. paniculatum*, *A. pallens*, and *A. oleraceum*), and several other species with limited distribution (e.g., *Allium macedonicum*, *A. tardans*, *A. podolicum*). Since the publication of *Flora Europaea*, further floristic exploration in the Mediterranean and the southeastern Europe led to discovering and describing of number of "local" species with narrow ranges (e.g., Brulo *et al.*, 2001, 2003a; Peruzzi, 2007; Tzanoudakis and Kypriotakis, 2008) that are members of this complex. Also some progress in taxonomic treatment of some less known and/or wrongly interpreted species of this complex had been made (e.g., Brulo *et al.*, 1996ab, 2003b, 2008; Aedo, 2010). The taxonomy of the section *Codonoprasum* was recently summarized in several regional monographs (Pastor and Valdes, 1983; Brullo *et al*., 2001; Jauzein and Tison, 2001; Aedo, 2010) but these treatments are not completely corresponding to each other.

Most of the species considered as members of this complex consist of either diploid or diploid & polyploid cytotypes. The only exclusively polyploid species of this complex has been considered until recently *Allium oleraceum* L. (but see Brullo *et al*., 2004, 2008). It is distributed much wider than other taxa of this complex (Meusel *et al*., 1965). It is common in the western, central, eastern Europe, and southern Scandinavia, and it extends southwards to the northern part of the Mediterranean. It is the only species covering whole northern part of the complex's distribution area. Morphologically, *A. oleraceum* is the only one species of the *A. paniculatum* complex having regularly bulbils within inflorescence and limited degree of sexual reproduction, and the presence of bulbils is generally considered as a crucial character that differentiate this taxon from the other representatives of this complex (Stearn, 1980; Pastor and Valdes, 1983; Brullo *et al*., 2001; Jauzein and Tison, 2001).

Allium oleraceum L. is represented by polyploid series consisting of tri-, tetra-, penta- and hexaploid cytotypes (2n = 24, 32, 40, 48; Duchoslav *et al*., 2010). Levan (1938) considered *A. oleraceum* to be an autopolyploid form of diploid *Allium paniculatum* that arose either by somatic doubling of chromosomes or fusion of unreduced gametes. However, reinspection of crossing experiments in Levan's paper showed that "synthetic" tetraploid *A. oleraceum* obtained therein by crossing plants from two distant diploid populations of '*A. paniculatum'* may be a result of interspecific hybridization through unreduced gametes (see Duchoslav *et al*., 2010).

Detailed screening of cytotype variation in *A. oleraceum* in the Czech Republic (Duchoslav *et al*., 2010) and Slovakia (Šafářová *et al*., 2011) showed obvious sharp contrasts both in composition and spatial patterns of cytotypes. So far published papers reported usually cytotype uniform tetra- and pentaploid populations over Europe and extremely rare records of tri- and hexaploids from Central and East Europe, except for the occurrence of hexaploids in Spain. On the other hand, we found (Duchoslav *et al*., 2010; Šafářová *et al*., 2011) complex spatial pattern of tetra-, penta-, and hexaploid cytotypes with high reprezentation of mixed populations consisting of all possible cytotype

combinations. The results also provided evidence for ecological differentiation among ploidy levels which contributed together with the prevalence of asexual reproduction and localised dispersal to the complex distribution pattern and local coexistence in *A. oleraceum* cytotypes (Šafářová and Duchoslav, 2010).

Complex distribution pattern of cytotypes found in the Czech Republic and Slovakia clearly reflects problems, errors or limitations in the collection and interpretation of the karyological data for the cytogeographic purposes. Casual sampling of low number of plants is usually able to detect the most frequent cytotypes but fail to detect rare ones (Burton and Husband, 1999; Halverson *et al.*, 2008) and inaccurately evaluate spatial pattern of detected cytotypes. But as Šafářová *et al.* (2011) showed, even such low-intensity sampling can give totally misleading results. Taking into account recently published data, at first the question arises whether our previous results could be extrapolated outside Central Europe. Secondly, knowledge of cytotype composition and genome size variation, especially over contact zones between *A. oleraceum* and its presupposed progenitors (i.e., other members of the *A. paniculatum* group), may allow for inferences about the evolutionary history of polyploidy in *A. oleraceum*. Holoploid and monoploid genome size are important adaptively relevant parameters of fundamental biological importance (Bennett, 1972; Bennett and Leitch, 2005, 2011; Leitch and Bennett, 2007) with potential as taxonomically significant traits at lower taxonomic levels (Murray, 2005; Šmarda *et al.*, 2008*b*) or allowing identification of both heteroploid and homoploid hybrids (e.g. Mahelka *et al.*, 2005). We hypothesize overall higher cytotype diversity and genome size variation of cytotypes of *A. oleraceum* could be the result of multiple origins via independent crosses between different members of this complex.

In this study, we addressed the following questions: (1) What is the diversity and frequency of cytotypes of *A. oleraceum* and pattern of their geographic distribution in Europe? Is there greater cytotype variation of *A. oleraceum* in the contact zones with presupposed progenitors than in more northerly located regions without such contacts? How frequent are mixed-ploidy populations and which cytotypes participate on their composition? (2) Are there any relationships of cytotypes with environmental factors? If so, do possible differences correspond with previous results that were obtained on regional scale? (3) What range and pattern of genome size variation occurs in the *A. oleraceum* on European scale? Is there any ecological or evolutionary interpretation of genome size variation on this scale?

Materials and methods

Plant material and sampling

For the chromosome number survey, data were extracted from 28 publications resulting in 153 localities. Our own samples were collected in 2004-2011 throughout Europe covering most of the area of distribution of *A. oleraceum*. In total, 201 populations of *A. oleraceum* were sampled (Appendix 2). In the central and western Europe, our sampling was relatively dense and many populations were sampled from France (41), Germany (33), Hungary (27), Austria (22), and Poland (16). Other countries were sampled less intensively: Italy (9), Lithuania (8), Switzerland (7), Romania (7), Slovakia (7), Spain (4), Croatia (3), Slovenia (3), Ukraine (3), Great Britain (2), Belgium (2), Denmark (2), Bulgaria (1), Netherlands (1), Norway (1), Russia (1) and Serbia (1). We did not collect samples from the Czech Republic and partly from Slovakia because detailed ploidy-level screening of 418 populations has been recently published elsewhere (Duchoslav *et al.*, 2010; Šafářová *et al.*, 2011). However, we extracted data from these papers and incorporated them into some analyses.

Plants were collected from the wide spectrum of habitats, including natural alluvial forests, mesic and open dry grasslands, rocks, ruderal stands and arable land. Each population sample consisted of mostly 3–30 plants depending on population size. Efforts were made to avoid collecting individuals growing close together. During sampling, area of population was estimated in square meters. Samples were transported to and planted in the garden of the Palacký University in Olomouc, Czech Republic. Our samples were completed by the 10 samples containing either seeds or bulbils that were obtained via 'Index seminum' from foreign botanical gardens and represented samplings from natural populations (see Appendix 2).

Environmental characteristics

At each sampled site the following environmental variables identical with those used in previous study (for details see Duchoslav *et al.*, 2010) were recorded: (i) Habitat type was assessed in the field and classified into one of seven common habitat types (rock; dry grassland; mesic & wet grassland; (semi)natural forest; scrub; planted *Robinia pseudacacia* forest; arable field & field margins). (ii) Habitat naturalness, i.e. vegetation at site was classified according to degree of anthropic impact as either 'human-impacted' (vegetation strongly influenced or created by man, typically with higher proportions of ruderal or alien species) or 'natural' (natural and seminatural vegetation without strong anthropic influence). (iii) Populations were classified into two categories according to their distance to the nearest arable field ('Presence of arable land'; distance to the nearest arable field either ≤ 20 m or > 20 m). (iv) Light conditions were assessed in the field according to visually estimated proportion of full sunlight falling at the ground during late spring (strong shade, half-shade, low shade, full insolation). (v) Altitude was estimated via GPS instrument on-site or later according to co-ordinates through the Google Earth application (Google Inc.). Furthermore, for description of site xericness, (vi) heat index (Parker, 1988) was computed. This index was calculated according to the following formula: heat index = cos (slope aspect-225)*tan (slope angle), where aspect is expressed in degrees azimuth and angle in degrees. Unfortunately, any detailed data concerning the environmental conditions of sites, where seeds for the purpose of seed exchange program (Index Seminum, see Appendix 2) were sampled, were not provided by collectors.

Set of climate layers (climate grids) – such as annual mean temperature, maximal temperature of warmest month, minimal temperature of coldest month, annual precipitation, precipitation of wettest month and precipitation of driest month – with a spatial resolution of a square kilometre were obtained from the WORLDCLIM database (http://www.worldclim.org) (Hijmans *et al.*, 2005). For each locality, climatic data were derived from this database through ArcView GIS 3.1 software (Environmental Systems Research Institute, Inc.).

Flow cytometry and chromosome counts

DNA ploidy levels were determined by flow cytometry (FCM hereafter) using the method of internal standardisation. The nuclei of the standard and the sample were isolated, stained and analysed together (Doležel, 1991). *Triticum aestivum* cv. *Saxana* was used as an internal standard and it was calibrated against plant reference standard *Secale cereale* cultivar Dankovske with 2C DNA 16.19 pg (Doležel *et al.*, 1998). The relative fluorescence intensity of stained nuclei was analysed using a Partec PAS instrument (Partec GmbH, Münster, Germany) equipped with an argon ion laser (535 nm). Young fresh and healthy leaves of the internal standard and *Allium* sample were chopped with a new razor blade in a Petri dish containing 1 ml of ice cold LB01 buffer (Doležel *et al.*, 1989). The suspension was filtered through a 42-μm nylon mesh. The nuclei suspension

was supplemented with RNAse and stained with propidium iodide (PI) (both 50 µg ml^{-1}). Histograms of fluorescence intensity were registered over 512 channels.

DNA ploidy levels were measured from 1549 survived plants during 2005–2011. In each sample, at least 2000 nuclei of both the standard and the test plant were analysed. The gain of the instrument was adjusted so that the G_0/G_1 peak of the standard was approximately on channel 50. The ploidy level of each sample was determined by the position of its G_0/G_1 peak relative to the G_0/G_1 peak of an internal standard. Plants with counted chromosomes were used for the specification of internal standard-sample position. The fluorescence ratios between the positions of sample and internal reference standard peaks were 1.18-1.29, 1.40-1.69, 1.74-1.98, 2.02-2.23, 2.37-2.59 and 2.64-2.77 for $3x$–$8x$ cytotype, respectively. PI staining yielded histograms with coefficients of variance (CV) for both the standard and the sample below 5 % in the majority of DNA-ploidy measurements except for octoploids (mean±s.e. CV of standard was 4.22 % ± 0.02, mean CV of samples were 4.04 % ± 0.06, 4.10 % ± 0.04, 4.19 % ± 0.03, 4.32 % ± 0.03, 4.87%±1.44 and 6.02% ±0.93 for $3x$–$8x$ cytotype, respectively). Several samples of various DNA ploidy level and samples with DNA content on the margin of the variation were subjected to classical chromosome counting. Chromosome number determination followed the same procedure as described by Duchoslav *et al*. (2010).

For the purpose of estimation of genome size variation, 112 populations and 434 individuals from our stock (see Electronic supplementary), covering ranges of respective cytotypes studied, were selected by stratified random procedure. At least three (in the case of heptaploids two) randomly selected plants per population were analysed. Before measurements, all samples were cultivated under standard homogeneous conditions of the experimental garden of the Department of Botany, Palacky University, for two or more years and finaly for three months in cold glasshouse. The following measurement strategy was chosen to ensure maximum validity of the results: (i) each sample was measured with the same operator at least three different times on different days to minimise potential instrumental drift; (ii) all measurements were done over one month (April-May 2007-2010); (iii) at least 5000 nuclei per sample were recorded; (iv) if some outlier was detected, this most remote value was discarded and the sample was re-analysed. Because of huge content of the cytosolic compounds, coefficients of variation for the G_0/G_1 peaks of the standard and *Allium* samples varied between 3 and 5% (mean CV of the standard was 4.11% ± 0.02, mean CV of samples were 4.03% ± 0.06, 4.10% ± 0.04, 4.09% ± 0.02, 4.31% ± 0.03, 4.09%±0.31, 6.02%±0.93 s.e. for $3x$–$8x$, respectively). Analysis of variation of repeated measurements showed that the approximate measurement inaccuracy did not exceed ±2.5% in 95% of accessions of all ploidy levels studied. Therefore, variation beyond arbitrary fluctuation (5.0%) should be considered as really existing. The absolute 2C DNA content of a sample was calculated based on the values of the G_0/G_1 peak means ((sample G_0/G_1 peak mean)/(standard G_0/G_1 peak mean)) × standard 2C DNA content (pg DNA) (Doležel and Bartoš, 2005). DNA content of the non-replicated monoploid genome (i.e. the 1Cx value *sensu* Greilhuber *et al*., 2005) was estimated as the amount of nuclear DNA divided by ploidy level. For the verification of intraspecific DNA variation and to avoid artefact in measurement, we simultaneously analysed two samples with markedly different DNA content within the same ploidy level. The appearance of separate peaks was considered as a proof for true differences in the amount of nuclear DNA (Greilhuber *et al*., 2007).

Statistical analyses

Because they were only found at a single site, the octoploids were excluded from all analyses. Statistical analyses were performed for four, partly different data sets: (1) DNA ploidy level ($3x$–$7x$) of 294 European populations, including 201 populations sampled by

us and 93 populations from Slovakia (Šafářová *et al*., 2011) connected with environmental data for each locality (n = 294; data-set 1). Data from the Czech Republic (Duchoslav *et al*., 2010) were used just for comparison because of different sampling designs used in the studies. (2) Several cultivated plants died before analysis that lead to only one analysed plant per population in some cases. For statistical testing of proportions of cytotype-uniform and mixed populations, only those populations from the data-set 1 with two and more plants analysed were involved (n = 277; data-set 2). (3) DNA ploidy level of the reduced data set including either 654 accessions (71 %) from the complete set of all available data (data-set 1 + published karyological data + data from Index Seminum + data extracted from Duchoslav *et al*., 2010 + Šafářová *et al*., 2011) related to climatic (data-set 3a) or 654 accessions (71 %) from the complete set of all available data (data-set 1 + data extracted from Duchoslav *et al*., 2010 + Šafářová *et al*., 2011) related to environmental data (data-set 3b). To decrease the influence of oversampling within some European regions on the results of statistical analyses, accessions were selected via geographic stratification procedure as follows: Europe were divided into grid with the size of grid cell 20 x 20 km. Separately within each cytotype, accessions were included into data set if just single population was sampled in the respective grid cell or one population was randomly chosen in the case of two and more sampled populations within respective grid cell. Of course, one should have in mind that the results are valid to the respective data-set and did not reflect overall climatic affinities of respective cytotypes. (4) Mean holoploid (2C) and monoploid (1Cx) genome size of selected populations of $3x$-$8x$ cytotypes related to geographic, climatic and ecological variables of the sites (data-set 4).

Common descriptive statistics and statistical tests were done in the NCSS 2001 software (Hintze, 2001). Contingency tables were used for the analyses of qualitative environmental variables. Because Shapiro-Wilk normality test revealed non-normality of environmental quantitative variables even after various transformations, non-parametric Kruskal-Wallis test followed by multiple comparison Dunn's test were used for the analyses of quantitative and ordinal data, respectively (Zar, 1996). Ecological and climatic variables of the data-sets 1 and 3 were subsequently subjected to constrained principal coordinate analysis (db-RDA; Legendre and Anderson, 1999) according to approach explained in details by Duchoslav *et al*. (2010).

Populations with measured genome size (data-set 4) were tested for the intra- and interpopulation variation in DNA content employing mixed model ANOVA in Statistica 9 (Statsoft Inc.). Overall test on repeated measurements of individuals were done with cytotypes as fixed factor, populations as random factor nested within cytotypes and individuals as random factor nested within populations. Then, similar tests on repeated measurements of individuals were done for each ploidy level with populations as random factor and individuals as random factor nested within populations. The range of the DNA content ('intra-ploidy variation') was calculated as the highest/lowest DNA content ratio found among individuals. Relationship between DNA content and environmental characteristics was tested using one-way ANOVA in the case of categorical variables or by Spearman´s correlation coefficient in the case of quantitative and ordinal variables. Separate tests were done for each cytotype. The spatial relation of the genome size (1Cx) of cytotypes with more analysed populations ($4x$-$6x$) was tested with Procrustes analysis using PROTEST software (Peres-Neto and Jackson, 2000) with significance test from Jackson (1995) based on 9999 permutations. Data settings followed approach used by Šmarda and Bureš (2006). Significant results of the test indicate that there is a geographical relationship of the genome size of cytotypes under comparison.

Results

DNA ploidy levels and chromosome numbers

In 155 previously published localities that were karyologically examined (see Supplementary data, available on-line), the most common tetraploid cytotype ($2n = 32$) was reported at 80 localities (52 %), followed by pentaploids ($2n = 40$; 59 localities; 38 %), hexaploids ($2n = 48$; 5 localities; 3 %) and triploids ($2n = 24$; 2 localities; 1 %). Majority of samples (95 %) consisted of single ploidy level, mixed populations consisting of $4x+5x$ cytotypes were detected on 8 sites, and those consisting of $5x+6x$ cytotypes were detected on 1 site.

Six different DNA ploidy levels ($2n = 3x$–$8x$) were found through the FCM analysis of 2454 individuals from 304 European populations collected through the years 2004-2011 (data-set 1) or obtained via 'Index seminum'. Two previously unknown DNA-ploidy levels – heptaploid and octoploid – were identified. Heptaploids were found as a sole cytotype in four populations and single DNA heptaploid plants were identified in one otherwise tetraploid and one pentaploid population, respectively. Octoploids were found in just one population. DNA ploidy levels inferred from FCM data were confirmed using chromosome counting (Figure 1). No aneuploid counts were found. Of the populations analysed by us, triploids occurred in 3.4 %, tetraploids in 26.5 %, pentaploids in 70.4 %, hexaploids in 12.6 %, heptaploids in 2.0 % and octoploids in 0.3 %.

There was marked diversity of cytotype composition in populations (Table 1). In 277 European populations sampled by us, majority of them comprised single cytotype, much less populations contained two and populations with three cytotypes were extremely rare. Among the populations consisting of a single ploidy level, pentaploids were found to be the most common (67%), followed by tetraploids (18%), hexaploids (9%), triploids (4%), heptaploids (2%) and octoploids (<1%).

More individuals were analysed from populations (data-set 2) revealed as cytotype-mixed (median = 9.5) than cytotype-uniform (median = 6) by FCM ($\chi^2 = 7.9$, $P = 0.005$). Also area of mixed populations (median = 250 m^2) were larger than that of uniform populations (median = 100 m^2; $\chi^2 = 8.83$, $P = 0.003$). Proportion of mixed populations were cytotype-dependent ($\chi^2 = 17.2$, $P < 0.001$). While populations containing triploids or pentaploids were almost cytotype-uniform (only 10 % or 21 % of their populations co-occured with other cytotype, respectively), those containing either tetra- or hexa- or heptaploids were more frequently mixed (43 % or 43 % or 33 %, respectively). Among populations consisting of two cytotypes (Table 1), tetra-, penta- and hexaploids occurred in all possible combinations and $4x+5x$ or $5x+6x$ combinations were found most often. On the other hand, triploids were found just once in mixture with pentaploids, and heptaploids once in mixture with tetra- and once with pentaploids. Populations consisting of three cytotypes included combination $4x+5x+6x$ only, that was detected in three cases. Any other mixed populations containing three or more cytotypes were not found. The detailed statistics are shown in Table 1.

Despite overall dominance of pentaploids in all our data-sets, proportion of mixed populations were higher in the Czech than in European (ex Czech) dataset ($\chi^2 = 4.94$, $P = 0.026$) and data-sets also showed strong differences in population's cytotype composition (Table 1). In the Czech populations, hexaploids dominated over tetraploids but the opposite was true for the rest of Europe. Both datasets also differed in the structure of cytotype mixtures: $5x+6x$ populations dominated within Czech cytotype-mixed sites whereas $4x+5x$ populations dominated within those in the rest of Europe.

Table 1. Cytotype composition of 602 *Allium oleraceum* populations sampled by us. Only populations with two or more analysed specimens were included into analysis. Data are separately presented for European populations (except for the Czech Republic; 'Europe ex CZ'; data-set 2), Czech populations ('CZ'; data-set 2), Czech populations (Duchoslav *et al.*, 2010; 'CZ') and collectively for European populations sampled by us ('Europe'). Populations were grouped and relative frequencies calculated according to the number of cytotypes per population (one, two, or three). Populations were further subdivided according to specific cytotypes present. Frequencies are shown in percentages (and absolute numbers).

|	Number of cytotypes per population			Populations containing one cytotype						Populations containing two cytotypes*						Populations containig three cytotypes*	Number of analysed plants	
	1	2	3	3x	4x	5x	6x	7x	8x	3x+5x	4x+5x	4x+6x	4x+7x	5x+6x	5x+7x	4x+5x+6x	(mean±s.d.)	n
Europe ex CZ	84(234)	14(40)	1(3)	4(9)	18(42)	67(157)	9(21)	2(4)	0(1)	3(1)	60(24)	5(2)	3(1)	28(11)	3(1)	100(3)	8.1±7.1	277
CZ	77(250)	22(71)	1(4)	0(0)	14(35)	57(142)	29(73)	0(0)	0(0)	0(0)	21(15)	8(6)	0(0)	70(50)	0(0)	100(4)	14.9±9.9	325
Europe	80(484)	18(111)	1(7)	2(9)	16(77)	62(299)	19(94)	1(4)	0(1)	1(1)	35(39)	7(8)	1(1)	55(61)	1(1)	100(7)	11.7±9.3	602

*Any other combinations of two/three/four cytotypes were not found

Cytotype distribution and ecology

The geographic distribution of cytotypes (including all previously published data) in Europe is shown in Fig. 1. Cytotype diversity was larger in the southern latitudes below 50° than in the northern latitudes where only tetra- and pentaploids were found. Triploids were spatially restricted to the south-eastern part of the Central Europe and the (south) eastern Europe. Due to scattered sampling in the eastern Europe, it is difficult to unambiguously comments on its contact zones with other cytotypes. There was, however, remarkable contact zone consisting of tri- and pentaploid populations (incl. one mixed $3x+5x$ population) but devoid of other cytotypes in Hungary. Moreover, one triploid population was found in contact zone with tetra-, penta- and hexaploids in the southern Slovakia (for details see Šafářová *et al.*, 2011). Both tetra- and pentaploids were widespread and mutually almost sympatric over area studied. In the zone of sympatry, mixed $4x+5x$ populations occured more or less frequently. There was, however, a zone devoid of tetraploids and dominated by pentaploids in the western part of Hungary continuing southwards to Croatia and Serbia (see also above). For hexaploids, the centre of distribution seems to be in Central Europe, but scattered populations occured in contacts with $4x$, $5x$ and $7x$ populations also in the (south-)western Europe. A striking boundary of hexaploid distribution run along the outer/inner ranges of the Western Carpathians and continues along the outer part of the Eastern Alps to the southern Austria and the northeastern Italy. Considering mixed populations consisting of hexaploids, only $5x+6x$ mixed populations were found frequently and occured over most range of hexaploids while $4x+6x$ populations were extremely rare and with one exception occured just in the Central Europe. Mixed $4x+5x+6x$ populations were found only in the Central Europe. Heptaploids (incl. uniform and mixed populations $4x+7x$ and $5x+7x$) were found only in the eastern and southern France; and just one octoploid population was found in Spain.

Comparisons of associations between cytotypes and selected environmental factors in two dataset, i.e. without (data-set 1) and with incorporation of samples from the Czech Republic (data-sets 3ab), suggest similar trends despite more significant results observed in the data-set 3. This was due to larger sample size in the later data-set. Cytotypes ($3x$-$7x$) did show weakly different associations with habitats, i.e. their requirements rather overlaped (Table 2, Fig. 2). This is especially true for tetra- and pentaploids that inhabit wide but very similar spectrum of habitats. On the other hand, increased frequency of hexaploids on habitat type 'arable field & field margins' was apparent in both dataset, even more outside the Czech Republic. Both tri- and heptaploids showed lower habitat variation but it could be partially caused by lower sample sizes. Cytotypes showed increasing occurrences in the proximity of arable land in the direction $3x \rightarrow 6x$ but this was significant only after incorporating data from the Czech Republic. Consistently in both data-sets, $3x$-$6x$ cytotypes showed increasing occurrences in human-impacted habitats, with triploids occuring predominantly in natural habitat types, tetra-, penta- and heptaploids equally in both natural and human-impacted habitats and hexaploids were more frequent in human-impacted habitats like field margins, road ditches, ruderalised sparse forests, disturbed grasslands, etc. (Fig. 2; Table 2). Sites of different cytotypes did not differ in light conditions and heat index (Table 2).
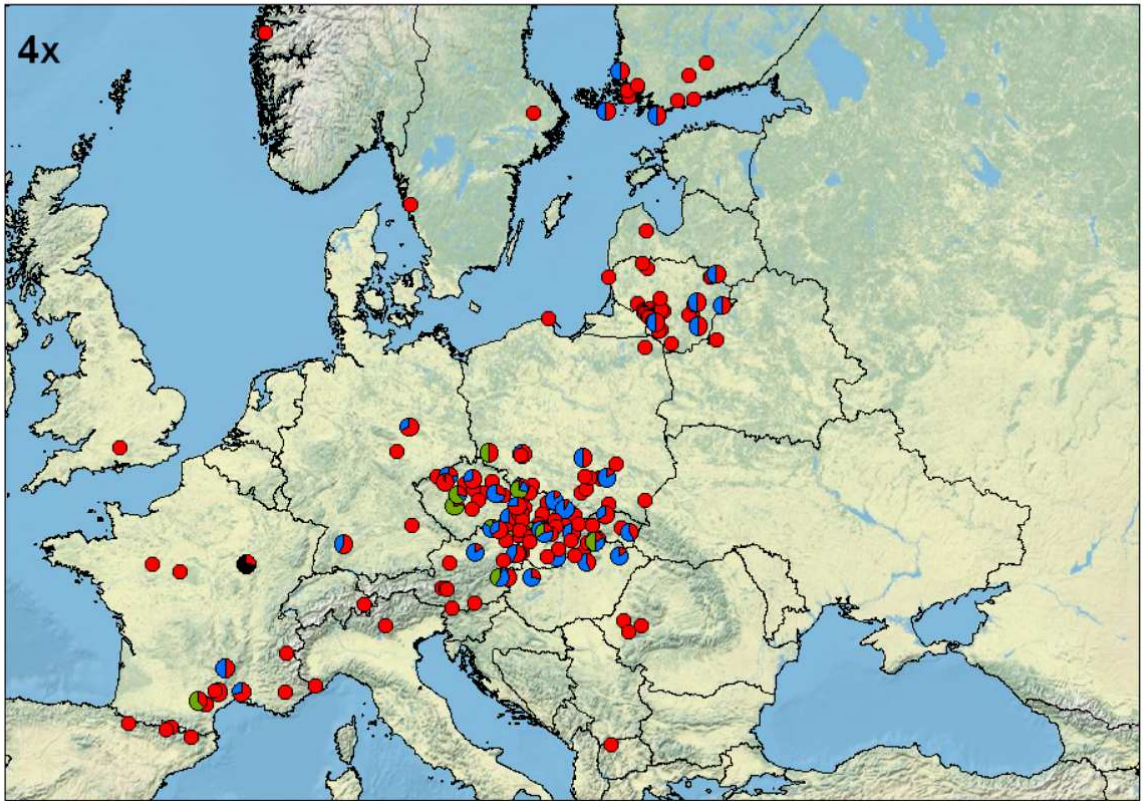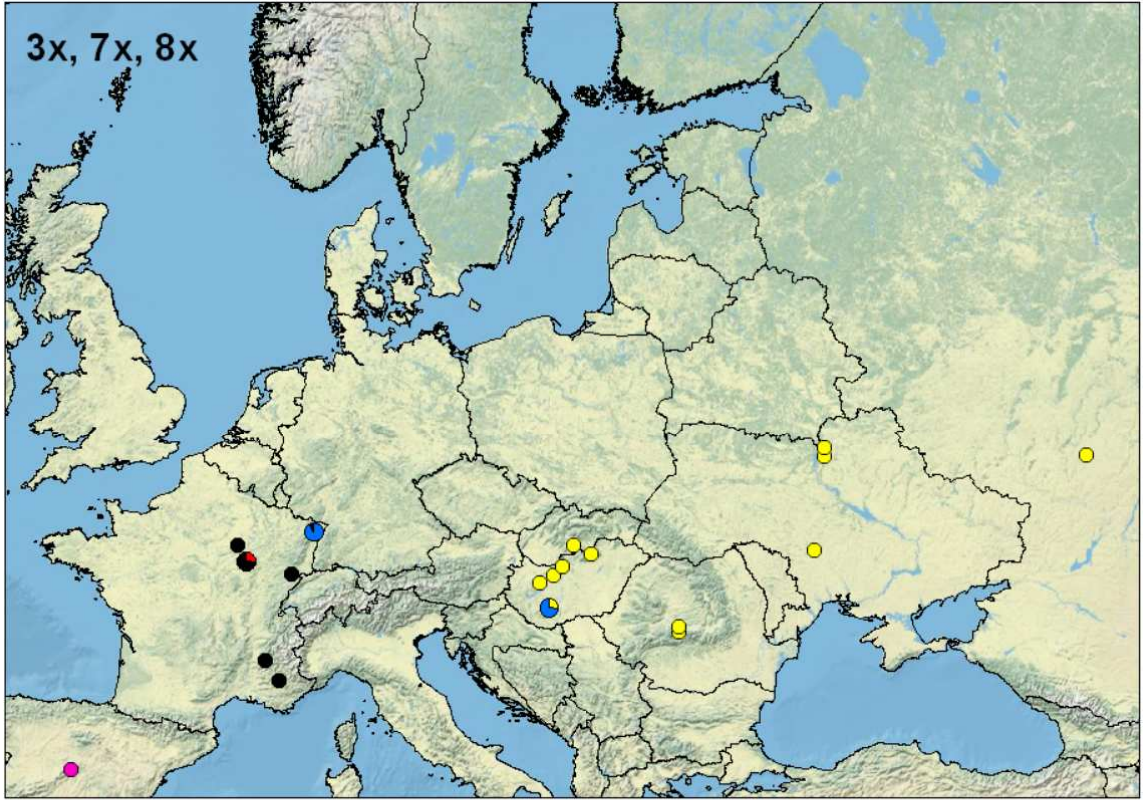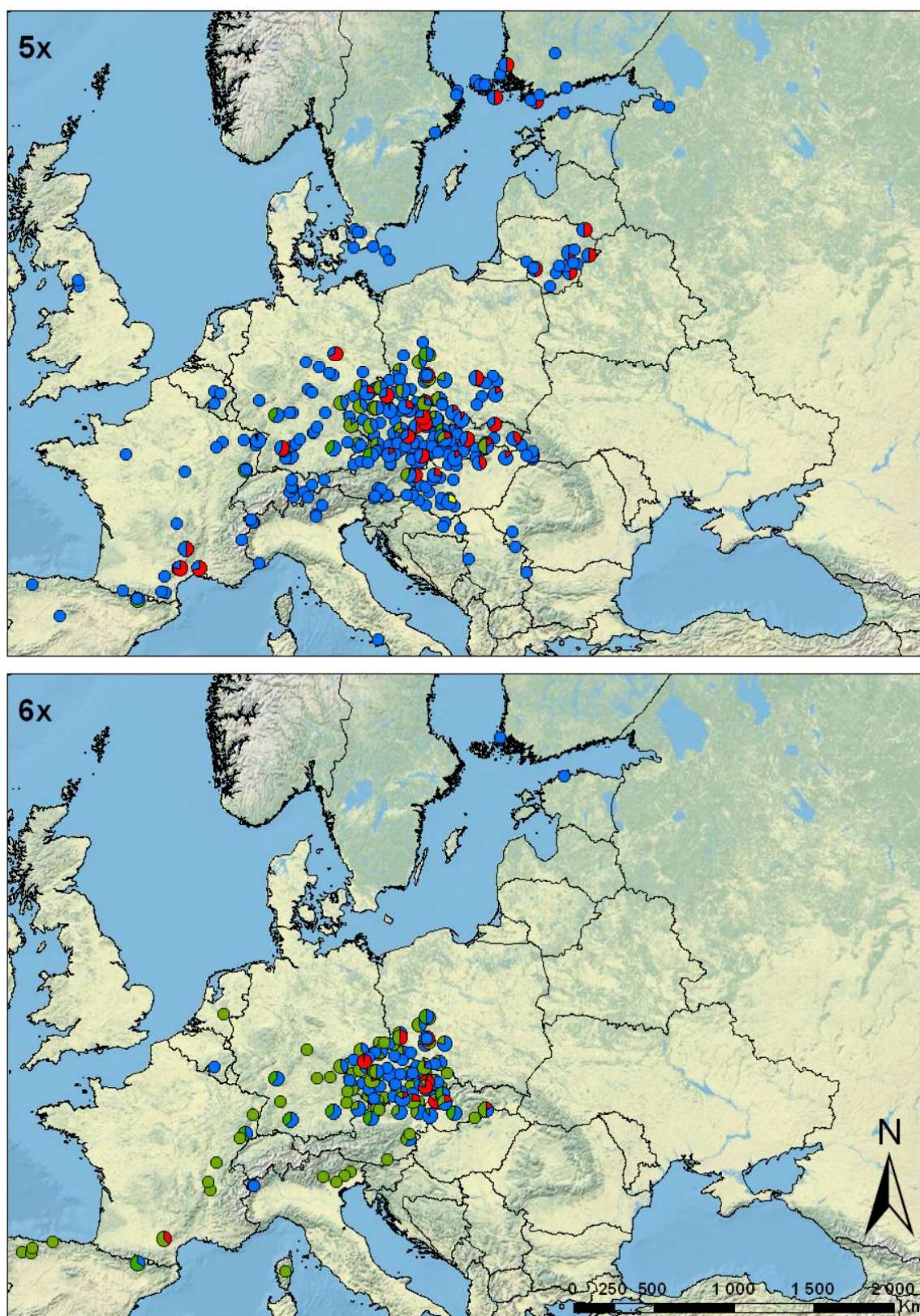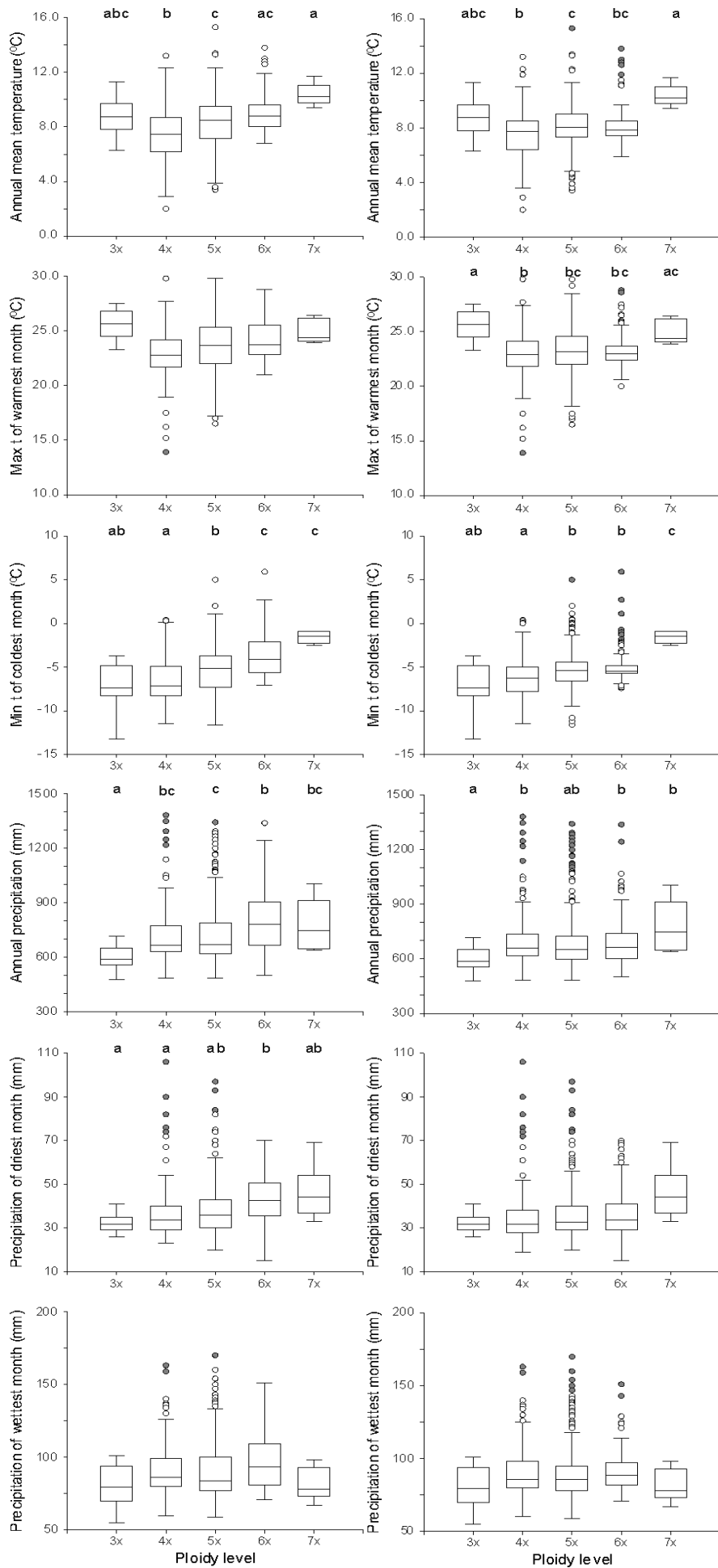
Fig. 1. Distribution of ploidy levels of *Allium oleraceum* in Europe (based on all available data; see Supplementary Information X-XX). Different ploidy levels are distinguished by different colours: 3x – yellow, 4x – red, 5x – blue, 6x – green, 7x – black, 8x – pink.
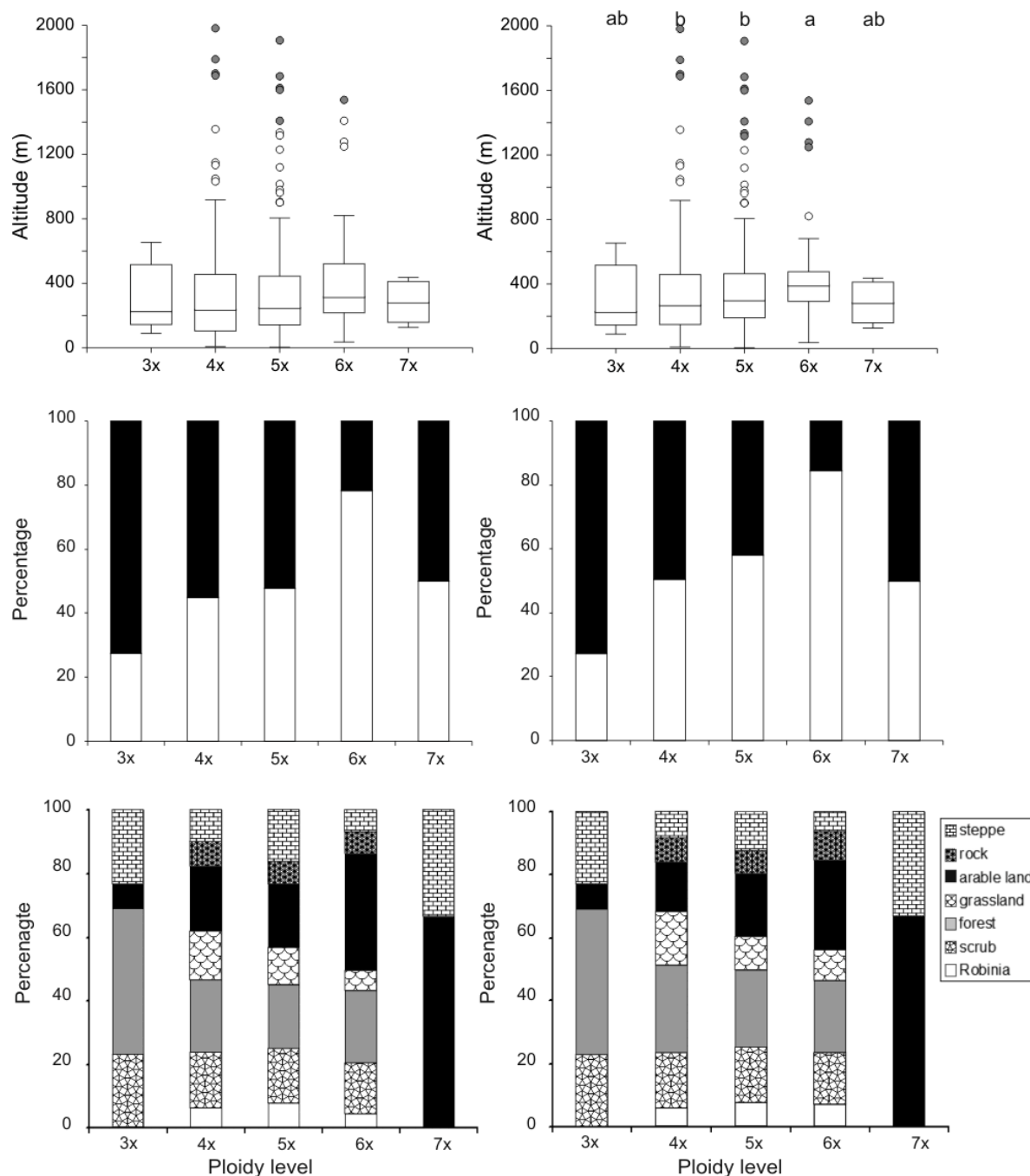
Fig. 2. Variation of selected climatic and ecological variables for ploidy levels of *Allium oleraceum* for different data-sets: on the left - European populations without samples from the Czech Republics (data-set 1) and on the right - European populations including samples from the Czech Republics (data-set 3a or 3b). Significant differences in medians between ploidy levels (Dunn's test; P < 0.05) are marked by different letters in rows above the box plots. One outlier value of annual precipitation for the population 06/98 (2351 mm) is not shown in the figure. Frequency distribution of ploidy levels in the natural (black) and human-influenced (white) type of the habitat types is shown in the bar chart. Octoploid population were excluded from the analyses.

Table 2. Summary of the associations between ploidy levels of *Allium oleraceum* and selected environmental and climatic variables on the area of Europe. Differences in environmental characteristics were tested for different data-sets: European populations without samples from the Czech Republics (Europe ex CZ 1 = data-set 1; Europe ex CZ 2 = data-set 3a without data from the Czech Republic) and European populations including samples from the Czech Republic (Europe 1 = data-set 3b; Europe 2 = data-set 3a).

Variable	Test*	D.F.	Test statistics	P	Variable	Test*	D.F.	Test statistics	P
Europe ex CZ					**Europe**				
Habitat type	CT	24	31.24	0.147	Habitat type	CT	24	**37.96**	**0.035**
Presence of arable land	CT	4	5.74	0.220	Presence of arable land	CT	4	**38.28**	**<0.001**
Habitat naturalness	CT	4	**15.40**	**0.004**	Habitat naturalness	CT	4	**42.11**	**<0.001**
Light conditions	K-W	4	5.28	0.260	Light conditions	K-W	4	5.45	0.244
Heat index	K-W	4	8.23	0.083	Heat index	K-W	4	8.82	0.066
Europe ex CZ and Literature					**Europe and Literature**				
Altitude	K-W	4	5.99	0.200	Altitude	K-W	4	**21.45**	**<0.001**
Annual mean temperature	ANOV	4	**9.30**	**<0.001**	Annual mean temperature	K-W	4	**28.97**	**<0.001**
Max *t* of warmest month	K-W	4	**25.94**	**<0.001**	Max *t* of warmest month	K-W	4	**27.84**	**<0.001**
Min *t* of coldest month	K-W	4	**50.49**	**<0.001**	Min *t* of coldest month	K-W	4	**48.39**	**<0.001**
Annual precipitation	K-W	4	**23.18**	**<0.001**	Annual precipitation	K-W	4	**13.10**	**0.011**
Precipitation of wettest month	K-W	4	**10.97**	**0.027**	Precipitation of wettest month	K-W	4	**13.39**	**0.010**
Precipitation of driest month	K-W	4	**22.08**	**<0.001**	Precipitation of driest month	K-W	4	8.12	0.087

Despite similar trends in both data-sets, significant differences in the median altitudinal distribution among cytotypes were observed in combined one. Tetra- and pentaploids showed the widest altitudinal range from the lowlands to alpine belt. On the other hand, triploids and heptaploids were found only in altitudes below ca 700 and 400 m, respetively. Generally, cytotypes showed different associations with climate, through minor differences were visible when comparing both data-sets (Table 2, Fig. 2). Triploids were found in regions with continental climate, i.e. with low precipitations and high summer and low winter temperatures. On the other hand, hexa- and heptaploids were largely found in regions with subatlantic climate, i.e. with mild winter temperatures and higher precipitations. Both tetra- and pentaploids were found in wide ranges of climatic conditions.

Though cytotypes under consideration have overlapping ecological niches, multivariate db-RDA on both data-sets showed that cytotypes are ecologically differentiated with the largest realised niche differences being observed between hexa- & heptaploids and other cytotypes (Monte Carlo permutation test of all canonical axes, *P* < 0.002 in all cases; Fig. 3). In summary: (i) Triploids almost avoided human-impacted (ruderal) vegetation and occured in areas with continental climate. (ii) Tetraploids occurred primarily in both shaded (forests) and open (semi)natural habitats. (iii) Pentaploids showed wide ecological amplitude without clear differentiation from other cytotypes through were frequently found in dry grasslands. (iii) Hexa- and partly also heptaploids inhabited usually open human-influenced habitats such as field margins and road ditches but also eutrophicated sparse forests with ruderal species and ruderalized woody vegetation outside forest in regions with mild winters.
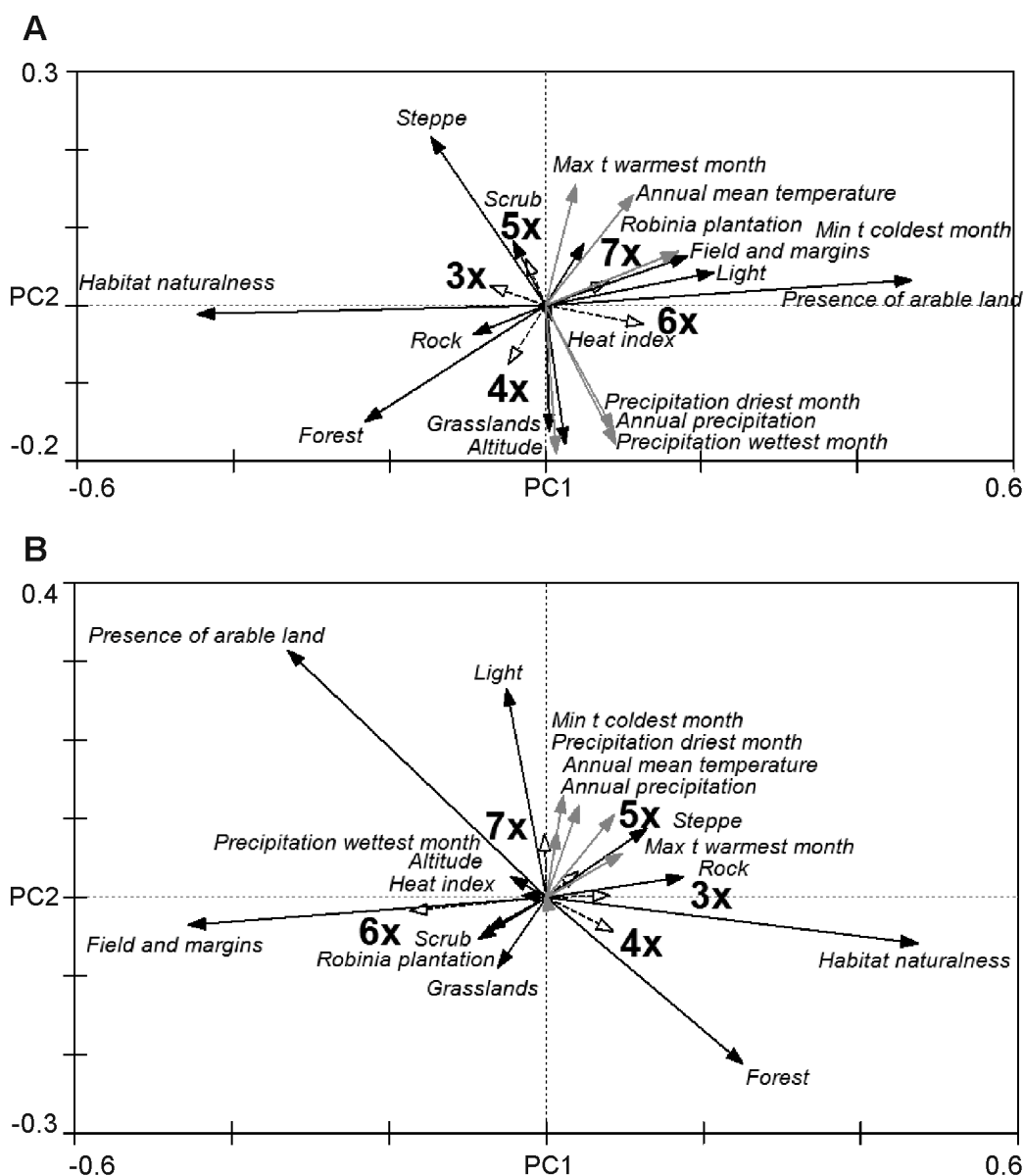
Fig 3. The first and the second axis of the constrained principal coordinate analysis of (A) European populations sampled by us (data set-1) except for samples from the Czech Republic and (B) all European populations sampled by us incorporating also Czech samples (datas set-3b). Principal coordinates axes was used as a species data, ploidy level (fuzzy coding) was used as an explanatory variable (dashed arrows). Vectors of the environmental (black arrows) and climatic variables (grey arrows) were used as supplementary data to help interpret the ordination.

Genome size variation

Significant effects of cytotype (F = 569.4, P < 0.001), population (F = 27.8, P < 0.001) but no significant effect of individual plant (= intrapopulation variation; F = 1.1, P = 0.166) on 2C DNA content were found by mixed model ANOVA. As experted, mean 2C DNA content significantly differed among any two cytotypes. Ratios of 2C DNA between tri- and tetra-, penta-, hexa-, hepta- and octoploids averaged 1.23 : 1.48 : 1.69, 2.00 and 2.17, respectively (Table 3, Figure 4a). Accordingly, the monoploid DNA amount (1Cx) was significantly dependent on ploidy level (ANOVA for 3x-7x cytotypes; F = 50.1, P < 0.001) and genome downsizing between two successive cytotypes was 7.8, 3.4, 5.0 and 4.9 % in 3x/4x, 4x/5x, 5x/6x and 7x/8x cytotypes, respectively. Only exception were heptaploids, who did not differ in 1Cx from hexaploids (Fig. 4b).

The intra-ploidy variation in genome size was relatively large in tetraploids (18.3 %) and pentaploids (13.2 %) and no 2C DNA outliers were observed. Divergence in estimated nuclear DNA content between populations was confirmed by simultaneous FCM analyses (Fig. 5). Smaller intra-ploidy variation in genome size was discovered in other cytotypes (≤ 7.5 %) but even decreased after elimination of populations with extreme DNA content, e.g. in hexaploids and triploids. Within tri-, tetra- and pentaploid cytotypes, majority (≥ 75 %) of total variance in genome size was attributable to interpopulation variation. On the other hand, interpopulation variation was apparently less pronounced in hexa- (45.8 %) and heptaploids (52.4 %) than in other cytotypes (Table 3).

Table 3. Variation in DNA content for ploidy levels of *Allium oleraceum* (2C DNA; pg) using standard *Triticum aestivum* cv. *Saxana* (2C = 34.2388 pg) as a unit value.

DNA ploidy level	Number of populations/ number of individuals analysed	2C DNA (pg;mean±s.d.)[$]	2C DNA range (min/max)	1Cx DNA (pg;mean)	Intra-ploidy variation (%)	Proportion of total variance in genome size attributable to interpopulation variation (%)
3x	8 / 20	42.49±0.92[a]	40.52 / 43.55	14.80	7.5	74.9*
4x	24 / 77	52.34±2.53[b]	47.96 / 56.74	13.10	18.3	92.5***
5x	50 / 219	63.04±1.83[c]	58.73 / 66.50	12.64	13.2	74.8***
6x	24 / 95	71.92±0.99[d]	69.10 / 73.66	12.00	6.6	45.8*
7x	5/10	85.36±1.94[e]	82.98 / 88.66	12.19	6.8	52.4*
8x	1/13	92.18±1.43[f]	90.14 / 94.56	11.40	4.9	-

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

$ Significant differences in mean 2C DNA between ploidy levels (Bonferroni test; $P < 0.05$) are marked by different letter in columnwise
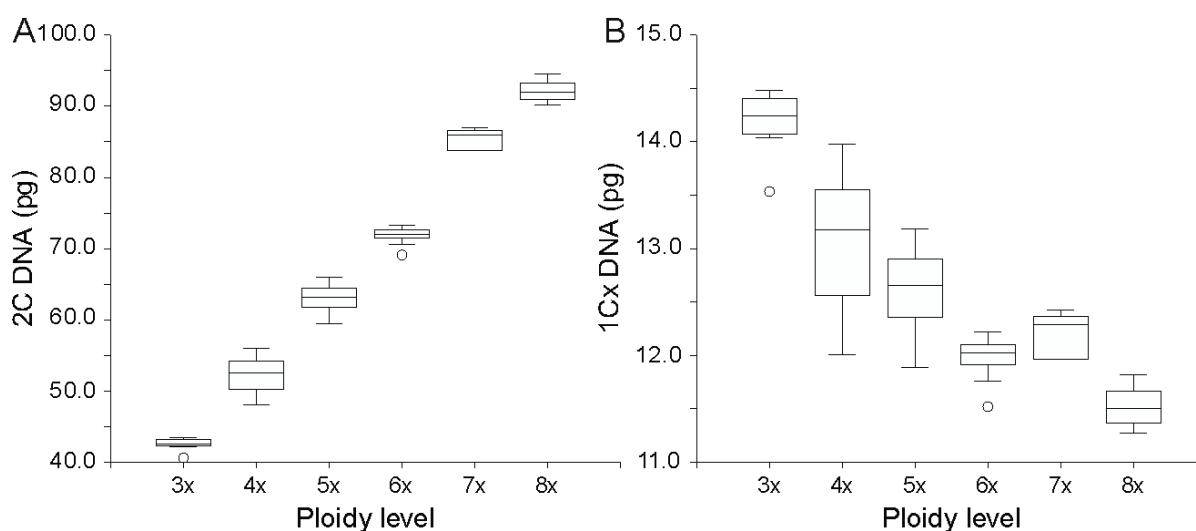

Fig. 4. Box plot of 2C DNA (pg) and 1Cx DNA (pg) of DNA triploids, tetraploids, pentaploids, hexaploids, heptaploids and octoploids of *A. oleraceum*.
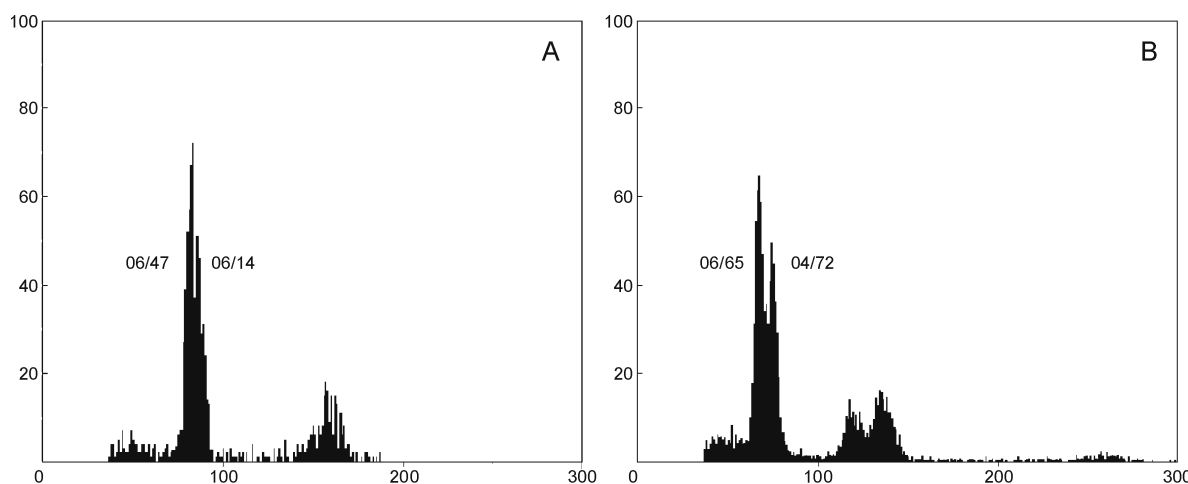
Fig. 5. Flow cytometric histograms documenting variation in genome size. A. Simultaneous analysis of pentaploid populations 06/47 from Italy (2C = 61.84 pg) and 06/14 from Hungary (2C = 65.71 pg). B. Simultaneous analysis of tetraploid populations 06/65 from France (2C = 49.17 pg) and 04/72 from Poland (2C = 55.55 pg).

Ecogeographic pattern of genome size variation

The relationships between geographic location, ecological, climatic characteristic and 2C DNA content of the populations were analysed (Table 4). In triploids, DNA content did not show any clear spatial pattern though the western populations (Slovakia, Hungary) had markedly homogeneous 2C DNA content. Positive correlations between altitude, precipitation and 2C DNA content are probably statistical artefacts with confouding altitude and geography. In tetraploids, 2C DNA content was correlated significantly with latitude and longitude, with the tendency of plants with larger genomes to occur in the north-eastern part of the studied range. DNA content of pentaploids was also correlated with longitude and latitude, increasing from (south)-west to the (north)-east part of the studied range. Geographical gradients observed in DNA content of tetra- and pentaploids matches well with the gradient of continentality, with plants with larger genomes occurring in more continental climate, On the contrary, 2C DNA content in hexaploids was not correlated with any geographical and climatic variables (Fig. 6). However, when two outlier values with apparently lower 2C DNA content were excluded from the analyses, 2C DNA content was correlated significantly with latitude and negativelly with longitude. Also correlation with minimal temperature of the coldest month was opposite to that found in tetra- and pentaploids, suggesting tendency to increase 2C DNA content in hexaploids in more subatlantic climate. Heptaploids showed bimodal distribution of 2C DNA content with populations with smaller genome in the northern France while those with larger genome both in the northern and southern France. Hence, significant correlations between several climatic variables and 2C DNA content are results of low sample size.

Concerning local-scale environmetal variables, only DNA content of pentaploids was related to the level of habitat naturalness and habitat type. Plants with lower DNA content occurred in natural habitats whereas those with higher DNA content occurred in human-impacted habitats.

A strong spatial coincidence of the monoploid genome size of tetra- and pentaploids, and penta- and hexaploids was revealed by Procrustean analysis (both P < 0.001). On the other hand, no spatial coincidence of the DNA content were found between tetra- and hexaploids (P = 0.242). Except for triploids limited to the eastern Europe only, monoploid genome size of cytotypes was similar in the (south)western Europe, but diverged towards the (north)east (Fig. 6, 7).

Table 4. Relationships between 2C DNA content in tri-, tetra-, penta-, hexa- and heptaploids of *Allium oleraceum* and geographical, climatic and ecological characteristics. r$_s$ Spearman´s rho, ANOVA on-way analysis of variance, KW Kruskall-Wallis test. In hexaploids, n = 24: entire data were used, n = 22: two outlier values with extremely low genome size were excluded (populations 08/4 and 11/107).

	Latitude °NL	Longitude °EL	Altitude (m)	Heat index	Annual mean t (°C)	Max t of warmest month (°C)	Min t of coldest month (°C)	Annual precipitation (mm)	Prec. of wettest month (mm)	Prec. of driest month (mm)	Habitat	Absence of arable land	Habitat naturalness
3x; n = 8 r$_s$											ANOVA		KW
test statistic	-0.48	-0.33	**0.95**	0.18	0.12	-0.38	-0.05	**0.79**	0.68	0.17	0.25	-*	0.44
P	0.233	0.420	**<0.001**	0.674	0.779	0.349	0.911	**0.021**	0.062	0.690	0.788	-*	0.505
4x; n = 24 r$_s$											ANOVA		
test statistic	**0.77**	**0.59**	-0.40	-0.08	-0.12	-0.04	-0.27	**-0.42**	-0.31	**-0.53**	0.89	0.49	3.17
P	**<0.001**	**0.003**	0.061	0.720	0.576	0.828	0.216	**0.049**	0.153	**0.010**	0.518	0.493	0.089
5x; n = 50 r$_s$											ANOVA		
test statistic	**0.37**	**0.74**	**-0.35**	-0.03	-0.04	**0.35**	**-0.62**	**-0.60**	-0.19	**-0.67**	**3.45**	3.17	**7.12**
P	**0.009**	**<0.001**	**0.013**	0.823	0.804	**0.014**	**<0.001**	**<0.001**	0.189	**<0.001**	**0.006**	0.081	**0.010**
6x; n = 24 r$_s$											ANOVA	KW	
test statistic	0.34	-0.11	0.167	-0.40	-0.10	-0.31	0.20	0.12	-0.06	0.22	1.12	0.16	1.52
P	0.103	0.616	0.437	0.056	0.639	0.140	0.354	0.582	0.768	0.296	0.388	0.695	0.230
6x; n = 22 r$_s$											ANOVA		
test statistic	0.14	**-0.44**	0.30	-0.35	-0.10	**-0.44**	**0.44**	0.26	-0.04	0.36	1.23	0.34	1.05
P	0.523	**0.041**	0.171	0.108	0.669	**0.040**	**0.043**	0.234	0.859	0.098	0.339	0.569	0.318
7x; n = 5 r$_s$											ANOVA		
test statistic	-0.700	0.200	0.700	-0.707	**0.900**	**1.000**	0.410	-0.300	0.100	-0.800	1.720	1.570	0.230
P	0.188	0.747	0.188	0.182	**0.037**	**<0.001**	0.493	0.624	0.873	0.104	0.281	0.299	0.667

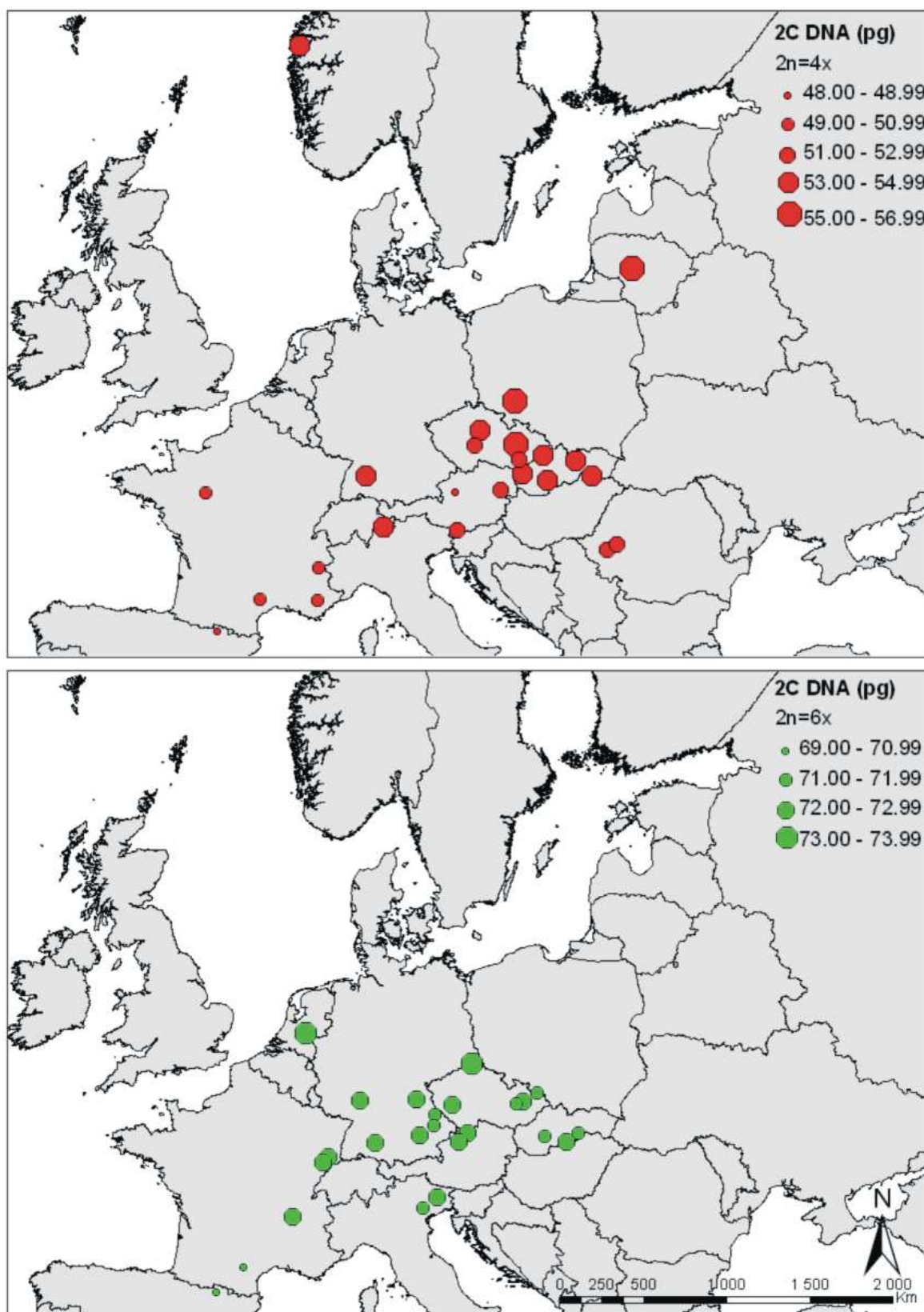* Populations occurred in natural habitats only.
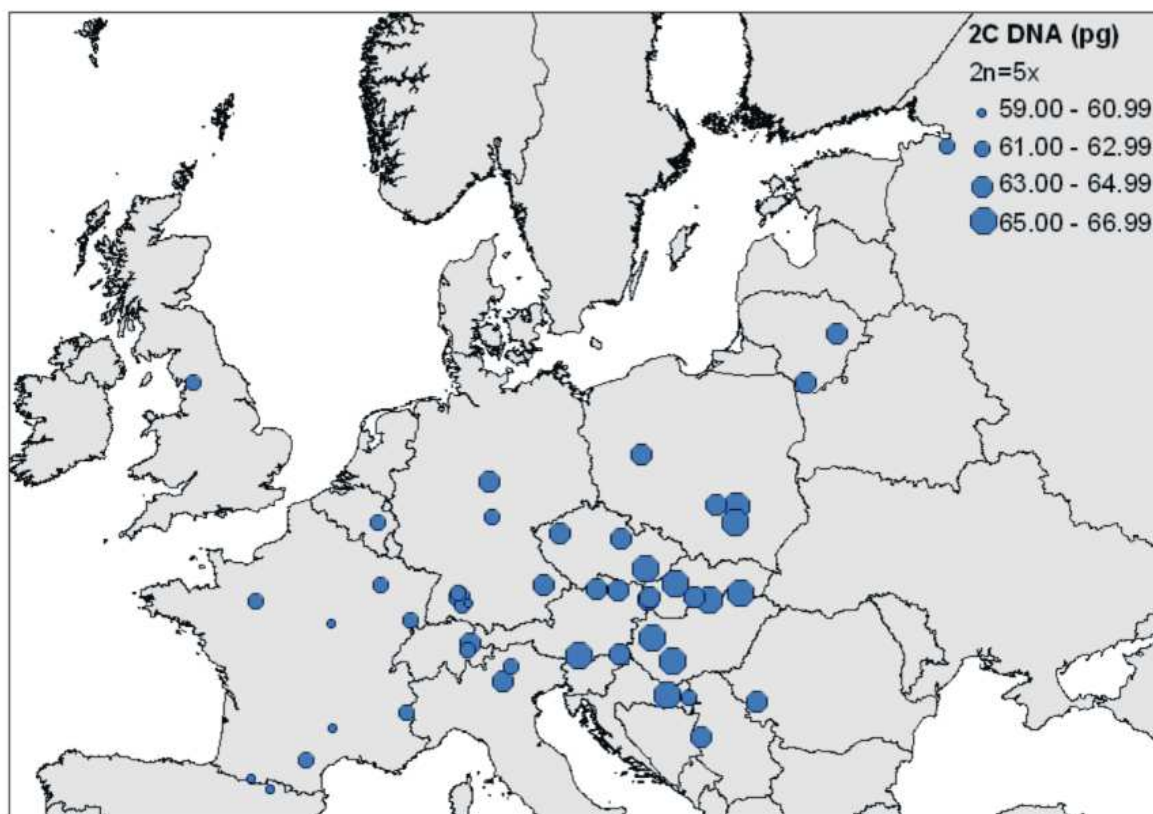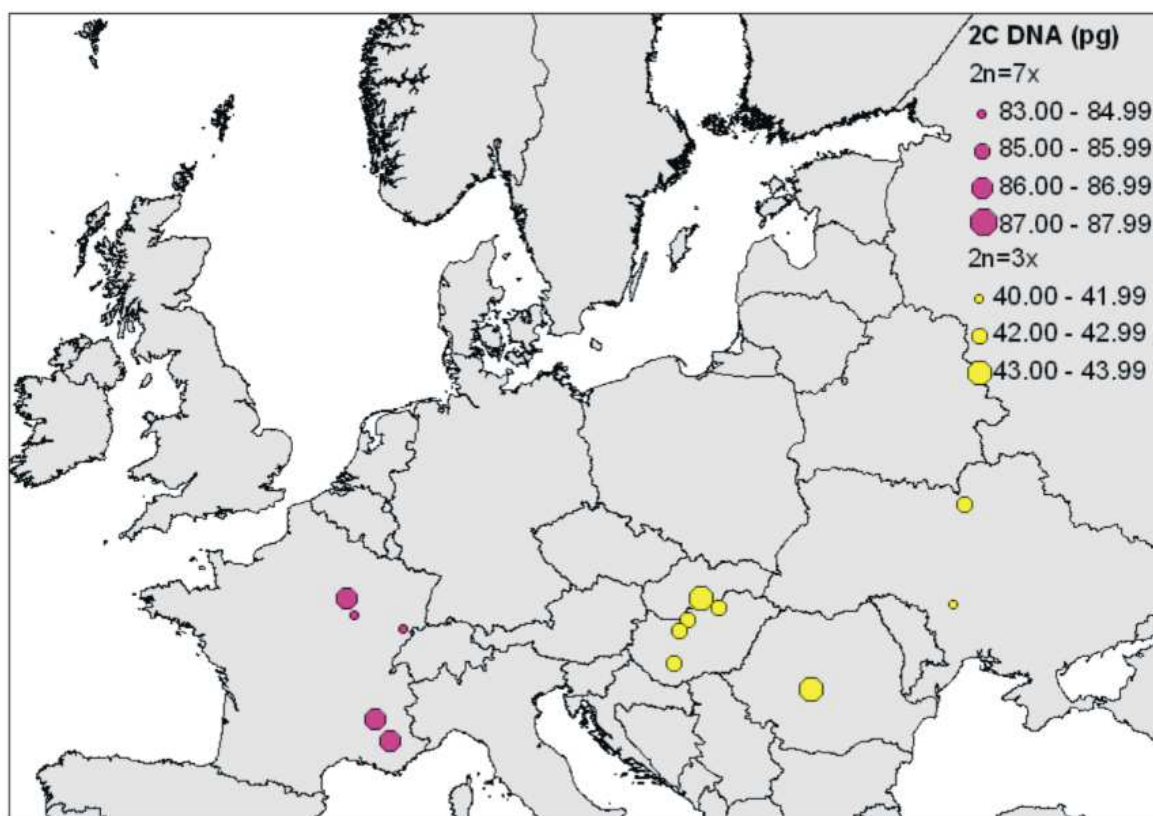
Fig. 6. Observed landscape pattern of 2C DNA (pg) content of selected populations of *Allium oleraceum*. The size of the symbol reflects the genome size. Note non-equidistant intervals of 2C DNA in some cases due to missing values.
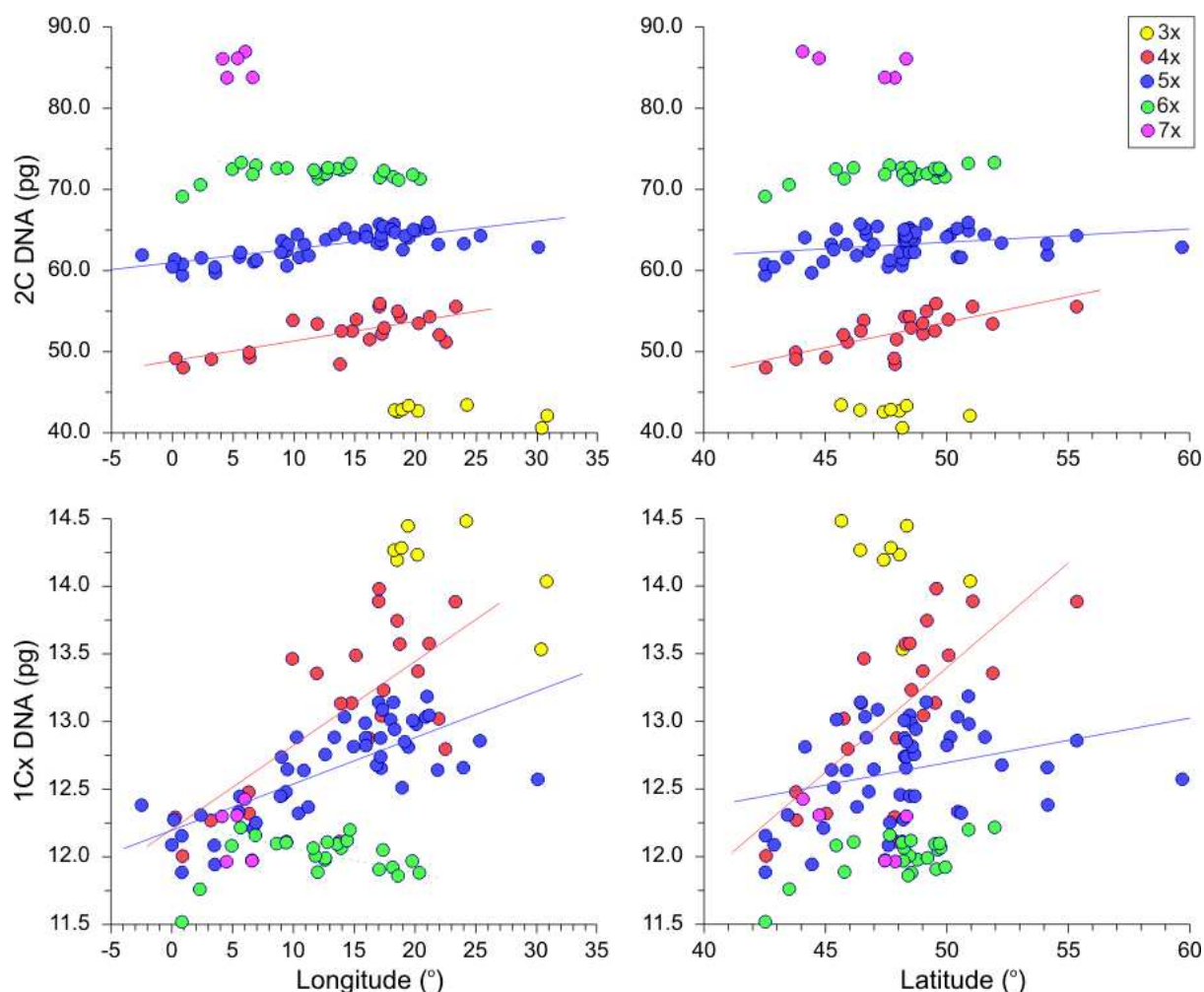
Fig. 7. Relationships between geographic variables (latitude, longitude) and genome size (2C, 1Cx) in ploidy levels of *Allium oleraceum*. Genome size was regressed on geographic variables and straight lines were fitted for each cytotype separately in the case of significant correlations (P ≤ 0.05; see Table 4). In hexaploids, dotted line was fitted on data after excluding two outlier values with extremely low genome size.

Discussion

Cytotype diversity

Chromosome numbers and DNA ploidy data were obtained for *A. oleraceum* plants from substantial parts of distributional area and together with previously published data listed also here we provided a fairly good overwiew of cytotype's distribution in Europe. However, only limited material from (south)eastern Europe was available. Therefore, more collections from these regions are required for unbiased insight into ploidy level variation across the whole of Europe.

In accordance with previous chromosome number records and DNA ploidy levels measurements, we here confirmed the presence of four cytotypes (3x–6x) within *A. oleraceum*. Triploids are the rarest ploidy level being confirmed from Hungary and Ukraine and newly recorded in Romania and Slovakia (see also Šafářová *et al*., 2011). Our data on tetra- and pentaploid distribution corresponds well with published karyological counts, fills gaps and extends their distributional range to the eastern Europe. Surprisingly, tetraploids were found less frequently by us than published karyological data implied. On the other hand, pentaploids are the most widespread ploidy level and occur throughout most of species range. Rare hexaploids were revealed by us for the first time for several western and central European countries, thus filling gap between previous reports from

Spain (Pastor, 1982; Pastor and Valdes, 1983) and Austria (Dobeš and Vitek, 2000), Czech Republic (Duchoslav *et al.*, 2010) and Slovakia (Šafářová *et al.*, 2011). Moreover, we found two unusual and previously unknown cytotypes corresponding to hepta- and octoploid levels that are the first such high counts for the section *Codonoprasum* and even represent extremely rare ploidy levels for whole genus *Allium* (Hanelt *et al.*, 1992; Goldblatt and Johnson, 2008). Such substantial intraspecific ploidy variation (six ploidy levels) is only rarely seen in other sexually reproducing plants, e.g. in *Cardamine yezoensis* (Marhold *et al.*, 2010) and *Ixeris nakazonei* with six cytotypes (Denda and Yokota, 2004), and *Senecio carniolicus* with eight cytotypes (Sonnleitner *et al.*, 2010).

Ecological differentiation

In *Allium oleraceum*, previous studies (Duchoslav *et al.*, 2010; Šafářová and Duchoslav, 2010, Šafářová *et al.*, 2011) revealed considerable variation in niche breath and optimum of tetra-, penta- and hexaploid cytotypes at both regional and local scales in the Czech and Slovak Republics. Accordingly, main differentiation among the cytotypes was related to the level of anthropic pressure and soil conditions with the largest difference being observed between tetraploids and hexaploids. When we expanded our analyses on the scale of Europe, we obtained somewhat similar results to those on more regional scales: tetra- and pentaploids showed greater breath of ecological niches than was observed in other cytotypes, both lower (triploids) and higher (hexa- and heptaploids). However, our results also corroborate previous findings that cytotypes did not have strong ecological boundaries and their niches overlap, resulting in frequent local sympatry (Šafářová and Duchoslav, 2010). There is presently consensus that ecological divergence between cytotypes is rather common (Otto and Whitton, 2000; Levin, 2002; Leitch and Leitch, 2008) but polyploids do not consistently tolerate harsher environmental conditions and do not always show a broader ecological niche than their diploid progenitors (Levin, 2002; Schönswetter *et al.*, 2007; Rivero-Guerra, 2008; Parisod *et al.*, 2010). Moreover, only limited number of studies compared ecology of higher cytotypes in polyploid series and results are also inconsistent (e.g. Brochmann and Elven, 1992; Sonnleitner *et al.*, 2010). One of the main reasons explaining such puzzling pattern is linked to the type of polyploidy when autopolyploidy, in contrast to allopolyploidy, does not inevitably produce transgressive traits to fuel adaptive ecological divergence (Hegarty and Hiscock, 2008; Parisod *et al.*, 2010), and the second is the age of established polyploids with evolutionary-older cytotypes combining both the effects of polyploidy per se and subsequent genic evolution (Ramsey and Schemske, 1998, 2002; but see Ramsey, 2011). When comparing observed ecological amplitude of *A. oleraceum* cytotypes with those of supposed di- or tetraploid progenitors (i.e. *A. paniculatum*, *A. podolicum*, *A. fuscum*, *A. dentiferum*; Stearn, 1980; Brullo *et al.* 1996, 2001, 2008; Dobrotchaeva *et al.*, 1999), *A. oleraceum* inhabits wide spectrum of habitats ranging from stressed and infertile rocky habitats though disturbed open habitats, nutrient-rich communities with dense vegetation to late succesional forest communities, overlaping thus narrower niches of supposed progenitors. Broad niche in tetra- and pentaploid cytotypes of *A. oleraceum* suggest that such cytotypes harbour either large plasticity of physiological or morphological traits or are composed of many adapted types, probably of repeated and/or different origin (Soltis *et al.*, 2010), partial ecological (and also geographical) similarity of tetra- and pentaploids suggest that gene flow may exist between them. Narrow niche in triploids can be simply result of limited number of sampled populations and hence did not reflect their real (and probably wider) ecological amplitude. However, their predominant occurence in dry forests and steppes coincide well with ecological demands of one supposed diploid progenitor *A. paniculatum* in Central Europe (Holub in Čeřovský *et al.* 1999; Ciocârlan, 2000). Hexaploids have the most different and narrower ecological niche from other cytotypes and commonly occur in

ruderalised, often weedy habitats in areas with subatlantic climate. Such different niche may suggest their superiority in intensively agriculturaly utilised landscapes where anthropogenic habitats prevail over natural vegetation. Their relative avoidance of continental climate may be an artefact due to recent expansion from subatlantic climatic zone, competitive exclusion in contact zones (see below) or suggest real climatic limits of hexaploids. Unfortunately, due to logistic and legal reasons we did not sampled soil samples to examine preferences of hexaploids for mineral-poorer, weakly acidic soils with higher concentrations of phosphorus found by Duchoslav *et al.* (2010) in the Czech Republic. Definition of ecological niche of the heptaploids is problematic because only limited number of populations was observed and all are geographically restricted to the eastern or southern France. Concerning habitats, heptaploids are partly similar to hexaploids with prevailing occurence on or near field margins and road ditches, i.e. disturbed and partially ruderalised habitats.

Felber-Girard *et al.* (1996) showed on example of di- and tetraploid *Anthoxanthum alpinum* that assesment of niche breadth of cytotype may differ depending on whether the ecological differentiation between cytotypes is studied across their whole ranges ('primary ecological differentiation') or is limited just to contact zones where it can represent a response to competition between cytotypes ('secondary ecological differentiation', Felber-Girard *et al.*, 1996). Also Ståhlberg and Hedrén (2009) observed that in pure populations consisting of either di- or tetraploids of *Dactylorhiza maculata* the ecological amplitude of both cytotypes was wider than in mixed populations. Unfortunatelly, no single-cytotype areas, at least at regional level, were observed in *A. oleraceum* and mixed populations are common. Hence, relatively weak habitat differentiation among cytotypes observed by us may be theoretically even lower when considering cytotype-pure areas.

Genome size variation

Our nuclear DNA values of five out of six cytotypes represent prime estimates. However, estimation of genome size in octoploids must be considered as preliminary because of high coefficient of variance obtained. Previously, only two nuclear DNA values for *A. oleraceum* had been gathered (see Bennett and Leitch, 2011), and both were determined for pentaploids using Feulgen densitometry. While the estimate of Baranyi and Greilhuber (1999; 2C = 60.37 pg) was in a good agreement with our estimations, the prime estimate 2C-value = 52.78 pg for pentaploids by Labani and Elkington (1987) is suspicious and should be discarded.

In general, the present study revealed considerable intraspecific variation in genome size. Major part of variation in genome size within each of $3x$-$7x$ cytotypes was attributable to interpopulation variation though it was apparently lower in tri-, hexa- and heptaploids (ca 5-8 %) than in tetra- and pentaploid cytotypes (ca 13-18 %). On the other hand, within-population variation was not proved to be significant. The simple explanation of the genome size variation is based on methodological artefacts, usually associated with instrumental drift and/or dissimilar levels of secondary metabolites that may have interfere with DNA fluorochromes (Greilhuber, 2005). Many examples of variation have been shown to be measurement artefact when repeatedly analysed (Greilhuber, 2005). There are, however, many reports documenting intraspecific or even intrapopulation genome size variation where appropriate methodology has been used (e.g. Reeves *et al.*, 1998; Pečinka *et al.*, 2006; Suda *et al.*, 2007*a,b*; Pavlíček *et al.*, 2008; Balao *et al.*, 2009; Šmarda *et al.*, 2008a; Zaitlin and Pierce, 2010). Considering the maximum genome size heterogeneity reported in other species, Šmarda and Bureš (2006) found 16.6 % in *Festuca pallens* Host, Slovák *et al.* (2009) found 23 % in *Picris hieracioides* L. and Cires *et al.* (2010) reported 20.3 % in *Ranunculus parnassifolius* s.l. *Allium oleraceum* belongs to hardly measurable plants with a huge content of mucilage that is mirrored in coefficients of variance (up to

4.31 % an 6.02 % on average in hexaploids and octoploids, respectively) for histograms of fluorescence intensity that were slightly higher than those proposed by Doležel *et al.* (2007) for genome size estimation. Consequently, higher coefficient of variance cause insufficient sensitivity of the method to distinguish two individual peaks when individuals from the same population are measured simultaneously (Benson and Braylan, 1994; Doležel and Göhde, 1995; Balao *et al.*, 2009). Other likely reasons behind undemonstrated within-population variation are small population samples with three individuals analysed, predominant asexual reproduction in *A. oleraceum* being mostly pronounced in tri- and hexaploids (Fialová, 2005; Ohryzek, 2007) and responsible for uniclonal structure of many populations (Staňková, 2005), and higher measurement error causing lower reproducibility of repeated measurements, especially in hexa- and heptaploids. However, we believe that intracytotype variation reported in the present study should be considered genuine, as (1) our samples were from fresh mature plants, grown under standardized environmental conditions and measured using standardized protocol that minimised measuring bias and (2) simultaneous analyses of samples of the same cytotype but with different 2C DNA always yielded histograms with one bifurcated or two separate peaks (Fig. 5), which is considered the most convincing evidence for real differences in nuclear DNA content (Greilhuber 2005).

Part of the variation in 2C DNA can be explained by aneuploidy, which has been rarely found through chromosome counting in seedlings from pentaploids by Fialová (1996) and Jandová (2010). Similarly, an intracytotype divergence in genome size in Asian polyploid *Cardamine* species (Marhold *et al.*, 2010) a *Senecio carniolicus* (Suda *et al.*, 2007c) was attributed to aneuploidy, although this was not recorded by direct chromosome counting. However, in adult *A. oleraceum* plants only euploid chromosome numbers are reported in literature (Fialová 1996, Karpavičienė, 2007; Duchoslav *et al.*, 2010) and have also been found in the 12 individuals analysed here, even in simultaneously measured plants with the greatest difference of genome size. Alternatively, variation may be partly related to variation in heterochromatin composition (Šmarda and Bureš, 2010), mostly of satellite DNA (ved Brat, 1965; Meagher and Vassiliadis, 2005; Biémont, 2008). Fialová (1996) reported apparent karyotype plasticity in *A. oleraceum* with various types and numbers of satellite chromosomes that are often poorly identifiable in metaphase figures. However, geographic component of genome size variation contradict this scenario.

Many studies suggest that variation in inter- and intraspecific genome size could be a product of local adaptation along ecological gradients (see Knight and Ackerly, 2002; Knight *et al.*, 2005 for review). As regards spatial and ecological correlates, the correlation of geographic location and climatic variables with genome size was strong in tetra- and pentaploids but weaker in other cytotypes of *A. oleraceum*. In triploids, however, observed correlations have limited value because only eight populations have been measured and surprisingly three populations with high genome size were sampled in higher altitudes than populations with lower genome size. In tetra- and pentaploids, genome size increased in SW–NE gradient and simultaneously increased with decreasing precipitation and increasing temperature extremes. Possibly, DNA content mirrors a gradient in climatic conditions from oceanic to more continental zones. Moreover, variation seems more or less continuous along these gradients at first look (Fig. 7). This may fit well with the results of MacGillivray and Grime (1995) that found positive relationship between genome size and frost resistance in British herbaceous plants. Correlations of genome size and gradient of continentality have also been reported previously for several plant species from Europe but no general relationship emerged and in most cases many other explanations, including surviving the glaciation and of post-glacial migration, were propoused. Similar to our results, Pečinka *et al.* (2006) and Šmarda and Bureš (2006) found increase in DNA content towards the east and southeast part of Central Europe in *Koeleria macrantha* var. *majoriflora* and *Festuca pallens*, respectively. In contrast, fluorescence intensity of

tetraploid *Lythrum salicaria* was negatively correlated with population location along the west-east gradient in Europe (Kubátová *et al.*, 2008). Longitudinal component of interspecific genome size distribution was found in *Hieracium* subgenus *Pilosella* (Suda *et al.*, 2007*b*) with positive correlation between genome size and longitude of sampling localities. On the contrary, negative correlation between interspecific genome size and longitude has been observed in central European species of *Cirsium* (Bureš *et al.*, 2004). Firstly, confusion about the relationship between genome size and continentality gradient may arise because most studies did not consider full geographic and/or ecological ranges of the studied plants which may show unimodal trend of genome size over whole environmental gradients as observed by Knight and Ackerly (2002) in the Californian flora, where species with large genomes tended to be excluded from extreme environments with shorter growing season (see Knight *et al.*, 2005). Also our analysis did not account for full geographical ranges of cytotypes because of limited availability of samples from the northern and east Europe and weak lack of fit of genome size of pentaploids is visible at higher latitudes/longitudes, suggesting partly divergent trend from one fitted by linear regression (Fig. 7). Secondly, in many studies that analysed interspecific variation in genome size, authors did not make a clear distinction between the effect of holoploid and monoploid genome sizes (*sensu* Greilhuber *et al.*, 2005) though some studies (Grotkopp *et al.*, 2004; Beaulieu *et al.*, 2007*a*; Münzbergová, 2009) suggest different relationships between plant traits and holoploid and monoploid genome size (but see Beaulieu *et al.*, 2007*b*; Knight and Beaulieu, 2008).

Most surprising is relative low variation of genome size in hexaploids. Low intraploidy variation was also observed in some other plants (e.g., Lysák *et al.*, 2000) but in spite of large variation found in tetra- and pentaploids is genome size stability in hexaploids striking. Duchoslav *et al.* (2010) proposed that common occurrence of hexaploids in the Czech Republic may represent evidence of a recent range expansion of a newly established type. Except for two exceptionally low 2C values measured in plants from hexaploid populations in Spain and France which may suggest different polyploidization event, our measurements support this hypothesis. Low genome size variation in hexaploids is probably maintained also by almost exclusively asexual reproduction via aerial bulbils and daughter bulbs (Ohryzek, 2007) and coincides with their low genetic variation (Staňková, 2005). Similar relationship between genome size variation and amount of genetic variation was observed e.g. in apomictic triploids of *Hieracium alpinum* (Mráz *et al.*, 2009). For this reason, we expected no relationships of genome size with ecogeographic variables. However, after removal of two extreme values from data set, quite reverse correlations of genome size of hexaploids was found with several ecogeographical variables than was observed in tetra- and pentaploids. Although significant, regression lines have small negative slopes and therefore this trend could not have any biological meaning. Alternatively, selection operates on hexaploids in different way as in tetra- and pentaploids.

There is, however, an alternative explanation for genome size variations observed, i.e. so-called 'orthodox' variation due to non-recognized phylogenetic components (Greilhuber, 1998, 2005). Chrtek *et al.* (2009) found positive correlation between genome size and longitude of sampling sites in *Hieracium* subgen. *Hieracium* but when phylogenetic pattern was taken into account, correlation almost disappeared. Thus, any correlation of genome size with ecogeographical variables was outweighted by the basal phylogenetic divergence into species of eastern and western European origin (Chrtek *et al.*, 2009). At the intraspecific level, Slovák *et al.* (2008) observed both lati- and longitudinal increase in genome size of *Picris hieracioides* over Europe which fit well with climatic gradients but authors inclined more to the opinion that the taxon encompasses several independent lineages with unique evolutionary histories. Bogdanovič *et al.* (2009) proposed in autumn-flowering species of *Allium* sect. *Codonoprasum* two speciation

centres in Europe: one in the western Mediterranean and the second one in the eastern Mediterranean area. Duchoslav *et al.* (2010) suggested the recurrent formation of particular *A. oleraceum* cytotypes and consider their polyphyletic origin as highly probable. Consequently, dominant east-west gradient in genome size observed by us in tetra-, penta- and hexaploids *A. oleraceum* may potentially fit with the hypothesis of their polyphyletic origin with an existence of at least two independent lineages: eastern and western. Presently, this hypothesis is supported by (i) uniform relationships of genome size in tetra- and pentaploids and partly also in hexaploids with ecogeographic variables in the western Europe, that contrast with (ii) apparent variation in genome size (2C an 1Cx) of tetra- and pentaploids in Central Europe, weakening of correlations with ecogeographical variables in Central and Eastern Europe.

When looking on variation in monoploid genome size, we found, with one exception, that ploidy levels of *A. oleraceum* differ on average each other in their monoploid genome size with 1Cx gradually decreasing (except for 7*x*) from tri- to octoploid cytotype. This might indicate downsizing of genomes after polyplodization, a general trend observed in angiosperms including various representatives of the genus *Allium* (Leicht and Bennett, 2004) due to several mechanisms from which deletion of repeated DNA sequences and the dynamics of transposable elements is considered at the most important (Bennetzen, 2000; Bennetzen *et al.*, 2005). However, there is no general trend to either downsizing or upsizing within polyploid species and absence of downsizing in polyploids usually suggest (very) young neopolyploids (Bancheva and Greilhuber, 2006; Weiss-Schneeweiss *et al.*, 2006; Cires *et al.*, 2009; Cosendai and Hörandl, 2010, but see Suda *et al.*, 2007*a*), probably of autopolyploid origin (Balao *et al.*, 2009). Therefore, it could seem reasonable to assume, that differences in monoploid genome size among ploidy levels in *A. oleraceum* indicate some genomic differentiation and rather ancestral origin than recent autopolyploidy. However, when geographic scale of variation of monoploid genome is taken into account, more complicated pattern emerged. In short, cytotypes have similarly sized monoploid genome size in the (south)western but not in the (south)eastern Europe (see Fig. 6, 7). The most parsimonous explanation for similarly sized 1Cx values of 4*x*–7*x* cytotypes from the (south)western Europe is their recent autopolyploid origin while differently sized genomes of eastern 3*x*–6*x* cytotypes could be either results of independent polyploidization events, including introgression with other taxa of the *A. paniculatum* group (see above) and/or ancient autopolyplodization. Unfortunately, it is unable to rigorously test this hypothesis since no data concerning genome size of potential diploid (or lower polyploid) progenitors of *A. paniculatum* group are presently available in literature (Bennett and Leitch, 2011). Hovewer, we recently made some preliminary measurements of genome size of several diploid populations of *A. paniculatum* and *A. fuscum* and tetraploid populations of *A. dentiferum* and *A. pallens* from southern Europe resulting in 1Cx values 14.6–16.3 pg for diploids and 10.85–11.02 pg for tetraploids, respectively (A. Váňová, M. Jandová and M. Duchoslav; unpubl. res.). These values are either apparently higher (diploids) or lower (tetraploids) than corresponding values of all cytotypes of *A. oleraceum* presented here. This, most likely, rules out hypothesis of recent autopolyploid origin of *A. oleraceum*, at least from these species. Yet, spatial relationships of genome size in tetra- and pentaploids and penta- and hexaploids may indicate an evolutionary link between ploidy levels, i.e. possibility of repeated recent autopolyploidization events, but similar situation may be also explained by adaptation of ploidy levels to similar environmental conditions (Šmarda and Bureš, 2006). This argues against simplified explanations. Pattern of genome size in tetra- and pentaploids also give a preliminary idea of two "hot zones", i.e zones where different types got into secondary contacts: western Central Europe at border between France and Germany and eastern Central Europe. Such zones are known as important biogeographic contact zones as a result of postglacial range re-colonization from different refugias (Hewitt, 1999, 2004;

Schmitt 2007) though we are aware of possibility that wide expansion of cytotypes can be connected also with more recent creation of secondary habitats by man-made changes including deforestation, introduction of grazing and cultivation of crop-plants.

There is also evidence suggesting that species or genotypes with smaller monoploid genome size are more invasive due to the effects of genome size on key life-history traits that enhance colonization potential, e.g. rapid plant development, generation time and production of propagules with greater dispersal potential (Grotkopp *et al.*, 2004; Lavergne *et al.*, 2010; Kubešová *et al.*, 2010). Contrary to expectations, our data concerning intracytotype variation suggest that populations of pentaploids and weakly but non-significantly also of tetraploids occurring in anthropic habitats have higher genome size than populations inhabiting natural habitats (Table 4) while in other cytotypes no relationships between genome size and habitat type was found. These relations unchanged even after correction for geographic pattern of habitat distribution (analysis not shown). Within all ploidy levels studied, we therefore did not corroborate hypothesis of genome size reduction enhancing invasive ability. Similar results obtained Kubátová *et al.* (2008) for native and invasive populations of *Lythrum salicaria*. We are, of course, aware that habitat classification used by us could not fully reflect plant invasiveness. At the interploidy level, high or low colonization potential of cytotypes with either medium (i.e. tetra- and pentaploids) or high/low (i.e. tri-/hexaploids) monoploid genome size, based on their present ranges, is also not consistent with this hypothesis. It seems therefore more probable that evolutionary history of respective cytotypes is responsible for their present pattern of distribution. However, tight affinity of hexaploids to anthropic habitats observed in the Czech Republic (Duchoslav *et al.*, 2010) and over Europe (this study) together with their quicker phenologic development (Jírová, 2007) suggest that their low monoploid genome size could enhance their colonization ability and potential future spread over Europe. Whether similar conclusion can be drawn for heptaploids is doubtful because only limited population samples are presently available.

Cytotype distribution patterns on broad scale

The large-scale distribution pattern of cytotypes throughout the Europe was found to be remarkably complex that fits well into and broaden results of detailed screening in the Czech Republic (Duchoslav *et al.*, 2010). While tetra- and pentaploids were found sympatric with other cytotypes and common over whole Europe, triploids vs. higher cytotypes (hexa-, hepta- and octoploids) were not nearly entirely in mutual contacts and occupied separate regions below 50 latitude. All cytotypes are also in contact with supposed diploid, sexually reproducing progenitors but entirely in the southern latitudes. Regions with high cytotype diversity are thus confined to unglaciated and also almost permafrost-free areas of the late Pleistocene while only tetra- and pentaploids are presently distributed also in northern areas that were glaciated during the last glacial maximum (Huntley and Birks, 1983). Somewhat similar distribution pattern has been described for several sexual–apomictic genera (e.g. *Antennaria* L., Bayer and Stebbins 1987; *Ranunculus auricomus* group, Hörandl, 2006; *Hieracium pilosella*, Mráz *et al.*, 2008) where polyploid apomicts tend to have larger ranges than (diploid) sexuals which suggest evolutionary advantages of polyploidy associated with apomixis in the colonization of deglaciated areas (geographical parthenogenesis). Hörandl (2009) showed that climatic changes may have triggered interspecific hybridization because fluctuations in distribution ranges have brought previously geographically or ecologically isolated sexual species together, which increases frequencies of new origins of asexuality which is further advantageous for re-colonization of previously devastated areas. Application of this model on *A. oleraceum* is however puzzling. Firstly, through *A. oleraceum* is not apomictic plant (*sensu* Asker and Jerling 1992), all its cytotypes predominantly reproduce uniparentaly, i.e.

produce huge and similar amount of asexual propagules (= bulbils; Fialová and Duchoslav, pers. observation). Bulbils are ecologically similar to seeds (see e.g. Ronsheim, 1994) but due to their higher amount of stored resources (Ohryzek, 2007; Karpavičienė and Karanauskaite, 2010) has higher fitness than seeds under low competition from surrouding vegetation, i.e. in open early-succesional vegetation (M. Fialová and M. Duchoslav, pers. observations). These characters favour *A. oleraceum* cytotypes over their supposed sexual diploid progenitors but cannot be considered as selectively enhancing one of them over other cytotypes. Moreover, two most northernly distributed cytotypes (tetra- and pentaploids) also produce order of magnitude more sexual seeds than other cytotypes which are mostly sterile (M. Fialová and M. Duchoslav, pers. observation). Such combination of sexual and asexual reproduction potentially give tetra- and pentaploids advantage over other cytotypes due to their better ability to adapt together with conservation of such genetically and ecologically different clones via asexual propagules, i.e. bulbils (= 'Frozen Niche Variation Model'; Vrijenhoek 1984, 1994). Several lines of evidence support the applicability of this model on *A. oleraceum*. Firstly, as we hereinbefore mentioned, both tetra- and pentaploids showed somewhat greater breath of ecological niches and higher genome size variation than other cytotypes. Secondly, Staňková (2005) found higher genotypic diversity of tetra- and pentaploids than in hexaploids in the population samples from the Czech Republic. Thirdly, Ronsheim (1997) observed existence of significant local adaptation of asexually produced bulbils to the parental microsite in functionaly similar *Allium vineale* and we suggest that the same mechanism works also in *A. oleraceum*.

However when the range of a cytotype is considered to be an indicator of its colonizing ability, evolutionary age of cytotypes could be a coufounding factor (Fawcett and de Peer, 2010). Because both hexa- and heptaploid cytotypes were found to have smaller ranges and confined to human- impacted habitats, it is probable that either they established later than lower ploidies and/or their spread was faciliated by antropic impact. As mentioned by Hörandl (2008), evolutionary and biogeographical history of taxa have had a major impact in shaping their ranges and a multidisciplinary approach will be necesasary for a full understanding of geographical patterns which may differ case by case.

Absence of triploids outside the range of supposed diploid progenitors together with near absence of cytotype-mixed populations containing triploids suggest that the fitness of triploid individuals seems to be too low to facilitate the polyploidization process (triploid block; Köhler *et al.*, 2010). It is supported by polen and seed sterility observed in triploid plants transfered and cultivated in the common garden (Jírová, 2007). On the other hand, triploids, like other cytotypes, maintain and spread by vegetative propagation via aerial bulbils (Fialová and Duchoslav, pers. obs.) and therefore it is unclear why their distribution is limited. Simple explanation supposes that triploids are common in the Eastern Europe but due to less intensive sampling are considered as rare cytotype with narrow niche. Other possible explanation may by their low genetic diversity resulting in narrow ecological niche. Duchoslav *et al*. (2010) suggested that triploids may arise from the fusion of reduced and unreduced gametes of diploid progenitor(s) or from crosses between diploid progenitor and tetraploid *A. oleraceum*. Knowledge of triploids of supposed progenitors, i.e. *A. paniculatum* (Tzanoudakis and Vosa, 1988) and *A. fuscum* (Özhatay, 1990), from Mediteranean and morphology of triploid *A. oleraceum* with characters typical for this species (small number of flowers and many aerial bulbils; Fialová and Duchoslav, pers. obs.) thus suggest rather allopolyploid origin of *A. oleraceum* triploids. However, many chromosome counts reported for members of *A. paniculatum* complex, especially for *A. paniculatum*, could belong to other species because of poorly understood taxonomy and frequent species misinterpretation and confusion both in the literature and herbarium collections (e.g. Brullo *et al*., 2008; Koçyiğit and Özhatay, 2010). Hence, we cannot exclude autopolyploid origin of triploid *A. oleraceum*, most probably

from diploid *A. paniculatum* s.s. Because genome size of triploids in eastern Central Europe is similar, we also suggest their common origin. On the other hand, apparently low genome size of one south-Ukrainian triploid population may suggest different polyploidization event.

Surprising absence of triploid *A. oleraceum* from other southern regions of Europe (Spain, France, Italy) is hardly explainable as sampling error because we sampled there more extensively than in the Eastern Europe. More probable their absence coincides with apparently lower diversity of supposed diploid progenitors belonging to the sect. *Codonoprasum* in the western Mediterranean area than in the eastern one (Brullo *et al*., 1997; Bogdanoviċ *et al*., 2008; Aedo, 2010), which in turn may decrease probability of triploid establishment and/or survival. The latter explanation is supported by newly discovered high polyploids (hepta- and octoploids) that were found just in the (south)western Europe. Such high cytotypes could originate via various pathways including auto- and/or allopolyploidy, with participation of higher cytotypes of related species of *A. paniculatum* complex. Their absence ouside the hexaploid range may suggest hexaploids are involved in their origin.

Tetra- and pentaploids are the most common cytotypes and seem to coexist over most part of Europe, though we found tetraploids surprisingly less common than literary data suggested. Šafářová *et al*. (2011) supposed that at least two types of tetraploids occur in Central Europe. Authors based their claims on contrasting altitudinal distibutions and habitat differences of tetraploids between Hercynian and Carpathian biogeographic regions. Also large-scale pattern of tetraploids in Central Europe (Fig. 1) support an existence of two migration routes: eastern and western. However, apparent variation in genome size of tetraploids in Central Europe without clear geographic component did not clearly corroborate this hypothesis. Still, high genome size variation found by us in tetraploids in Central Europe suggest that the cytotype is composed of fairly heterogeneous units potentially originating via various mechanisms including secondary contacts between partially genetically different tetraploids or reccurent establishment from other cytotypes via fusion of various forms of gametes (unreduced, reduced and partially reduced) or even via hybridization of different diploid progenitors as was previously experimentaly documented by Levan (1938). Spatial pattern of triploids suggest that they may play role in origin of eastern tetraploids but based on our data no direct evidence for origin of tetraploids via triploid bridge is available. Lower genome size of the western tetraploids together with their flower colors differing from those observed in the eastern tetraploids (Fialová and Duchoslav, unpubl. results) suggest their different origin.

Similar distributional pattern of tetra- and pentaploids and spatial relationships of their genome sizes probably indicate an evolutionary link between ploidy levels, i.e. a possibility of existence of (i) primary hybrid zone (*sensu* Petit *et al*. 1999) and (ii) at least two pentaploid types: western and eastern. Somewhat similar situation (i.e. the "primary hybrid zone" hypothesis) suggested recently Trávníček *et al*. (2011*a*) as plausible explanation of complex spatial patterns of cytotypes of *Hieracium echioides*. However, we previously suggested (Duchoslav *et al*., 2010; Šafářová and Duchoslav, 2010) that due to absence of "donor" hexaploid cytotype within such 4x+5x mixed populations or even in their wider surrounding, is origin of pentaploids hard to explain and mixed-ploidy populations of tetra- and pentaploids thus represent results of secondary contacts betwen cytotypes. Yet, Jandová (2010) recently analysed progenies (seeds and seedlings) from tetraploid plants originating from wild pure tetraploid and cytotype-mixed populations and in both cases she detected, albeit with low frequencies, generation of pentaploids. Generation of pentaploids in tetraploid populations may also partly explain why we observed significantly higher proportion of mixed populations with presence of tetraploids than those with presence of pentaploids. Also similarly sized genome size in some tetra-

and pentaploid populations may support this hypothesis. Presently, we can only speculate how common this mechanism of pentaploid generation is and how it works.

Surprisingly, we found one large area ranging from the western part of Hungary continuing southwards to Croatia and Serbia dominated by pentaploids and additionaly with rare occurence of triploids. On the other hand, no other cytotype was found there (incl. tetraploids). We can just speculate about origin of such zone but it is clear from distributional data, that either competitive exclusion and/or rather environmental conditions of this zone limit spread of tetra- and hexaploids.

Hexaploids show specific distribution range being absent from eastern and southeastern Europe. Moreover, it seems that they avoid high altitudes with cold climate, being absent from e.g. Alps and Hercynian mountains. Sharp boundary of their distribution in the eastern Central Europe coincides with analogical situation observed in several other polyploid complexes, e.g. *Vicia cracca* (Trávníček *et al*. 2010), *Pilosella officinarum* (Mráz *et al*. 2008), and *Knautia arvensis* (Kolář *et al*., 2009). Remarkable contact zone of tri- and hexaploids in the southern Slovakia can be thus considered as secondary and based on cytogeographical pattern, hexaploids may spread into Central Europe from two southern refugias: southwestern Europe (France, Spain?) and Italy. However, situation is probably more intricated. Relative stability of genome size and similar morphology with mostly absence of flowers and thus predominant clonal reproduction (Ohryzek, 2007; M. Fialová and M. Duchoslav, unpubl. res.) of Central European hexaploid populations may suggest their single origin and quick spread through agricultural landscape. On one hand this fit well with similarly sized monoploid genome sizes in majority of southernly occuring hexaploids. However, this southern hexaploids (i.e. from Italy and France) differ from Central-European hexaploids morphologically, having many flowered inflorescence (M. Fialová and M. Duchoslav, unpubl. res.). Moreover, two hexaploid populations from southern France and Spain with apparently lower DNA content may have experienced different evolutionary history and/or independent polyploidization event. Whether other hexaploids reported by Pastor (1982) from Spain belong to the same type with lower DNA content or represent another type is not known presently.

Bogdanoviċ *et al*. (2009) proposed in autumn-flowering species of *Allium* sect. *Codonoprasum* two speciation centres in Europe: one in the western Mediterranean and the second one in the eastern Mediterranean area. Brullo *et al*. (1997) showed that the populations occurring in these two centers also show different ploidy levels; the eastern species are all diploid (2n = 16), while the western ones are tetraploid (2n = 32). Bogdanoviċ *et al*. (2009) thus considered eastern autumn-flowering diploid species as palaeoendemics currently restricted to refugial sites while tetraploids from the western Mediterranean as palaeopolyploids, probably originating from interspecific hybridizations. While this could be an attractive hypothesis explaining also pattern in cytotype distribution of *A. oleraceum*, karyological data on supposed progenitors of *A. oleraceum* over Europe contradict this scenario: published data on chromosome counts of members of *A. paniculatum* group (Goldblatt and Johnson, 1979) suggest higher cytotype variation in the eastern and not in the western Mediterranean. Therefore, a multimethod approach, with an emphasis on molecular analyses, will be necessary in order to gain deeper insights into the evolutionary history of this polyploid complex and to elucidate its taxonomy.

Mixed-ploidy populations

High frequency of mixed-ploidy-level populations has been recently observed in several plant species (Burton and Husband, 1999; Suda, 2002; Keeler, 2004; Suda *et al*., 2007a; Halverson *et al*., 2008; Kao, 2008; Marhold *et al*., 2010; Sonnleitner *et al*., 2010; Trávníček *et al*., 2011*ab*; Šingliarová *et al*., 2011) despite theoretical arguments that the coexistence of multiple cytotypes within populations should be unstable and may represent

only a transient stage following generation or immigration of a divergent cytotype (Levin, 1975). Though many theoretical and empirical studies (Fowler ad Levin, 1984; Felber, 1991; Rodriguez, 1996; Thompson and Lumaret, 1992; van Dijk and Bijlsma, 1994; Li *et al.*, 2004; Baack, 2005; Rausch adn Morgan, 2005; Kao, 2007, 2008, Halverson *et al.*, 2008) suggested many adaptive or non-adaptive mechanisms allowing persistence of mixed-cytotype populations (see introduction), we still have little empirical data exploring mechanisms influencing polyplodization and the maintenance and spread of polyploids (Kao and Parker, 2010). Concerning *A. oleraceum*, we found 15.2 % populations comprising two or three cytotypes that is not as common as reported by Duchoslav *et al.* (2010) from the Czech Republic (23 %). Lower frequency of mixed populations found in the present (and also in many other) research may be simply result of lower average number of analysed plants per population that decrease probability of detection of minor cytotype(s). This was clearly showed by studies on *Allium oleraceum* (Šafářová and Duchoslav, 2010) and *Senecio carniolicus* (Sonnleitner *et al.*, 2010), that resulted in an rise of proportion of mixed-ploidy populations when sample size was appropriatelly increased. To overcome problems with oversampling of identical genet by sampling of neigbouring plants, we systematically sampled plants in span at least 1 m from each other over whole area of population. Therefore we believe that this partly balances for lower number of sampled specimens per population. Moreover, since area of cytotype-mixed populations were larger than that of uniform ones and because of, though minor, niche differences among cytotypes, we suggest that coexistence of cytotypes in these mixtures may be partly result of ecological sorting in heterogeneous environment (Fowler and Levin, 1984), a explanation proposed previously by Duchoslav *et al.* (2010).

Cytotype mixed populations of *A. oleraceum* were found across the entire species range and occured in majority of regions where two or more cytotypes grow sympatrically and intensive sampling was carried out. Furthermore, all cytotypes form also their own populations suggesting rather long term stability of all cytotypes and not a scenario of continued polyplodization via production of unreduced gametes as proposed, for example, for hexaploids and higher-ploidy cytotypes of *Cardamine yezoensis* (Marhold *et al.*, 2010) and so-called secondary cytotypes ($3x$, $5x$, $7x$, $8x$) in *Senecio carniolicus* (Sonnleitner *et al.*, 2010) that do not spread from mixed populations and do not develop their own populations. We also confirmed all previously reported combination of cytotypes of *A. oleraceum* composing mixed populations (Duchoslav *et al.*, 2010) including unusual combination 4x+5x and 5x+6x mixtures and newly also 3x+5x, 4x+7x and 5x+7x mixtures. It seems, in concert with our previous studies, that the existence and spatial distribution of majority of these mixtures are hardly explainable as a result of the interploidy crosses or representing primary origin of higher cytotype within a lower cytotype population but are better explained by secondary contacts among cytotypes. Similar results obtained Kao (2007, 2008) for polyploids in *Arnica cordifolia*. In *A. oleraceum*, mechanisms supporting "secondary contacts hypothesis" are (Duchoslav *et al.*, 2010, Šafářová and Duchoslav, 2010): (i) partial similarities of ecological niches among cytotypes allowing their co-occurence in proximity, (ii) prevailing vegetative reproduction allowing plants to escape reproductive costs due to their minority status, (iii) local dispersal allowing formation of cytotype-homogenenou clumps that decrease intercytotype competition, and (iv) disturbance of localities and antropic dispersal of propagules (e.g. via transport of hay and cereals, soil preparation) increasing probability of contacts of various cytotypes. Yet, several lines of evidence (Jandová, 2010) suggest that tetra-, penta- and hexaploid cytotypes of *A. oleraceum* are able to generate sexual seeds with variable chromosome numbers. Therefore, we must admit an existence of primary zones but we can not accurately assess their roles in creating cytotype mixtures. Presently, we study cytotype composition of seeds and seedlings that originated from populations with various composition of cytotypes.

Based on analyses of recently published papers on cytotype composition in various perenial species (Duchoslav *et al.,* 2010; Šafářová and Duchoslav, 2010; Sonnleitner *et al.*, 2010; Trávníček *et al.*, 2011*ab*; this study) we suggest that for future research on cytotype diversity in plants (i) sample size should be proportional to population size and (ii) at least 30 individuals per population, if available, should be sampled to detect with at least 95% probability minority cytotype considering its relative frequency ~15% within population and truly random sampling. To detect rare cytotypes (~ 5%) with at least 95% probability, we suggest sample size ~ 100 individuals. Because of prevailing clumped distribution of individuals in plants and difficulty to do random sampling which is usually replaced by systematic or preferential samplings, these recommended sample sizes could be somewhat lower in reality.

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4. Prezentace na konferencích

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5. Metodické postupy

Stanovení DNA ploidní úrovně a absolutního obsahu 2C DNA (pg) u druhu *Allium oleraceum* L. za použití barviva propidium iodid (PI)

Rostliny sloužící jako srovnávací materiál pro základní stanovení poměrů vzdáleností píků vzorku a standardu byly podrobeny přímému počítání chromozomů z kořenových špiček (obr. 1).

Samotné měření vzorků na průtokovém cytometru probíhá v několika následných krocích:
1) příprava rostlinného materiálu
   a) rostliny ve stejné fenologické fázi (nejlépe měřit během časných jarních měsíců)
   b) nejméně měsíc před zahájením měření absolutního obsahu DNA pěstovat rostliny ve standardizovaných podmínkách (skleník, fytotron)
2) odběr rostlinného materiálu
   a) rostliny v dobrém fyzickém stavu, nestresované klimatickými podmínkami (mráz, sucho), bez patrného napadení škůdci nebo houbovou chorobou
   b) v případě měření v rozpětí více let, měření provádět ve stejném ročním období (nejlépe během časných jarních měsíců; březen nebo duben)
   c) v případě měření 2C DNA odběr rostlinného materiálu provádět ve stejnou denní dobu
   d) odebírat cca 5 cm materiálu z horní části mladého listu česneku
   e) listy uložit do navlhčené buničité vaty a uzavíratelného igelitového sáčku
   f) skladovat v ledničce, týž den změřit
3) příprava vzorku – metoda vnitřního standardu (Doležel 1997)
   a) 2 cm pletiva listu a 1 cm pletiva standardu (*Triticum aestivum* cv. *Saxana*)
   b) společně homogenizovat v Petriho misce v 1 ml LB01 pufru pH 7.5 (Doležel et al. 1989)
   c) k homogenizaci používat vždy zcela novou ostrou žiletku
   d) filtrace přes nylonové sítko (42 µm)
   e) k roztoku jader pipetovat 50 µl RNA-zy a 50 µl barviva Propidium iodid (PI)
   f) vzorek 20s míchat na třepačce
   g) 30 minut vzorek ponechat reagovat uložen na ledu
   h) před měřením vzorek krátce promíchat na třepačce
4) měření
   a) na 512 kanálové stupnici, standard nastaven na kanál 50
   b) pro stanovení DNA ploidní úrovně měřit nejméně 2000 jader, při stanovení obsahu 2C DNA 5000 jader, CV 2 – 3%(4%)
   c) stanovení obsahu DNA (2C DNA) na základě poměru vzdálenosti píků standardu a vzorku za použití vzorce:

$$2C\ DNA\ (pg.) = \frac{\text{Poloha G1 píku vzorku x obsah 2C DNA (pg.) standardu}}{\text{Poloha G1 píku standardu}}$$

   d) obsah DNA pro *Triticum aestivum* cv. *Saxana* byl stanoven na základě průměru dat z opakovaného měření se *Secale cereale* (2C DNA 16.19 pg) (Doležel et al. 1989)

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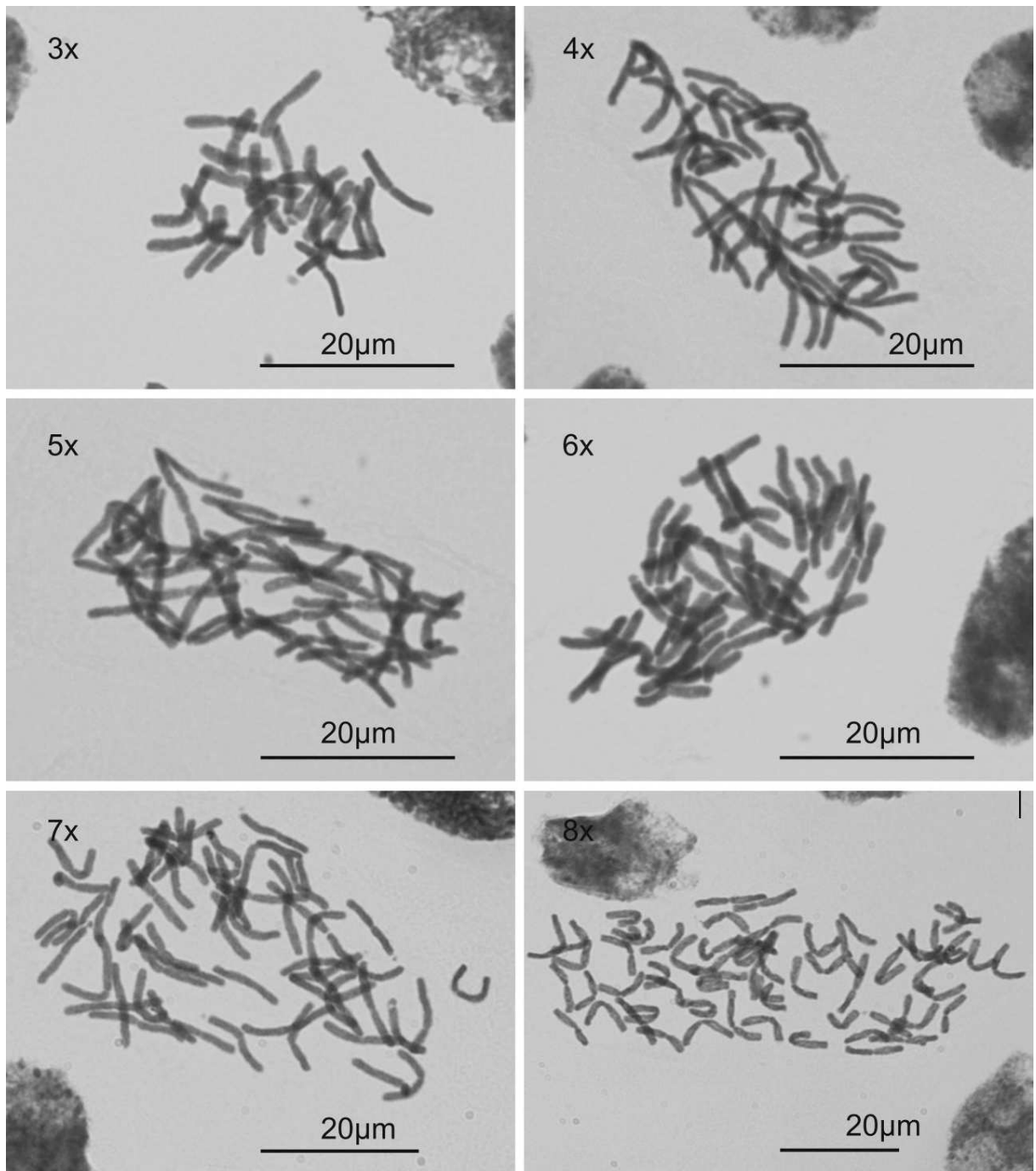
Obr. 1. Vybrané metafáze různých ploidních úrovní *Allium oleraceum*: 3x=2n=24, 4x=2n=32, 5x=2n=40, 6x=2n=48, 7x=2n=56, 8x=2n=64.

6. Závěr

Údaje o počtech chromozomů a DNA ploidních úrovní se staly základem pro studium polyploidního komplexu *Allium oleraceum*. Tento komplex byl studován na třech různých prostorových škálách: na území Evropy, na úrovni středu areálu druhu (podrobný průzkum České a Slovenské republiky) a na úrovni jednotlivých populací.

DNA ploidní úroveň byla u všech jedinců stanovena metodou průtokové cytometrie. Základem pro stanovení ploidie průtokovou cytometrií se staly hodnoty zjištěné analýzou jedinců se známým počtem chromozomů. V polyploidním komplexu *Allium oleraceum* byly potvrzeny čtyři doposud známé cytotypy: triploidní 2n = 24, tetraploidní 2n = 32, pentaploidní 2n = 40 a hexaploidní 2n = 48. Nejrozšířenějším cytotypem je pentaploidní následován tetraploidním, vyskytují se napříč celým areálem druhu. Hexaploidi se vyskytují řidčeji a byli nově objeveni na území řady států střední a západní Evropy. Triploidi se ukázali být velmi vzácní, byli zjištěni pouze na území Maďarska, Ukrajiny, Rumunska a Slovenska. Dále byly zjištěny dva nové cytotypy: heptaploidní 2n = 56 (Francie) a oktoploidní 2n = 64 (Španělsko).

Na úrovni celého areálu druhu i na úrovni podrobněji zkoumaného území České a Slovenské republiky byla zjištěna ekologická diferenciace cytotypů. Tetra- a pentaploidi mají ve srovnání s ostatními cytotypy širokou ekologickou niku a vyskytují se v širokém rozmezí klimatických faktorů. Naopak, hexaploidi se nejvíce odlišují od ostatních cytotypů a velmi často se vyskytují na ruderalizovaných lokalitách, podobné chování vykazují i heptaploidi. Triploidní populace byly zjištěny jen na přirozených stanovištích s klimatem odpovídajícím kontinentálním charakteristikám. Přesto, niky všech ploidních úrovní se výrazně překrývají a ve srovnání s pravděpodobnými předky ze skupiny *Allium paniculatum,* cytotypy *Allium oleraceum* osídlují daleko širší spektrum stanovišť.

Převažují populace cytotypově homogenní, pokud se vyskytly populace smíšené, pak byly ve většině případů tvořeny pouze dvěma cytotypy, jen výjimečně třemi, nikdy nebyla nalezena populace zahrnující více jak tři ploidní úrovně. Celkem 15% všech analyzovaných populací obsahovalo více jak jeden cytotyp, smíšené populace se vykytovaly na celém území Evropy. Koexistenci cytotypů na společné lokalitě lze částečně vysvětlit ekologickou diferenciací nik, pravděpodobně se však převážně jedná o sekundární kontakt populací jednotlivých cytotypů. Vzhledem ke schopnosti produkovat sexuálně vzniklé potomstvo však nelze vyloučit ani primární vznik polyploidů.

Na úrovni podrobné škály vykazovala z 21 detailně prověřovaných smíšených populací na území České Republiky dle typu použitého testu téměř polovina (48%) nebo nadpoloviční většina (62%) nenáhodnou distribuci jednotlivých cytotypů na lokalitě. Cytotypy byly prostorově nenáhodně uspořádány častěji v heterogenním prostředí, což by znovu mohlo ukazovat na jejich ekologickou diferenciaci. Zároveň však byly 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. Existenci cytotypově smíšených populací tedy nelze samu o sobě vysvětlit diferenciací nik mezi cytotypy, je třeba zvážit další faktory, jako jsou: vegetativní způsob rozmnožování a s ním spojené lokální šíření, vysoká populační hustota druhu v krajině a různé ekologické charakteristiky lokalit.

Byla zjištěna rozsáhlá vnitrodruhová variabilita ve velikosti jaderné DNA. Hlavní část této variability byla způsobena rozdíly ve velikosti jaderné DNA mezi populacemi, variabilnější se ukázali být tetra- a pentaploidi než tri-, hexa- a heptaploidi. U tetra- a pentaploidních populací stoupá velikost genomu severovýchodním směrem, společně s poklesem srážek a nárůstem teplotních extrémů. Velikost genomu u hexaploidů je poměrně homogenní a vykazuje jen velmi malou intraspecifickou variabilitu na západo-východním gradientu. Velikost monoploidního genomu jednotlivých cytotypů v západní Evropě si je navzájem podobná, narozdíl od Evropy východní, kde se liší. To může být

vysvětleno jejich odlišným původem, cytotypy (4x-7x) ze západní Evropy mohou být případem recentně vzniklých polyploidů, zatímco východní cytotypy (3x-6x) případem opakovaně vzniklých polyploidů doplněných introgresí rodičovských druhů ze skupiny *Allium paniculatum*.

Tetra- a pentaploidi jsou nejčastějšími cytotypy, které se společně vyskytují na většině území Evropy, jejich podobné rozšíření stejně jako gradient velikosti genomu naznačují vzájemnou evoluční příbuznost. Hexaploidní populace nebyly zjištěny v (jiho)východní Evropě, ale poměrně běžně se vyskytovaly na území střední Evropy (hl. České Republiky). Nízká vnitrodruhová variabilita a značná morfologická podobnost může znamenat jejich poměrně recentní původ následovaný rychlým šířením, naopak populace z jižní Evropy (Španělsko, Francie) s nižší velikostí genomu mohou představovat jiný případ polyploidizace. Nízká vnitrodruhová variabilita hexaploidů je bezesporu udržována převažujícím způsobem vegetativního rozmnožování.

Další pokračování této práce bude spočívat v nasazení molekulárních technik a odkrytí populační struktury a příbuzenských vztahů v rámci komplexu, včetně domnělých rodičů a dále ve studiu variability potomstva cytotypově smíšených i homogenních populacích.

7. Summary

Chromosome numbers and DNA ploidy data were obtained for *Allium oleraceum* plants from Europe and provided a good overview of cytotype's distribution in Europe. *Allium oleraceum* L. polyploid complex was studied on three different spatial scales; on the scale of the Europe, local scale of the center of complex distribution (Czech Republic, Slovak Republic) and on the region scale of the individual population (in the Czech Republic).

DNA ploidy level was investigated by flow-cytometry method. The bases of all measurements were individuals with exactly known numbers of chromosomes. Four ploidy levels were confirmed in *Allium oleracum* L. complex: triploid 2n=24, tetraploid 2n=32, pentaploid 2n=40 and hexaploid 2n=42. Pentaploids are the most widespread ploidy level and they occur throughout most of species range. Second most common types are tetraploids. Rare hexaploids were revealed for the first time for several western and central European countries. Triploids are the rarest ploidy level being confirmed from Hungary, Romania, Slovakia and Ukraine. We found two previously unknown cytotypes - heptaploids 2n=56 (France) and octoploids 2n=64 (Spain).

Both, on the scale of Europe, as well as on local scale (Czech and Slovak Republic), cytotypes significantly differ in environmental requirements. Tetra- and pentaploids showed greater breath of ecological niches than was observed in other cytotypes. Triploids prefer natural habitats in continental type of climate. Hexaploids and heptaploids occured in human-impacted habitats in mild climate regions. Triploids were found just in natural habitats in continental type of climate. However, niches of the all ploidy levels are overlapped. Furthermore, when comparing observed ecological characteristic of *A. oleraceum* cytotypes with supposed progenitors, *A. oleraceum* inhabits wider spectrum of habitats.

In Europe we found 15 % populations with more than one cytotypes. Mixed ploidy populations of *A. oleraceum* were found across the entire species range in majority of regions. These coexistence of cytotypes may be partly result of ecological sorting in heterogeneous environment but is better explained by secondary contacts among cytotypes. Further, tetra-, penta- and hexaploid cytotypes of *A. oleraceum* are able to generate sexual seeds with variable chromosome numbers. Therefore, we must take into account existence of primary origin polyploids.

Plants of 21 populations were examined on regional scale. Depending on the statistical test (Mantel test or average distance test) used, either 47.6% or 61.9% investigated populations were spatially segregated. Cytotypes were structured at sites with heterogeneous environments, this may implies that the cytotypes are ecologically differentiated. Nevertheless, structured and even non-structured populations were discovered at sites with heterogeneous and also with homogenous environments. This suggests that it is not possible to explain existence of the mixed ploidy population using only the niche differentiation. 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.

These study revealed intraspecific variation in genome size. Major part of variation in genome size within each of 3*x*-7*x* cytotypes was attributable to interpopulation variation which was apparently lower in tri-, hexa- and heptaploids than in tetra- and pentaploid cytotypes. In tetra- and pentaploids, genome size increased in SW–NE gradient and simultaneously increased with decreasing precipitation and increasing temperature extremes. In hexaploids is reltive low varation of genome size and is weakly correlated with W–E gradient. Dominant east-west gradient in genome size observed by us in tetra-, penta- and hexaploids *A. oleraceum* and differences in monoploid genome size among cytotypes may potentially fit with the hypothesis of their polyphyletic origin and recurrent

formation with an existence of at least two independent lineages: eastern and western. Two contact zones between cytotypes are suggested: western Central Europe and eastern Central Europe.

Tetra- and pentaploids are the most common cytotypes over most part of Europe, their similar distributional pattern and spatial relationships of their genome sizes probably indicate an evolutionary link between ploidy levels. Hexaploids being absent from eastern and southeastern Europe, but they commonly occur in the central Europe (Czech Republic). Hexaploids may represent evidence of a recent expansion of a newly established type of polyploids. Relative stability of genome size and similar morphology of Central European hexaploid populations may suggest their single origin and quick spread through agricultural landscape. Low genome size variation in hexaploids is probably maintained also by almost exclusively asexual reproduction. Low 2C values measured in plants from hexaploid populations in Spain and France may suggest different polyploidization event.

Future extension of this research would be to employ molecular technique. Determine population structure of the complex and reveal cytotype composition of seeds and seedlings that originated from populations with various composition of cytotypes.