Autotetraploids of *Vicia cracca* show a higher allelic richness in natural populations and a higher seed set after artificial selfing than diploids

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**Key Results and Conclusions**

- Background and Aims Despite the great importance of autopolyploidy in the evolution of angiosperms, relatively little attention has been devoted to autopolyploids in natural polyploid systems. Several hypotheses have been proposed to explain why autopolyploids are so common and successful, for example increased genetic diversity and heterozygosity and the transition towards selfing. However, case studies on patterns of genetic diversity and on mating systems in autopolyploids are scarce. In this study allozymes were employed to investigate the origin, population genetic diversity and mating system in the contact zone between diploid and assumed autotetraploid cytotypes of *Vicia cracca* in Central Europe.

- Methods Four enzyme systems resolved in six putative loci were investigated in ten diploid, ten tetraploid and five mixed-ploidy populations. Genetic diversity and heterozygosity, partitioning of genetic diversity among populations and cytotypes, spatial genetic structure and fixed heterozygosity were analysed. These studies were supplemented by a pollenination experiment and meiotic chromosome observation.

- Key Results and Conclusions Weak evidence of fixed heterozygosity, a low proportion of unique alleles and genetic variation between cytotypes similar to the variation among populations within cytotypes supported the autopolyploid origin of tetraploids, although no multivalent formation was observed. Tetraploids possessed more alleles than diploids and showed higher observed zygotic heterozygosity than diploids, but the observed gametic heterozygosity was similar to the value observed in diploids and smaller than expected under panmixis. Values of the inbreeding coefficient and differentiation among populations (\( F_{ST} \)) suggested that the breeding system in both cytotypes of *V. cracca* is mixed mating with prevailing outcrossing. The reduction in seed production of tetraploids after selfing was less than that in diploids. An absence of correlation between genetic and geographic distances and high differentiation among neighbouring tetraploid populations supports the secondary contact hypothesis with tetraploids of several independent origins in Central Europe. Nevertheless, the possibility of a recent *in situ* origin of tetraploids through a triploid bridge in some regions is also discussed.

**Key words:** Autopolyploidy, allozymes, genetic diversity, heterozygosity, fixed heterozygosity, genetic differentiation, \( F \)-statistics, inbreeding depression, mating system, artificial pollination, meiotic chromosomes, *Vicia cracca*.

**INTRODUCTION**

Polyploidy has played a key role in the evolution of angiosperms (Van de Peer et al., 2009; Soltis et al., 2009; Jiao et al., 2011). Regarding natural polyploids, much attention has been devoted to allopolyploids (originating through the process of interspecific hybridization and genome doubling), especially those that have emerged during the last 150 years, i.e. neopolyploids (e.g. Soltis et al., 2004; Abbott and Lowe, 2004; Aïnouche et al., 2004). In contrast, autopolyploids arising by genome doubling *per se* were considered for a long time to be of marginal significance and even unable to survive in nature (Stebbins, 1985). However, a few case studies showed that autopolyploidy has important evolutionary and ecological consequences in natural populations (Soltis et al., 2007; Parisod et al., 2010). Autopolyploids, for example, may occupy larger areas than their diploid ancestors (Van Dijk et al., 1992), have shifted or broadened ecological niches (Johnson et al., 2003), be superior competitors (Maceira et al., 1993) or be more resistant to pathogens (Oswald and Nuismer, 2007).

Recent data show that autopolyploids are more common than once claimed, but the frequency of naturally occurring autopolyploids is still poorly understood. Moreover, autopolyploidy is in many cases inferred only on the basis of morphology (listed in Soltis and Soltis, 2000; Parisod et al., 2010) and convincing evidence about the type of polyploidy based on polysomic inheritance or molecular data is lacking. Sometimes it is even necessary to reassess supposed autopolyploids as being actually allopolyploid (Mráz et al., 2012). The first step in the study of polyploid complexes should thus be to confirm the supposed type of polyploidy.

Several hypotheses have been proposed to explain why autopolyploids are so common and so successful. Genetic mechanisms for autopolyploid success include increased dosage of functional genes, which allows the development of new phenotypes, increased allelic diversity due to overdominance and heterosis, and reduced expression of deleterious recessive alleles due to higher heterozygosity (Bever and Felber, 1992). In fact, several empirical studies have confirmed that autopolyploids exhibit a higher level of allele richness and heterozygosity (Soltis and
Nevertheless, consistent evidence of adaptive benefits of autopolyploidy due to accelerated mutation and diversification rates of duplicated genes is still lacking (Parisod et al., 2010). It seems, however, that meiotic irregularities caused by multivalent formation after genome doubling reduce fertility less than expected (Ramsey and Schemske, 2002) and that genome restructuration may occur very rapidly and restore the diploid-like behaviour of autopolyploid genomes (Eilam et al., 2009). An additional hypothesis for the evolutionary success of polyploids considers the transition of the mating system from outcrossing in diploids towards self-fertilization in polyploids (Rausch and Morgan, 2005; Barringer, 2007). Basically, polyploids can tolerate the loss of self-incompatibility because they exhibit less inbreeding depression (Husband and Schemske, 1997; Rosquist, 2001), although the advantage of selfing depends mainly on the degree of dominance (reviewed in Parisod et al., 2010).

Contact zones are often formed in polyploid complexes comprising several cytotypes (e.g. Suda et al., 2007; Mráz et al., 2008; Trávníček et al., 2011; Münzbergová et al., 2013). The dynamics of contact zones depend mainly on the viability of intercytotype hybrids and the evolution of reproductive barriers. Assortative mating should enhance the reproductive success of newly arising cytotypes in mixed-ploidy populations; otherwise they will be subjected to frequency-dependent selection and become extinct (minority cytotype exclusion; Levin, 1975; Rodriguez, 1996). Weiss et al. (2002) proposed that the development of such isolating mechanisms might be encouraged by previous differentiation of cytotypes due to allopatry. Hence, genetic differentiation of cytotypes in contact zones is one of the principal factors determining the further coexistence and fate of individual chromosomal races (Petit et al., 1999).

One of the intensively studied diploid–polyploid plant groups is *Vicia cracca* agg. (Fabaceae). This aggregate contains several morphologically similar, although karyologically well separated, species with partially overlapping geographic ranges, including *V. cracca* L. s.s., *V. oreophila* Zertova, *V. incana* Gouan, *V. dalmatica* A. Kern. and *V. tenuifolia* Roth. Whereas *V. cracca* and *V. oreophila* share the base chromosome number $x = 7$, other species are derived from $x = 6$ (Hanelt and Mettin, 1989).

*V. oreophila* is a tetraploid ($2n = 4x = 28$) occurring locally in Central European mountain systems (Zertová, 1962; Chrtková, 1995). *Vicia cracca* s.s. is native in Eurasia and now widespread in the Northern Hemisphere. It was first reported in Ontario in 1860 (Aarssen et al., 1986) and was introduced to Alaska in 1909 (Klebesadel, 1980, from where it has spread along disturbed areas in Alaska, Canada and the northern USA, and is considered as invasive there (http://akweeds.uaa.alaska.edu/, http://plants.usda.gov/). It is a climbing polycarps perennial herb that typically occurs in meadows, along roads and river banks, and at forest margins on rather fertile, moist soils. Vegetative reproduction is possible by the spread of below-ground runners (Klebesadel, 1980). The mating system has not yet been determined with certainty (Zhang and Mosjidis, 1998; Jaaska, 2005). The species is supposed to be predominantly allogamous (Hanelt and Mettin, 1989), although some experiments suggest that selfing is also possible (Rousi, 1973). There are three different ploidy levels in *Vicia cracca* s.s.: diploid ($2n = 2x = 14$), rare triploid ($2n = 3x = 21$) and tetraploid ($2n = 4x = 28$); a few aneuploids were also reported (Rousi, 1961; Chrtková-Zertová, 1973; Roti-Michelozzi, 1984). An autopolyploid origin of *V. cracca* tetraploids is hypothesized in view of the karyotype similarity (Rousi, 1961; Dvořák et al., 1977) and great morphological similarity (Rousi, 1973) of the two cytotypes, the existence of vigorous aneuploid individuals (Rousi, 1961; Chrtková-Zertová, 1973) and identical monoploid genome sizes of both cytotypes (Trávníček et al., 2010). The distribution of cytotypes was intensively studied in Europe and the intercytotype contact zone of assumed secondary origin was detected in central Europe, with tetraploids prevailing in the west and north and diploids in the east and south (Trávníček et al., 2010). Information about the distribution of cytotypes in Asia and in the non-native range in North America is scarce. Both major cytotypes as well as triploids were recorded in a few cases from Asia, whereas only tetraploids were published from North America (listed in Trávníček et al., 2010).

The present study used allozyme polymorphism in the diploid–tetraploid complex of *V. cracca* to address the following specific questions: (1) Do patterns of genetic diversity based on allozyme markers correspond to the assumption of an autopolyploid origin of *V. cracca* tetraploids? (2) Do tetraploids exhibit higher genetic diversity than diploids? (3) What is the predominant reproduction mode in both cytotypes of *V. cracca* and is there a difference in selfing rate between cytotypes? (4) Is the intercytotype contact zone in central Europe indeed of secondary origin?

**MATERIALS AND METHODS**

**Sampling of plant material**

Plants of *Vicia cracca* were sampled during the years 2007–2009 along the border between the Czech Republic and Slovakia, which represents the intercytotype contact zone (Fig. 1, Supplementary Data Table S1). A total of 636 plants (323 diploid, 305 tetraploid and eight triploid individuals) from 25 populations were collected. Ten populations comprised only diploid plants, ten populations comprised entirely tetraploid plants and five populations were composed of both diploid and tetraploid plants (denoted here as mixed populations). Two diploid populations involved also a few triploid plants (Fig. 1). A distance of at least 5 m between individual plants was kept if possible to avoid sampling of sib progeny. Populations were delimited by a stand, but there were no sharp boundaries between single stands. This widespread species rather should be considered to form metapopulations in which a mosaic of appropriate stands is present in the landscape. The closest populations examined in this study were 0.5 km apart. The mean distance between diploid populations (80 km) was the same as that between tetraploid populations, excluding the three most distant populations (populations 1–3 in Fig. 1) (70 km; $P = 0.14$). The DNA ploidy level of plant samples was estimated by flow cytometry according to the method described by Trávníček et al. (2010).

**Pollination experiments**

Pollination experiments were made to test the self-compatibility of *V. cracca*. Plants used in these experiments were collected in the wild, transplanted into pots and grown in garden beds for at least 1 year. Experiments were done during one vegetation season. In total, 18 diploid plants from eight
population and 17 tetraploid plants from six natural populations were involved in the experiment. Plants were pollinated artificially with a paintbrush (using a separate brush for each plant). From each plant we selected between one and nine inflorescences (out of a total of 1–150 inflorescences per plant). Pollen was transferred from one mature flower to the nearest mature flower within each inflorescence until all flowers mature at that time were pollinated. Since individual flowers in inflorescences come into bloom successively, single inflorescences were treated up to four times in order to make sure that all flowers within the selected inflorescences would have been pollinated. After artificial pollination, nylon bags were used to prevent pollination by insects. Developed pods were collected after ripening. The numbers of full and empty pods and the numbers of well-developed and aborted seeds were summarized. Well-developed seeds were scarified with sandpaper and sown in soil to test their ability to germinate. Because of the small size and arrangement of *V. cracca* flowers, emasculation is difficult to accomplish without flower damage. However, apomixis has not been recorded in any *Vicia* species; Buyukkartal (2008) only mentioned the probability of apomixis in related *Trifolium pratense*.

Statistical analyses of results from pollination and germination experiments were performed using the GLM procedure in S-PLUS 2000 (MathSoft, 1999). In all cases, one data point in the analyses was represented by one plant, i.e. the data from all the flowers/pods were summed within each plant. First, we tested the dependence of pod set on the ploidy level using GLM with the binomial distribution of the dependent variable; this analysis was done on a subset of plants in which at least one seed had arisen. Finally, we linked the number of germinated seeds to the number of non-germinated seeds and tested the dependence of germination rate on the ploidy level again using GLM with the binomial distribution of the dependent variable. These analyses were performed with the binomial distribution of dependent variables because when we treated the number of developed pods as a proportion of the overall number of treated flowers, the number of well-developed seeds as a proportion of the total number of arisen seeds and the number of germinated seeds as a proportion of the number of sown seeds the data did not fit the normal distribution (even after data transformation).

Additionally, the number of ovules produced by diploids and tetraploids was investigated, since this property could affect the result of self-pollination experiments. Ovules were counted in three young flower buds per mother plant from 31 diploid plants and 35 tetraploid plants planted in a common garden. The calyx, standard and wings were removed from the flower buds, the keel and ovary were cut open and ovules were counted using a stereo microscope. The dependence of the mean number of ovules on the ploidy level was tested using GLM with the Poisson distribution of the dependent variable.

### Enzyme analyses

Living plants were collected in nature, stored in a car refrigerator and processed within 36 h after collection. Approximately 60 mg of fresh leaf tissue was processed; the extraction protocol followed that described by Mandák et al. (2005). Extracts were centrifuged under cooling for 10 min at 15 000 r.p.m. and clear supernatants were stored at −75 °C for up to 2 years until
electrophoresis. The electrophoresis conditions were as described by Mandáková and Münzbergová (2008). Four enzyme systems were analysed: shikimate dehydrogenase (SDHD, EC 1.1.1.25), 6-phosphogluconate dehydrogenase (6-PGDH, EC 1.1.1.44), aspartate aminotransferase (AAT, EC 2.6.1.1) and leucine aminopeptidase (LAP, EC 3.4.11.1). The staining procedures followed Vallejos (1983) with the modifications described by Mandák et al. (2005). For 6-PGDH, 10 mg of 6-phosphogluconic acid, 5 mg of NADP, 30 mg of MgCl₂, 5 mg of thiazolyl blue tetrazolium bromide and 1 mg of phenazine methosulfate were combined and dissolved in 30 ml of 0·1 M Tris–HCl (pH 8·4). Gels were incubated in the dark at 32 °C until bands appeared. All gels were then thoroughly rinsed in distilled water, dried between two cellophane sheets and stored.

The isozyme loci were labelled I or II, I being the slowest, and the alleles were labelled a–j, a being the fastest. For all enzymes, banding patterns were examined for relative band intensities, which were interpreted as corresponding to genotypes of different allelic dosages. 6-PGDH and AAT were treated as dimeric enzyme systems according to Weeden and Wendel (1989).

Analysis of genetic variation within populations

To compare genetic variation within diploid and tetraploid populations, the following statistics were computed: the average number of alleles per locus (A), Shannon’s index of diversity (S) (Shannon, 1948), the observed proportion of (zygotic) heterozygotes (Hₒ) and the expected proportion of heterozygotes (Hₑ) with correction for population size 2N/(2N−1). The expected proportion of heterozygotes for tetraploids was computed as for diploids (Hₑ = 1−∑pᵢ²) (Hardy and Vekemans, 2001). In this case, the expected proportion of heterozygotes is not the expected proportion of individuals bearing more than one allele at a locus but the expected proportion of (diploid) gametes bearing two distinct alleles (O. J. Hardy, Université Libre Bruxelles, Belgium, pers. comm.). The number thus represents expected genetic heterozygosity. Additionally, observed genetic heterozygosity, Hₒ, was computed for tetraploids. Observed genetic heterozygosity is the frequency of heterozygous (diploid) gametes within the gamete pool that is inferred to have given rise to the (tetraploid) zygot genotype frequencies. In practice, Hₒ = 1−[f(iii) + 1/2 × f(iij) + 1/3 × f(ijj) + 1/6 × f(ijjk)], where f(iii) is the frequency of zygot with four alleles identical in state, etc., in a given locus and population (Moody et al., 1993). Assuming panmixia and no double reduction, the expectation is that Hₑ = Hₒ in tetraploids, given tetrasomic inheritance (Moody et al., 1993). The above-mentioned statistics were averaged across populations. Data normality was checked using the Shapiro–Wilk test (StatSoft, 2004). Statistical differences between mean values of individual cytotypes were inspected using t-tests with the same variances; differences between mean observed and expected values within cytotypes were tested using paired t-tests in S-PLUS 2000 (MathSoft, 1999).

Heterozygosity in tetraploids

In order to reveal the type of the polyploidization event (autopolyploidization or allopolyploidization) in V. cracca, we assessed the type and frequency of heterozygotes encountered in tetraploids. Tetrasomic inheritance in autotetraploids results in the formation of balanced as well as unbalanced heterozygotes in all possible combinations, because alleles at a given locus on the homologous chromosomes segregate at random. In allotetraploids, two sets of homologous chromosomes pair independently of each other, resulting in disomic inheritance as if they were included in a diploid. If the parental genomes of an allotetraploid are homoyzogous for different alleles, all gametes of such an allotetraploid will be heteroallelic and all offspring will be heterozygous, a condition called fixed heterozygosity (Roos and Gottlieb, 1976; Soltis and Soltis, 1993; Ramsey and Schemske, 2002; Berglund et al., 2006).

We then summed the numbers of homozygotes, balanced heterozygotes (AABB), unbalanced heterozygotes (AAAB or ABBB) and heterozygotes involving three and four alleles over all tetraploid individuals (305 in total). We used a polynomial calculator (available at http://xjrjunque.nom.es/precis/polycalc.aspx?ln=en) to calculate the expected proportions of homozygotes and different types of heterozygotes among the tetraploid individuals under Hardy–Weinberg equilibrium. Polynomial equations expressing Hardy–Weinberg equilibrium with an appropriate number of alleles for the single loci examined in this study are provided in Supplementary Data Table S2. Differences between expected and observed proportions of genotype categories were evaluated with χ²-tests.

Analysis of genetic variation among populations

Two approaches were used to analyse the genetic structure among populations. First, partitioning of the genetic diversity within each cytotype into within-population and among-population components was assessed according to Nei (1973). Total gene diversity (Hₑ), mean genetic diversity within populations (Hₛ) and among populations (DₑST) were computed for both cytotypes separately, and when diploids and tetraploids were grouped together. Total gene diversity was then decomposed into the gene diversity among populations within individual cytotypes as GₑSTwithin = [(DₑST(2i) + DₑST(4i))/2]/HₑT(2i) and between cytotypes as GₑSTbetween = [HₑT(2i+4i)]/(HₑT(2i) + HₑT(4i))/2]. Assuming the same population size in both cytotypes, the total gene diversity in tetraploids should be HₑT with exp = 2HₑT(2i)/(1 + HₑT(2i)) (Moody et al., 1993).

Inbreeding within populations and the level of differentiation among populations were evaluated also using F-statistics (Wright, 1951) estimated in SPAGeDi 1.2 (Hardy and Vekemans, 2002). In this program, F-statistics are based on allele identity and are types of kinship coefficients: they can intraclass correlation coefficients of allelic states for genes within individuals relative to all populations (FₑT), genes within individuals relative to a population (FₛT), and genes within populations relative to all populations (FₑST). The estimation procedure is based on a nested ANOVA following Weir and Cockerham (1984), in which populations are weighted according to their sample size. In addition to standard F-statistics, SPAGeDi also computes the intrapopulation correlation coefficient of relationship RₑST. Ronfort et al. (1998) demonstrated its convenient property of being independent of the ploidy level, the selving rate and the presence of double reduction in polyploids, contrary to the kinship coefficient FₑST. Statistical randomization tests were made by permuting the genes, individuals and locations 10 000 times. Both
Nei’s (1973) and Wright’s (1951) statistics of partitioning of genetic diversity were averaged across loci. Data normality was checked using the Shapiro–Wilks test in STATISTICA 7 (StatSoft, 2004). Statistical differences between mean values of individual cytotypes were tested using t-tests with the same variances in S-PLUS 2000 (MathSoft, 1999).

**Analysis of spatial genetic structure**

For the analysis of spatial genetic structure, tetraploid populations 1–3 were omitted because they were geographically too far from the contact zone of the cytotypes. Differentiation between pairs of populations as a function of their geographic distance was assessed using \( \rho_{ST}/(1 - \rho_{ST}) \) ratios determined for diploid population pairs, tetraploid population pairs and intercytotype pairs using SPAGeDi. Isolation by distance was inspected using the regression of these ratios on geographic distance according to Hardy and Vekemans (2001). Statistical significance was tested using the Mantel test with 999 permutations. To illustrate graphically the relationship between pairwise \( \rho_{ST}/(1 - \rho_{ST}) \) ratios and geographic distance, these inter-population ratios were classified into five 40 km wide distance classes and intraclass means were displayed. Normality of the data was inspected using Shapiro–Wilks test and differences between mean values in the three types of inter-population ratios (diploid–diploid, tetraploid–tetraploid and diploid–tetraploid) were tested using the non-parametric Kruskal–Wallis test and the multiple comparisons procedure in STATISTICA 7 (StatSoft, 2004).

**Meiotic chromosome behaviour**

Immature inflorescence buds (~0.5 cm long) were collected from a tetraploid population in bushy vegetation along the river Odra in Ostrava, Nová Ves, Czech Republic (coordinates: 49° 48’ 45.96” N, 18° 13’ 35.31” E). Buds were fixed immediately in a solution of ice acetic acid and 96 % ethanol (3:1) and kept at 4 °C until making squash preparations. Individual flower buds (~0.2 cm long) were extracted from the inflorescences and macerated for a few seconds in a solution of concentrated HCl and ethanol (1:1) and rinsed in water. Anthers were then placed on a slide and squashed in a drop of lacto-propionic-orcein. Chromosomes were observed under 12 500-fold magnification using a Neubauer microscope equipped with an immersion objective. Chromosome pairing was assessed based on approximately 50 cells observed for each diakinesis and metaphase.

**RESULTS**

**Pollination experiments**

In total, 910 and 839 flowers within 64 and 62 inflorescences on 18 diploid and 17 tetraploid plants were artificially pollinated. Pollination was more successful in tetraploids than in diploids; pod set was 5 % in tetraploids and 2.9 % in diploids (P = 0.02). Overall seed set (the proportion of all seeds from the number of pollinated flowers from mother plants in which at least one pod had developed) was also higher in tetraploids (21.3 %) than in diploids (8.8 %) (P < 0.001). The proportion of well-developed seeds was similar in both cytotypes: 54.8 and 43.5 % of all seeds were well developed in diploids and tetraploids, respectively (P = 0.21). Germination rate also did not differ significantly between diploids and tetraploids (91.3 and 75 %, respectively (P = 0.09). The mean number of ovules produced by diploids and tetraploids was similar (\( \bar{x}_{2} = 5.1 \), s.d. 0.9; \( \bar{x}_{4} = 4.9 \), s.d. 0.8; P = 0.16).

**Enzyme analyses**

Enzyme electrophoresis resolved six putative loci. Only one locus was observed for 6-PGDH and SHDH, while two loci could be distinguished for LAP and AAT. All loci were polymorphic. Banding patterns for LAP and SHDH were as expected for a monomeric enzyme system with one or two bands for diploids and one to three bands for tetraploids. 6-PGDH and AAT exhibited banding patterns expected for a dimeric enzyme, i.e. a three-banded phenotype when two alleles were expressed and a six-banded phenotype when three alleles were expressed, with heterodimeric bands resolved between the two respective homodimeric bands. The banding pattern for LAP I was complicated due to the formation of secondary bands by alleles \( a \)–\( d \). In addition, the slower form of the allele \( a \) co-migrated with the faster form of the allele \( b \); the slower \( b \) form co-migrated with the faster \( c \) form; and the slower \( c \) form co-migrated with the slower \( d \) form. Because of the secondary bands, diploids showed up to four-banded patterns and tetraploids up to five-banded patterns for this locus. The most complex patterns were expressed by the locus AAT II. There were only three alleles, but each produced one secondary band. Since this enzyme is dimeric, the dimers formed by subunits of the same allele, each representing a different mobility form, were resolved as extra bands with an intermediate position. As a result, homozygotes showed three-banded phenotypes. Moreover, individual bands on gels frequently merged into a single band because of a small distance between alleles, and the faintest bands could not be distinguished. Nonetheless, homozygous patterns and the position of the richest bands were sufficient guidelines to determine the allelic configuration of heterozygotes. Example images of banding pattern of single loci and description of the band interpretations are shown in Supplementary Data Fig. S1.

**Genetic variation within populations**

A total of 40 distinct alleles were observed (eight, six, nine, ten, four and three for the loci 6-PGDH, LAP I, LAP II, SHDH, AAT I and AAT II, respectively). Of these, 33 were shared by diploid and tetraploid cytotypes. Three alleles were found in diploids only [LAP II-h (populations 8, 10 and 11), LAP II-i (population 25) and SHDH-j (populations 8 and 9)] and four alleles were found in tetraploids only [6-PGDH-a (population 24), 6-PGDH-b (populations 3, 13, 14, 17 and 18), 6-PGDH-h (populations 4, 12, 14, 15 and 16) and AAT I-b (populations 9 and 18)]. The cytotype-specific alleles were always rare, with population frequency lower than 0.03 (with the exception of 6-PGDH-a in tetraploid plants from population 24 and SHDH-j in diploid plants from population 9, in which the population frequency was 0.11 and 0.07, respectively). Triploids did not possess any unique alleles and they shared alleles with sympatric diploid counterparts. However, these alleles were also common in tetraploids.
Population means of statistics of genetic diversity and heterozygosity are shown in Supplementary Data Table S3. Tetraploids possessed on average more alleles per locus than diploids (Supplementary Data Table S3). Shannon’s index was similar for the two cytotypes (Supplementary Data Table S3). The observed proportion of (zygotic) heterozygosity in tetraploids was 2-fold higher than in diploids, although expected (gametic) heterozygosity in tetraploids and expected (zygotic) heterozygosity in diploids were similar (Supplementary Data Table S3). The mean observed proportion of heterozygotes was significantly different from the expected proportion of heterozygotes in diploids ($P = 0.02$) as well as in tetraploids ($P < 0.001$). However, the mean observed gametic heterozygosity in tetraploids ($H_e'$) was similar to the mean observed (zygotic) heterozygosity in diploids ($P = 0.84$) and even lower than the expected (gametic) heterozygosity under Hardy–Weinberg equilibrium ($P < 0.001$) (Supplementary Data Table S3).

In tetraploids, both balanced and unbalanced heterozygotes were observed. For all loci except LAP I, unbalanced heterozygotes were more frequent than balanced heterozygotes (Table 1). The number of balanced heterozygotes for LAP I was more than three times higher than the number of unbalanced heterozygotes and two times higher than expected under Hardy–Weinberg equilibrium. Complex heterozygotes with three and four alleles were also detected for all loci except AAT I, where only individuals with three alleles were observed. Just three alleles were recorded at the AAT II locus, hence complex heterozygotes with four alleles were not possible at this locus. Furthermore, there was a marked excess of homozygotes balanced by the lack of complex heterozygotes, especially those with four alleles (Table 1). A significant excess of homozygotes with regard to the values expected under Hardy–Weinberg equilibrium was observed also in diploids, although not so large; the difference was 2- to 7-fold for individual loci (data not shown).

Genetic variation among populations

The coefficient of gene differentiation was similar and quite low in both cytotypes, indicating that most isozyme variation was partitioned within rather than between populations (Table 2). The observed total gene diversity in tetraploids was same as the expected value. The total gene diversity for both cytotypes combined was $H_{T(2x+4x)} = 0.444$, 8.8% of which was distributed between cytotypes and 8.2% among populations within cytotypes.

Both the inbreeding coefficient ($F_{IS}$) and the fixation index ($F_{ST}$) were similar in diploids and tetraploids (Table 3). In contrast to $F_{ST}$, the difference between cytotypes in $F_{ST}$ was more than 2-fold. When omitting the most distant populations 1–3, the mean value of $F_{ST}$ for tetraploids was similar to the value in diploids, whereas the mean value of $F_{ST}$ remained significantly higher than in diploids (Table 3).

Spatial genetic structure

Mean $\rho_{ST}/(1 – \rho_{ST})$ ratios (± s.d.) in both tetraploid–tetraploid population pairs (0.178 ± 0.132) and diploid–tetraploid population pairs (0.137 ± 0.088) were larger than in diploid–diploid population pairs (0.095 ± 0.070) ($P < 0.001$ in both cases); the difference between diploid–tetraploid population pairs and tetraploid–tetraploid population pairs was not significant ($P = 0.37$). This was also true for the geographically closest populations (0–39 km, Fig. 2) when testing the differences separately in several distance classes. In the remaining geographic distance classes (40–79, 80–119, 120–159 and 160–199 km) the mean $\rho_{ST}/(1 – \rho_{ST})$ ratios were statistically the same in the three types of pairwise comparison ($P > 0.09$ in all cases). Correlation among $\rho_{ST}/(1 – \rho_{ST})$ ratios of geographic differentiation and geographic distance was non-significant in all cases ($P > 0.16$). Genetic differentiation between diploids and tetraploids within sympatric populations of *V. cracca* was not lower than differentiation between allopatric populations (data not shown).

Genetic variation in diploids

Approximately 50 cells from young anthers of tetraploid *V. cracca* were observed at each diakinesis and metaphase.
Table 2. Partitioning of genetic diversity in diploid and tetraploid populations of *V. cracca* into within- and among-population components according to Nei (1973)

<table>
<thead>
<tr>
<th>Locus</th>
<th>Ploidy level</th>
<th>$H_S$</th>
<th>$H_T$</th>
<th>$D_{ST}$</th>
<th>$G_{ST}$</th>
<th>$H_{T4-exp}$</th>
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</thead>
<tbody>
<tr>
<td>6-PGDH</td>
<td>2</td>
<td>0.328</td>
<td>0.359</td>
<td>0.031</td>
<td>0.087</td>
<td></td>
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<td></td>
<td>4</td>
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<td>0.386</td>
<td>0.030</td>
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<tr>
<td>LAP I</td>
<td>2</td>
<td>0.503</td>
<td>0.565</td>
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<td>4</td>
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<tr>
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<tr>
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<tr>
<td></td>
<td>4</td>
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<td>0.287</td>
<td>0.019</td>
<td>0.066</td>
<td>0.430</td>
</tr>
<tr>
<td>Mean ± s.d.</td>
<td></td>
<td>0.406 ± 0.128</td>
<td>0.440 ± 0.140</td>
<td>0.034 ± 0.017</td>
<td>0.075 ± 0.021</td>
<td></td>
</tr>
<tr>
<td>6-PGDH</td>
<td>2x + 4x</td>
<td>0.342</td>
<td>0.375</td>
<td>0.032</td>
<td>0.086</td>
<td></td>
</tr>
<tr>
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<td>0.538</td>
<td>0.607</td>
<td>0.069</td>
<td>0.114</td>
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<tr>
<td>LAP II</td>
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<td>0.566</td>
<td>0.036</td>
<td>0.064</td>
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<tr>
<td>SHDH</td>
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<td>0.576</td>
<td>0.061</td>
<td>0.106</td>
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<tr>
<td>AAT I</td>
<td>2x + 4x</td>
<td>0.246</td>
<td>0.262</td>
<td>0.016</td>
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<tr>
<td>AAT II</td>
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<td>0.067</td>
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<tr>
<td>Mean ± s.d.</td>
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<td>0.444 ± 0.157</td>
<td>0.039 ± 0.022</td>
<td>0.083 ± 0.023</td>
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</table>

Total gene diversity ($H_T$), mean genetic diversity within populations ($H_S$) and among populations ($D_{ST}$) and the coefficient of gene differentiation ($G_{ST}$) were computed for both cytotypes separately and when diploids and tetraploids were grouped.

Expected value of total gene diversity for tetraploids ($H_{T4-exp}$) according to Moody et al., (1993) is shown.

Differences between diploid and tetraploid means were tested by two sided $t$-tests with the same sample variances.

Mean $H_{T4-exp}$ was tested against observed mean $H_T$ in tetraploids.
multivalent formation was recorded; example microphotographs are shown in Supplementary Data Fig. S2.

### DISCUSSION

#### Type of polyploidy

The allozyme markers supported the autopolyploid origin of tetraploid *V. cracca*. First, there was only weak evidence of the fixed heterozygosity that is expected for allopolyploids (only one locus out of the six investigated, while in all other loci unbalanced heterozygotes prevailed). Secondly, tetraploids possessed almost the same suite of alleles as diploids. The per locus percentage of alleles unique to tetraploids (4/37)/6 = 0.02 %) was comparable to the number reported in the autopolyploid *Heuchera micrantha* (0.02 %) (*Ness et al.*, 1989), *Vaccinium oxyccocos* (0.01 %) (*Mahy et al.*, 2000) and *Aster amellus* (0.04 %) (*Mandáková and Münzbergová*, 2008). However, all the unique alleles are rare and their absence in the other cytotypes is probably due to failure to detect them with the relatively small sample size. Furthermore, the genetic variation between cytotypes was almost the same as the variation among populations within cytotypes.

In contrast to the expectations for autopolyploids, no multivalents were observed at meiosis. However, *Svěšníková’s* (1937) work, which was doubted by *Rousi* (1962), remains the only study that has reported quadrivalent formation during meiosis in tetraploid *V. cracca*. Nevertheless, several taxa showing polysomic inheritance demonstrate that autopolyploids need not form multivalents at meiosis (*Soltis and Soltis*, 1993; *Qu et al.*, 1998; *Hardy et al.*, 2001). A controlled pollination experiment with mother plants of known genotypes should therefore be carried out to discriminate between tetrasomic and digenic–disomic inheritance in order to confirm definitively the autopolyploid origin of *V. cracca*.

#### Genetic diversity and heterozygosity

Tetraploids showed higher genetic diversity than diploids in terms of allelic richness and observed (zygotic) heterozygosity ($H_o$). For both measures, the values obtained for *V. cracca* were among the highest values obtained from allozyme data and published for autotetraploid taxa (listed in *López-Pujol et al.*, 2007). However, observed gametic heterozygosity ($H'_e$), which considers heterozygotes with two or three identical alleles (AABB, ABBB, AABB) as partially homozygous and so decreases their weight (*Moody et al.*, 1993), was similar to observed heterozygosity in diploids and even showed some degree of heterozygote deficiency when compared with expected heterozygosity under Hardy–Weinberg equilibrium. Nevertheless, the significantly higher number of alleles per locus found in this study theoretically might be evolutionarily advantageous for tetraploids, given the possibility of higher phenotypic variation.

#### Breeding system

The coefficient of gene differentiation ($G_{ST}$) for both cytotypes was closest to the value published by *Hamrick and Godt* (1996) for long-lived perennial species with outcrossing ($G_{ST} = 0.094$) and much lower than that reported for long-lived perennials with mixed mating ($G_{ST} = 0.145$). Our pollination experiment

<table>
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<tr>
<th>Locus</th>
<th>F&lt;sub&gt;ST&lt;/sub&gt;</th>
<th>F&lt;sub&gt;IS&lt;/sub&gt;</th>
<th>F&lt;sub&gt;IT&lt;/sub&gt;</th>
<th>P&lt;sub&gt;ST&lt;/sub&gt;</th>
<th>P&lt;sub&gt;IT&lt;/sub&gt;</th>
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</thead>
<tbody>
<tr>
<td>6-PGDH</td>
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<td>0.078</td>
<td>0.032</td>
<td>0.014</td>
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<tr>
<td>LAP I</td>
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<td>0.207</td>
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<td>LAP II</td>
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<tr>
<td>AAT I</td>
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<td>0.044</td>
<td>0.032</td>
<td>0.014</td>
<td>0.013</td>
</tr>
</tbody>
</table>

*Table 3. Wright's (1951) F-statistics and the P<sub>ST</sub>-statistic (Ronfort et al., 1998) for diploid and tetraploid populations of *V. cracca*.*

Values statistically not different from zero (after 10 000 random permutations of locations, individuals and genes) are in italics. Differences between diploid and tetraploid means were analysed using two-sided *t*-tests with the same sample variances.

*Values calculated for tetraploids excluding the most distant populations (populations 1–3).*
proven that self-fertilization is possible in V. cracca, but leads to large number of undeveloped pods. Selfing might take place also in natural populations of V. cracca, as indicated by the positive values of the inbreeding coefficient (albeit non-significant in tetraploids). Alternatively, the positive values of the inbreeding coefficient could be due to biparental inbreeding and/or, in the case of tetraploids, due to loci that do not segregate under perfect tetrasomic inheritance. Relatively low values of $F_{ST}$ indicate substantial gene flow between populations within both cytotypes. However, values of $\rho_{ST}$ suggest greater differentiation among populations and in the case of tetraploids it approximates the above-mentioned value of $G_{ST}$ published by Hamrick and Godt (1996) for long-lived perennials with mixed mating. Since Meirmans and Van Tienderen (2013) have shown that $\rho_{ST}$ is robust against uncertainties related to the exact level of tetrasomic inheritance, we tend to prefer this estimator. So, we conclude that the breeding system in both cytotypes of V. cracca is mixed mating with prevailing outcrossing, likewise in the case of diploids and autotetraploids of Chamerion angustifolium (Ozimec and Husband, 2011).

Furthermore, our results on artificial self-fertilization correspond to the hypothesis that polyploids tolerate a higher rate of autogamy, because tetraploids produced more pods and seeds although they have the same number of ovules as diploids. Unfortunately, comparable empirical data on inbreeding depression in autoployploid taxa are scarce. A smaller reduction in seed set in selfed autotetraploids compared with selfed diploids was recorded in C. angustifolium (Husband and Schemske, 1997). No reduction in fecundity after self-fertilization was observed in tetraploid Anthericum liliago (Rosquist, 2001). In contrast, Galloway and Etterson (2007) detected very high inbreeding depression in autotetraploid Campanulastrum americanum. Additionally, it is apparent that differences in the ability to accept self pollen appear in V. cracca already at the point of pollination, since after fertilization the ratio of aborted to developed seeds was the same in the two cytotypes.

We expect that the lower inbreeding depression indicated by the higher seed set in artificially self-fertilized tetraploids might be associated with an increase in selfing rate in natural tetraploid populations. Thus, the coefficient of inbreeding would be higher in tetraploids relative to diploids in natural populations. Nevertheless, mean values of $F_{IS}$ were similar in the two cytotypes of V. cracca. This could be explained by the fact that heterozygosity declines more slowly in polyploids than in diploids (Bever and Felber, 1992; Moody et al., 1993). Alternatively, we might have sampled fewer inbred individuals than was their true frequency in natural populations due to their reduced growth and survival. Another explanation for similar values of $F_{IS}$ in the two cytotypes of V. cracca may be that larger flowers of tetraploid plants can increase herkogamy and hence decrease the selfing rate (Rausch and Morgan, 2005). This is supported by the fact that tetraploids of V. cracca have larger flowers than diploids (Eliášová, 2008).

**Character of the contact zone**

Primary contact zones are characterized by a high frequency of mixed-ploidy populations comprising several cytotypes (Singliarová et al., 2011), whereas secondary contact zones can be distinguished by parapatric distribution of cytotypes with only a few mixed populations and a low frequency of intercytotype hybrids (Petit et al., 1999). The distribution pattern of cytotypes of V. cracca in Central Europe suggests the latter (Trávníček et al., 2010). Nevertheless, at the European scale, the distribution pattern could show a more complex scenario. Indeed, many European contact zones involving diploids and autoplopoloids were first thought to result from secondary contact, but recent results indicate that both types of contact zones are present within these polyploid complexes [Dianthus sect. Plumaria (Weiss et al., 2002); D. broteri (Balao et al., 2009); Melampodium spp. (Stuessy et al., 2004); Knaudia arvensis agg. (Kolář et al., 2009); Allium oleraceum (Duchoslav et al., 2010); and Aster amellus (Castro et al., 2012; Münzbergová et al., 2013)].

A secondary contact zone with patches of newly originating tetraploids seems also to be the case in V. cracca. In the case of a secondary contact zone where triploid block prevents intercytotype mating, we would expect $\rho_{ST}(1 - \rho_{ST})$ ratios in between-cytotype pairs of populations to be larger than within-cytotype inter-population ratios and that isolation by distance will occur only within individual cytotypes. These assumptions were not met in V. cracca, however. Our data did not show isolation by distance in diploids or tetraploids. We suppose that there could be a human impact on the genetic structure through seed flow in the cultural landscape. Nevertheless, genetic differentiation within tetraploids was greater than within diploids and between...
conclusions

our data complement previous observations and provide additional evidence for the autopolyplid origin of V. cracca tetraploids. They also show a higher level of genetic diversity in terms of allelic richness in tetraploids compared with diploids, supporting the idea that polyploids have greater ability to adapt to changing environment. The mating system in both cytotypes was mixed, with prevailing outcrossing; however, tetraploids showed a higher seed set after artificial selfing. This suggests that polyploids may be more resistant to inbreeding than diploids. There was only weak evidence for a recent origin of tetraploids in sympatric populations of the two cytotypes. The greatest differentiation among the closest tetraploid populations rather suggests a secondary contact zone of the two cytotypes of V. cracca with tetraploids of several independent origins in Central Europe. Nevertheless, we discuss the possibility of a current local origin of tetraploids through a triploid bridge in some regions.

overall, the study thus indicates that V. cracca in Central Europe is of autopolyplid origin and forms predominantly a secondary contact zone. The survival of tetraploids in the area may be supported by their greater ability to adapt to changing environments because of greater allelic richness and greater ability to resist the effects of inbreeding.

supplementary data

supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: list of V. cracca populations examined for this study. Table S2: polynomial equations used for calculation of the expected proportions of homozygotes and different types of heterozygotes among the tetraploid individuals under Hardy–Weinberg equilibrium. Table S3: population means of statistics of genetic variation of homozygotes and different types of heterozygotes among diploid and tetraploid populations of V. cracca. Fig. S1: example images of gels with banding pattern descriptions. Fig. S2: microphotographs of mitotic chromosome configuration.

acknowledgements

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