Evaluation of cytotype and morphological variability of *Vicia cracca* L. (Fabaceae) in central Europe

Zhodnocení cytotypové a morfologické variability druhu *Vicia cracca* L. (Fabaceae) na území střední Evropy

Diploma thesis

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Prague 2008

Diplomová práce **Anežky Eliášové** byla obhájena na katedře botaniky PřF UK v Praze dne 22.9.2008 a ohodnocena klasifikačním stupněm **výborně**.

I declare that I compiled this Thesis on my own and that all the results presented come solely from my original work. All literature and other sources I used to compile the Thesis are listed in the References and properly cited in the text.

Eva Ľšová

Acknowledgement

I would like to express my gratitude to my supervisor Mgr. Ing. Pavel Trávníček for general help and proposal of this diploma thesis; to all colleagues that helped me with collecting plants; to RNDr. Jiří Neustupa, Ph.D., for consultations on geometric morphometrics; to members of the Isozyme Laboratory at the Institute of Botany, Czech Academy of Sciences, for processing of plant material; to Mgr. Bohumil Mandák, Ph.D., for kind help with statistical evaluation of isozyme data; and last but not least to my husband for helpful comments, revisions of English and especially for a great deal of generosity and support.

Content

Summary

I conducted a biosystematic study of the polyploid complex of *Vicia cracca* with 2n = 2x = 14 and 2n = 4x = 28 occurring in the central Europe. Flow cytometry, isozyme analyses, crossing experiments, and geometric morphometrics were employed for the first time to study distribution, reproductive system and morphological variability in this polyploid complex. Using this complex approach, new evidence for the hypothesised autopolyploid origin of tetraploids was obtained. In the area of the former Czechoslovakia, tetraploids and diploids exhibit basically parapatric ranges. Tetraploids are rarely found in Slovakia whereas pure diploid populations were not found in the Czech Republic. A narrow zone of several mixed-ploidy populations along the Czech-Slovak border was recorded. This contact zone is probably of a secondary origin and is maintained by a balance between dispersal rates of the cytotypes and a frequency-dependent selection against inter-ploidy hybrids. A strong triploid block between cytotypes was proved by pollination experiments. Triploids resulting from fusion of reduced and unreduced gametes within diploid populations were very rare (0.1 %) and occurred in a restricted area in northwest Slovakia. Gene flow between cytotypes via the formation of tetraploid seeds by diploid mother plants seems to be possible but also extremely rare. Analyses of isozyme variation suggest that both cytotypes of *V. cracca* exhibit mixed breeding system with prevailing outcrossing. Cytotypes are slightly but significantly different with regard to morphology, especially in flower characters. Besides the quantitative differences, there were also some changes in the shape of floral structures correlated with the ploidy level. Impact of the natural selection on the reinforcement of morphological differences between cytotypes in sympatric populations was tested. Based on my results, recognition of the two diploid races of *V. cracca* delimited previously does not seem to be justified.

KEYWORDS: *Vicia cracca* polyploid complex, autotetraploid, flow cytometry, isozymes, geometric morphometrics, triploid block, minority cytotype exclusion.

Souhrn

Tato práce je biosystematickou studií polyploidního komplexu vikve ptačí (*Vicia cracca*) se dvěma cytotypy (2n = 2x = 14 a 2n = 4x = 28) vyskytujícími se ve střední Evropě. Ke studiu rozšíření cytotypů, způsobu reprodukce a morfologické variability v tomto polyploidním komplexu byly poprvé použity moderní metody jako průtoková cytometrie, analýza isoenzymových dat a geometrická morfometrika. Tento komplexní přístup přinesl nové důkazy o předpokládaném autopolyploidním původu tetraploidů. Rozšíření cytotypů na území bývalého Československa je v zásadě parapatrické. Tetraploidní populace se zřídka nacházejí i na Slovensku, zatímco v České republice nebyly recentně nalezeny žádné čistě diploidní populace. Úzká kontaktní zóna se smíšenými populacemi kopíruje česko-slovenské hranice. Jedná se pravděpodobně o sekundární kontaktní zónu, která je udržována frekvenčně závislou selekcí proti vzniku triploidních hybridů. Silný triploidní blok byl rovněž prokázán experimentálně. Triploidní jedinci vznikající v rámci diploidních populací splynutím neredukované a redukované gamety jsou velmi vzácní (0,1 %) a byli nalezeni jen na malém území na severozápadě Slovenska. Genový tok mezi cytotypy prostřednictvím tetraploidních semen vznikajících na diploidních rostlinách je také možný, i když extrémně vzácný. Isoenzymová variabilita naznačuje, že oba cytotypy kombinují jak autogamii, tak alogamii, přičemž alogamie je převládajícím způsobem rozmnožování. Cytotypy jsou jemně, nicméně prokazatelně morfologicky odlišné, zejména v květní morfologii. Kromě kvantitativních rozdílů byly mezi cytotypy zaznamenány i jisté změny tvaru jednotlivých květních struktur. Rovněž byl testován vliv selekce na míru morfologické diferenciace jedinců odlišné ploidie vyskytujících se ve smíšených populacích. Má data naznačují, že není důvod rozlišovat dvě dříve vymezené rasy diploidů.

KLÍČOVÁ SLOVA: polyploidní komplex *Vicia cracca*, autotetraploid, průtoková cytometrie, isoenzymy, geometrická morfometrika, triploidní blok, vyloučení minoritního cytotypu.

Introduction

Polyploidy is one of the most important and attractive aspect of angiosperms evolution. During the past century, the estimates of frequency of polyploidy in flowering plants have been increasing from 30-35 % up to 70-80 % in dependence on the expanding volume of knowledge and the approach individual authors had used (reviewed by Soltis et al., 2003). Otto & Whitton (2000) estimated that polyploidy represents about 2-4 % of all speciation events in angiosperms. Considerations that perhaps all flowering plants had experienced at least one round of polyploidisation have emerged recently with up-to-date findings of genetics and genomics.

A great boom in a study of polyploidy has set in with introduction of the flow cytometry technique to the plant research. Now we are able to analyse ploidy level of thousands of individuals in weeks. Importance for applied disciplines such as food industry, biotechnology, horticulture etc., contributes also by a great deal to the interest of researchers, particularly if we realise that 15 of 21 world principal crops are of polyploid origin (Bennett 2004).

The frequency of autopolyploids in nature was underestimated for a long time. The main reason for that is a great morphological similarity of autopolyploids and their diploid progenitors and inclination of botanists to the strict taxonomic species concept (Soltis et al. 2007). Secondly, observing mitotic tetravalents as a requirement for the species being counted as a autopolyploid led sometimes to incorrect conclusions because autopolyploids can form even bivalents during mitosis (Qu et al. 1998). Engagement of genetic markers to the study of plants have contributed to the findings that autopolyploids are much more prevalent in natural populations than proposed by Stebbins (1971) and Grant (1981). Although the rate of origin of autopolyploids is lower than that of allopolyploids (hybrids produce a great proportion of unreduced gametes, by fusion of which polyploids arise), it is still higher than the rate of gene mutation (Ramsey & Schemske 1998). Hence, autopolyploids do represent an important mean of sympatric speciation. In the following text, if not specified, polyploids refer to autopolyploids.

Many polyploid complexes of assumed non-hybrid origin are known, but only few were investigated in such details of all aspects of the origin and establishment of new cytotypes as *Tolmiea menziesii* (Pursh) T. & G., *Galax urceolata* (Poiret) Brummitt, *Chamerion angustifolium* (L.) Holub and *Heuchera grossulariifolia* Rydb. Individual cytotypes of the

species mentioned above have separate distribution, are reproductively isolated to some extent and are ecologically and morphologically differentiated. Hence, they fulfil criteria for separate species as defined by several different species concepts (Soltis et al. 2007). Our current knowledge about traits of autopolyploids contradicts earlier tendency of considering autopolyploids inferior to allopolyploids and unable to survive in natural conditions (Stebbins 1985). In some cases, autopolyploids may be distributed even in larger geographic areas than their ancestors; this holds for *Arrhenatherum elatius* (L.) J. Presl et C. Presl (Petit et al. 1997), *Plantago media* L. (Van Dijk et al. 1992) or *Asplenium ceterach* (syn. *Ceterach officinarum* Willd.) (Trewick et al. 2002) polyploid complexes. This fact can reflect superior colonisation abilities of polyploids that allow them to exploit habitats previously unavailable to their diploid progenitors (Maceira et al. 1993). In contrast to allopolyploids, autopolyploids do not have fixed heterozygosity, but they nonetheless have higher heterozygosity than diploids due to tetrasomic inheritance and can maintain three or four alleles at a single locus. Thus, there is a potential for a genetic basis for the success of autopolyploids in nature (Soltis et al. 2003).

Establishment of newly arising polyploids depends on avoiding mating with sympatric diploids to escape the minority cytotype disadvantage or exclusion. The minority cytotype disadvantage consists in ineffective mating with related cytotype resulting in nonviable hybrids (Levin 1975). Polyploids can become reproductively isolated by several means: (1) they can break down self-incompatibility system and evolve self-fertilisation. (2) They can speciate in allopatry or (3) in sympatry through niche differentiation, (4) through different phenology or (5) due to phenotypic divergence and hence assortative mating mediated by different pollinator fidelity.

To study ways by which cytotypes become isolated, contact zones where plants of different ploidy coexist are especially suited (Petit et al. 1999?). Primary contact zones bear witness of proceeding speciation through natural selection for between-cytotype divergence in morphology (e. g. *Heuchera grossulariifolia*, Nuismer & Cunningham 2005) or phenology (*H. grossulariifolia*, Segraves & Thompson 1999, *Arrhenatherum elatius*, Petit et al. 1997). On the other hand, the secondary contact zones originate in areas where lineages of different ploidy level come into contact after expansion from distinct places of origin or glacial refuges. These contact zones are often characterised by low frequency of intercytotype hybrids and of mixed populations and by absence of niche differentiation between two cytotypes. This situation seems to occur in *Centaurea jacea* L. (Hardy et al. 2000), *Aster amellus* L. (Mandáková & Münzbergová 2006) and *Plantago media* (Van Dijk & Bakx-Schotman 1997) in Europe. A balance between dispersal rates and frequency-dependent selection against

hybrids maintains coexistence of cytotypes in close proximity in such cases. This tension can take place on only a few meters, like for example in *Ranunculus adoneus* (Baack 2004). In some cases (e.g. in *Anthoxanthum alpinum*, Felber-Girard et al. 1996), nevertheless, niche differentiation also can contribute to the maintenance of the secondary contact zones. So the dynamics of contact zones is very complex and comprises many factors.

In contrast to overwhelming opinion that intercytotype mating prevents the establishment of polyploids, some recent studies have shown that triploids (or other odd-numbered diploid-polyploid hybrids) may actually facilitate fixation of tetraploids (Husband 2004). Depending on their fertility and the ploidy of their functional gametes, triploids may contribute to production of tetraploid progeny through backcrosses with diploids or matings with other triploids. Assuming that the triploids are viabile, this way of tetraploid formation through a process called the triploid bridge is more likely than through the union of two unreduced gametes (Ramsey & Schemske 1998). Indeed, literature available on wild species of mixed ploidy supports the presence of triploids in nature, albeit often at low frequencies (Husband 2004). So, by influencing the rate of recurrent polyploid formation, triploids may enhance the establishment of a dynamic equilibrium, with diploids in the majority and tetraploids in the minority following Felber's theoretical model (1991). Although the data on triploid fitness and gamete composition suggest that the triploid bridge alone may not account for the evolution of autotetraploids in *C. angustifolium*, triploids probably contribute to the prevalence of mixed-ploidy populations in this species (Husband 2004). In sporadic cases not only triploids originated through union of reduced and unreduced gametes, but also triploids from intercytotype matings may participate in recurrent polyploid formation (Peckert & Chrtek 2006).

Minority cytotype disadvantage may be overwhelmed or at least ameliorated by self-fertilisation (Rodríguez 1996). A major negative consequence of selfing, though, is inbreeding depression. It is supposed that autopolyploids may exhibit lesser inbreeding depression than diploids, owing to the presence of multiple gene copies and the associated reduction in the rate of homozygote formation. In agreement with this notion, allozyme markers document higher genetic diversity and higher proportion of heterozygotes in tetraploids than in diploids (Mahy et al. 2000, Ness et al. 1989). Furthermore, low inbreeding depression relaxes a pressure against the evolution of selfing. Indeed, data on angiosperm polyploids support the hypothesis that polyploids have, on average, higher rates of self-fertilisation than their diploid relatives (Barringer 2007). It was shown that greater tetraploid selfing rates and lower inbreeding depression decrease the critical threshold for tetraploids to spread to fixation and

also reduce the minimum rate of unreduced gamete production required for tetraploids fixation (Rausch & Morgan 2005).

Several studies have brought evidence of greater size of polyploid individuals, their organs or cells (Segraves & Thompson 1999, Stebbins 1971). Many of the gigas traits of polyploids may be a direct consequence of increased DNA content. Furthermore, phenotypic divergence between cytotypes may occur as a result of increased or altered gene expression as it has been proven in synthetically produced polyploids (Ramsey & Schemske 2002). Increased gene dosage allows polyploids to harbour three or more alleles per locus, and hence also exhibit greater overdominance than a diploid (Bever & Felber 1992).

Another possibility is that phenotypic divergence is a consequence of natural selection for increased assortative mating or competition between cytotypes for pollinators (Segraves & Thompson 1999, Nuismer & Cunningham 2005). Floral morphology plays the most important role in the interactions between insects and plants. Tools of the classical morphometrics are certainly useful for taxonomists, but insect perceive the flower as a complex organ. Geometric morphometrics has proven as a very successful tool for analysing the evolution of complex morphological structures. Gómez et al. (2006) employed geometric morphometrics to demonstrate natural selection for zygomorphic flowers in *Erysimum mediohispanicum*: plants with zygomorphic flowers had received more pollinator visits then those with actinomorphic flowers.

Individuals from mixed-ploidy populations should be included in the study of morphological differences because it is assumed that differences between cytotypes, if any, would have a genetic basis, as both cytotypes were subjected to common environmental conditions (Hardy et al. 2000). Moreover, if we assume that polyploids originated locally from diploids would be less divergent than those from secondary contact zones, a degree of differences between cytotypes of mixed-ploidy and cytologically pure populations can indicate the origin of contact zones.

Vicia cracca agg.

The genus *Vicia* L. comprising approximately 190 species (ILDIS, 2005) is on the basis of a phylogenetic analysis of the plastid gene *mat*K a paraphyletic taxon with *Lathyrus* L., *Pisum* L. and *Lens* L. nested within it, and together with *Vavilovia* A. Fedorov forms a monophyletic tribe *Vicieae* Adans. (Steele & Wojciechowski 2003). The paraphyly of *Vicia*

was repeatedly confirmed by a phylogenetic analysis based on the nuclear ribosomal internal transcribed spacer (nrITS) sequences (Choi et al. 2006). The genus is widely distributed in the temperate zone of the northern hemisphere, and in extratropical South America. The most striking species diversity is to be found in the Mediterranean region and the Caucasus, but the centre of origin is still controversial (Van de Wouw et al. 2001).

There are two subgenera traditionally recognised: subgen. *Vicia* and subgen. *Cracca* (Dumort.) Peterm. (syn. *Vicilla* (Schur) Rouy). The latter contains approximately three quarters of species of the entire genus and is traditionally divided into 17 sections according to Kupicha (1976) with slight modifications suggested by Leht (2005) and Jaaska (2005). It is a rather heterogeneous assemblage characterised by several primitive or (compared to the subgen. *Vicia*) less-derived character states (perennial growth, many-flowered, long-peduncled racemes, simple stipules without nectariferous spots, more ancestral symmetric karyotypes with mainly metacentric chromosomes, and the presence of a nonproteinogenic amino acid canavanine in the seeds), although they are shared always by only a part of the taxa in the subgen. *Cracca* (Hanelt & Mettin 1989). The distribution of the subgen. *Cracca* covers almost the total area of the genus, and distributional limits are mostly made up by *Cracca* species.

The basic chromosome numbers in the genus are x = 5, 6, or 7, with x = 7 as probably the most ancestral number (Hanelt & Mettin 1989). Polyploidy plays a limited role in the evolution of the genus (mere 19 out of 144 species with known chromosome numbers are polyploids) and is restricted only to the subgen. *Cracca*, mainly to the sects. *Cracca* Dumort. and *Cassubicae* Radzhi (Hanelt & Mettin 1989).

The section *Cracca* is the broadest and the most variable one of the subgen. *Cracca*. Since the delimitation of the sect. *Cracca* by Kupicha in 1976, several phylogenetic analyses were performed. Whereas the analysis based on isozyme data proved the monophyly of this section (Jaaska 2005), cladistic and phenetic analyses based on morphological data showed more complicated situation with species of the sects. *Variegatae* Radzhi and *Panduratae* Kupicha distributed throughout the sect. *Cracca* (Leht 2005). Nonetheless, results of both studies are not consistent with Kupicha's placement of *V. hirsuta* (L.) S. F. Gray in the sect. *Cracca*. Choi et al. (2006) consider the sect. *Cracca* as a monophyletic group sharing a laterally compressed style as an apomorphic character.

The section *Cracca* includes also *Vicia cracca* agg. This aggregate contains very similar species with partially overlapping geographical ranges as *Vicia cracca* L. s. str., *V. oreophila* Žertová, *V. tenuifolia* Roth, *V. incana* Gouan,and *V. dalmatica* A. Kern. *V. cracca* is native in

the whole Eurasia but its current distribution is much wider, because it was introduced to North America, Australia and New Zealand. *V. tenuifolia* has somewhat more southern distribution in Eurasia in comparison to the previous species. *V. incana* is distributed in the Mediterranean region, western and central Europe (Heywood & Ball 1968) and in Lithuania and Ukraine (Tzvelev 1987). *V. dalmatica* was observed in Germany, western, south-eastern and southern Europe, Ukraine and south-western and southern Asia. *V. oreophila* occurs locally in central European mountain systems (Žertová 1962; Chrtková 1995).

They are all perennial herbs, though *V. cracca* and *V. incana* are climbing herbs whereas the other two are non-climbing. Morphological characters as the number of leaflets, length of inflorescence with respect to the subtending leaf or blade/claw standard length ratio are not sufficiently reliable to distinguish the species. However, the species differ in the number of chromosomes, since *V. cracca* has 2n = 2x = 14 or 2n = 4x = 28, *V. oreophila* is tetraploid with 2n = 28, *V. incana* and *V. dalmatica* possess 12 chromosomes and *V. tenuifolia* has 2n = 4x = 24.

Nevertheless, in addition to aneuploid plants, there are some confounding chromosome numbers in the karyological literature: 2n = 12 for *V. cracca* (Sakamura 1914, Sveshnikova 1927, both in Senn 1938, Rousi 1961) and for *V. tenuifolia* (Činčura 1963, Rousi 1963) and on the other hand 2n = 14 for *V. incana* and 2n = 28 for *V. tenuifolia* (Roti-Michelozzi 1992). Later, Rousi (1973) reached the conclusion that, according to karyotype comparison, 12-chromosome strains of *V. cracca* reported by her in 1961 and of *V. tenuifolia* studied by Činčura (1963) should be considered as *V. incana*. Rousi mentioned that *V. incana*, when grown in garden conditions, lost much of the hairiness density, so plants did not give the impression of a typical *V. incana*. She noted further that the 12-chromosome strain of *V. tenuifolia* reported by her in 1963 resembled morphologically *V. dalmatica*. Rousi foreshadowed as early as in 1961 the feasible evolution of the 24-chromosome lineage from the 12-chromosome one by centric fusion of two chromosome pairs in the 14-chromosome lineage and subsequent polyploidization. Whereas Rousi (1973) maintained that 7- and 6-chromosome lineages are two well-differentiated and distinct evolutionary lineages, Roti-Michelozzi (1992) supported the hypothesis that the *V. cracca* group as a whole is still evolving. There are also disagreements as to whether there is involved in the origin of *V. tenuifolia* only *V. incana* (Roti-Michelozzi 1992) or both 12-chromosome diploids, i.e. *V. incana* and *V. dalmatica* (Rousi 1973). Dvořák et al. (1977) is of an opinion that if *V. tenuifolia* developed by allopolyploidy, the parental species must have had very similar karyograms.

An autopolyploid origin of *V. cracca* tetraploids is hypothesized with regard to the essentially similar karyotypes of the two cytotypes (Rousi 1961, Dvořák et al. 1977), meiotic chromosome behaviour (Rousi 1962), great morphological similarity (Rousi 1973) and existence of vigorous and morphologically normal aneuploid individuals with 2n = 27 and 2n = 30 (Rousi 1961) and 2n = 18-26 noted by Chrtková-Žertová (1973a).

Geographic distribution in central and northern Europe of both cytotypes was investigated in detail by Chrtková-Žertová (1973a; 1973b), but only scarce information from other parts of the species range is available. Data published to date are summarized in Fig. 1 and Tab. 1. Chrtková-Žertová distinguished three races of *V. cracca*: a widely distributed tetraploid race and lowland and mountain diploid races. She recorded a very interesting pattern since tetraploids occurred mostly in the Czech Republic and diploids more eastwards with a contact zone on the Czech-Slovak borders (Fig. 6B).

V. cracca occurs in many forms on the territory of the Czech Republic, nonetheless most of them depend on the conditions of the habitat and thus represent only inconstant ecomorphoses without any higher systematic value (Chrtková-Žertová 1973a). Very variable characters like the height of plants, length of internodes, number of stems, size and shape of leaflets or hairiness changed rapidly when plants were cultivated in garden conditions. On the other hand, size of the flowers and pods and the number, shape and size of seeds did not show a great variability, if any. The number of leaflet pairs and the length of inflorescences and its ratio to the length of the subtending leaf seemed to be the only constant characters that showed some differences between races. However, Chrtková-Žertová (1973a) concluded that it is impossible to distinguish individual plants in all cases. Rousi (1973) reached the same conclusion; nonetheless she described diploids with smaller leaves, flowers and seeds.

Zhang & Mosjidis (1998) used isozymes to rapidly predict the mating system of *Vicia* species. They supposed that within-accession variability lower than among-accession variability corresponds to self-fertility. According to this, *V. cracca* seems to be a self-fertilizing species. On the other hand, Jaaska (2005) observed frequent heterozygous isozyme phenotypes within *V. cracca* accessions suggesting extensive outcrossing. Nevertheless, in both studies population samples were not used, so results may be confounding. Rousi (1973) placed two diploid individuals among tetraploids and let insects pollinate them. Although the seed set was very poor and seeds were mostly shrivelled and undersized, the progeny consisted of 11 diploids, one triploid and one tetraploid hybrid. Both triploid and tetraploid hybrids grew up into vigorous plants. The tetraploid was normally fertile, whereas the triploid was almost sterile. The results suggested that diploids are at least partly self-compatible and

that hybridisation between two cytodemes in nature may result in tetraploid individuals. Some triploid plants were found also in nature (Chrtková-Žertová 1973a; Roti-Michelozzi 1992).

In my study of the polyploid complex *V. cracca* I addressed the following questions:

Is *V. cracca* really an autotetraploid? What is the current distribution of cytotypes in the central Europe and what have changed over the last 40 years? Where do mixed-ploidy populations occur? What is the origin of the contact zone? Do intercytotype hybrids occur in nature? Are there some morphological differences between cytotypes? Are tetraploids and diploids mutually more differentiated in mixed populations than in pure populations? Is *V. cracca* a self-compatible species? Are there differences in the selfing rate between cytotypes? Does a triploid block play a role in a mixed-ploidy population dynamics?

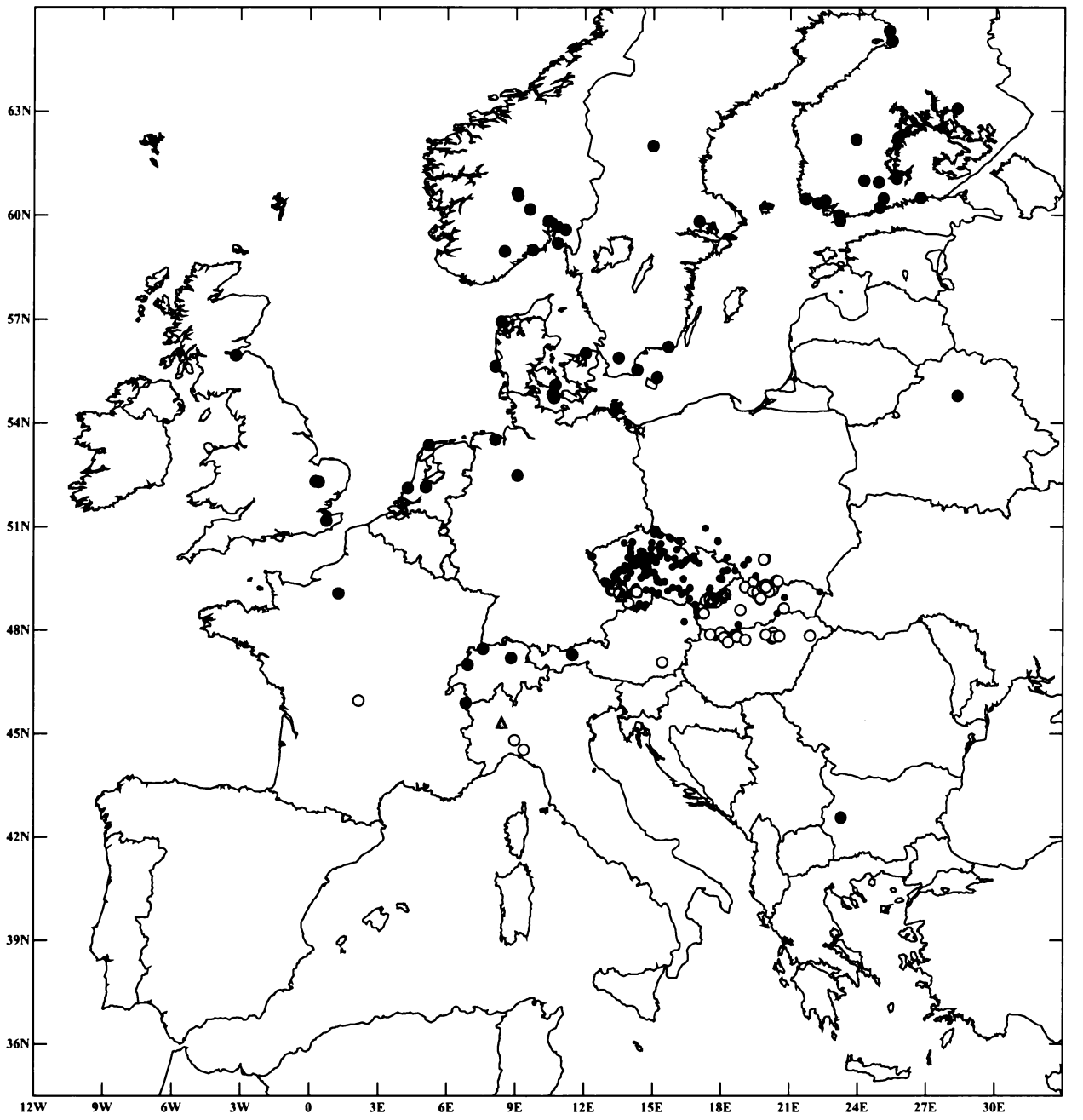


Fig. 1. Distribution map of *Vicia cracca* cytotypes in Europe based on published chromosome counts (see Table 1 for references). Open circles, diploids; black circles, tetraploids; grey triangles, mixed (2x + 4x) populations. Triploids are not shown. The symbol size of tetraploids is reduced in areas with a plenitude of chromosome records to aid legibility. The map was created by P. Trávníček on the basis of data from my literature survey.

Country	Ploidy level(s)	Reference(s)
Afghanistan	4x	Podlech & Dieterle 1969
Austria	2x, 4x	Rousi 1961, 1962; Chrtková-Žertová 1973a
Bulgaria	4x	Kuzmanov 1975
Byelorussia	4x	Semerenko 1989
Canada	4x	Ledingham 1957; Mulligan 1961; Rousi 1961; Tomkins & Grant 1978
Czech Republic	2x, 3x, 4x	Chrtková-Žertová 1973a; Dvořák et al. 1977
Denmark	4x	Rousi 1961; Chrtková-Žertová 1973b
Finland	4x	Rousi 1961; Chrtková-Žertová 1973b; Arohonka 1982
France	2x, 4x	Rousi 1961
Germany	4x	Rousi 1961; Chrtková-Žertová 1973b
Hungary	2x, 4x	Baksay 1954; Rousi 1961; Chrtková-Žertová 1973a
Iceland	4x	Rousi 1961
Italy	2x, 3x, 4x	Gadella & Kliphuis 1970; Roti-Michelozzi 1984, 1992; Roti-Michelozzi & Allione 1987
Japan	2x	Huziwara & Kondo 1963
Mongolia	2x	Měsíček & Soják 1969, 1995
Netherlands	4x	Rousi 1961; Gadella & Kliphuis 1963; Hommel & Weiffering 1979
New Zealand	4x	Rousi 1961
Norway	4x	Chrtková-Žertová 1973
Poland	2x, 4x	Ryka 1954; Rousi 1961; Chrtková-Žertová 1973a
Russia (Asian part)	2x, 3x, 4x	Rousi 1961; Belaeva & Siplivinsky 1975, 1977, 1981; Krasnoborov et al. 1980; Nikiforova 1984, 1990; Sokolovkaya et al. 1989; Krasnikov & Schaulo 1990; Volkova et al. 1999
Russia (European part)	4x	Yefimov 1987, 1988
Slovakia	2x, 4x	Činčura 1963, 1981; Chrtková-Žertová 1973a, Dvořák et al. 1977; Hallonová 1982
Sweden	4x	Rousi 1961; Chrtková-Žertová 1973b; Lökvist & Hultgård 1999
Switzerland	4x	Rousi 1961
Turkey	2x	Şahin & Babaç 1990; İnceer & Hayirlioğlu 2005
United Kingdom	4x	Rousi 1961

Tab. 1. Summary of published data on ploidy levels (chromosome counts) of *Vicia cracca*. The table was created by P. Trávníček on the basis of data from my literature survey.

Material and methods

Sampling of plant material

Plants were sampled during years 2006-2008 especially in the Czech Republic and in Slovakia. Additional samples originated mostly from the northern part of Austria and two samples were from Germany. A total of 6 613 plants from 261 populations were collected. For information about geographic coordinates and altitude of localities, number of individuals analysed, DNA ploidy levels, and collector(s) see Appendix 1. 1-156 plants (25.3 on average) per population and one leaf per plant were collected. At least 5-m distance among individual plants was kept as far as possible to avoid sampling of sib progeny. A fine-scale cytotype distribution was mapped in one mixed-ploidy population with similar frequency of both ploidies (no. 189, CZ – Horní Suchá). Distributional maps were prepared using DMAP for Windows, ver. 7.2e (Alan Morton, Windsor, UK).

Composition of mixed-ploidy populations

To test the prediction that tetraploids should be in low frequencies in the mixed populations if they are arising from diploids *de novo*, mixed populations were divided into five categories according to the percentage of diploid and tetraploid individuals (0-20 %, 21-40 %, 41-60 %, 61-80 % and 81-100 %), and analysed using a chi-squared goodness-of-fit test in PAST ver. 1.75 (Hammer et al. 2007) to determine if there were differences in the distribution between cytotypes and if there was deviation from a uniform distribution within individual cytotypes.

Segregation of cytotypes in a mixed-ploidy population

The segregation of diploid and tetraploid plants in a mixed population was tested by determining the ploidy level (i.e., same vs. different) of two nearest neighbours for all individuals. Deviations from the random distribution were assessed by the Fisher's exact test (S-Plus 6.2 for Windows professional edition).

Flow cytometry

DNA ploidy levels of plant samples were estimated by flow cytometry in the Laboratory of Flow Cytometry, Průhonice, Czech Republic. Young, intact leaf tissue of the analysed plant(s) and an appropriate amount of the leaf tissue (half in the case of supposed tetraploids) of the internal reference standard (*Pisum sativum* 'Ctirad', 2C = 8.84 pg; Greilhuber & Ebert 1994) were chopped together with a sharp razor blade in a plastic Petri-dish containing 0.5 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5% Tween 20; Otto 1990). The crude suspension was filtered through a 0.42 μm nylon mesh to remove tissue debris and then was left for approximately half an hour. Isolated nuclei were stained with 1 ml of Otto II buffer (0.4 M $Na_2HPO_4.12H_2O$) supplemented with AT-selective fluorochrome 4',6-diamidino-2-phenylindole (DAPI) and ß-mercaptoethanol at final concentrations of 4 μl/ml and 2 μl/ml, respectively. Relative fluorescence intensity of at least 3 000 particles was recorded on a PA-II flow cytometer (Partec GmbH, Münster, Germany) equipped with a mercury lamp for UV excitation. Resulting histograms were evaluated by the FloMax software (Partec GmbH, Münster, Germany), and DNA ploidy levels were determined on the basis of sample/standard ratio. Usually, bulk samples of up to 10 plants (one leaflet per each) were measured. A very good congruency between the number of nuclei in particular peaks and the number of analysed individuals with different ploidy levels allowed estimation of the cytotype proportion in mixed samples with a high accuracy. Only histograms with coefficients of variation (CVs) of G_0/G_1 peaks of both the bulked sample and the standard below 3.5 % were considered. If the quality of analyses did not meet this criterion, all plants from the bulked sample were analysed separately (e.g., to detect potential between-plant differences in fluorescence intensity).

Chromosome counts

To confirm the reliability of ploidy estimates, FCM results were supplemented by conventional chromosome counts. After disrupting a seed coat with a sand paper, seeds were stored on a wet filter paper for two days at 5°C in order to induce germination. Actively growing root tips of germinating seedlings were pre-treated with 0.002 M 8-hydroxyquinoline for 2 hours, fixed overnight in a 1:3 mixture of ice acetic acid and 96% ethanol, macerated for 80 s in 1:1 HCl:96% ethanol at room temperature, and stained with acetocarmine. Chromosomes were observed under 1000-fold magnification using an Olympus BX61

microscope equipped with an immerse objective. Four diploids (i.e., 3 plants from population no. 192 and 1 plant from population no. 234) and four tetraploids (i.e., one plant from each population 68, 84, 124, and 192) were analysed. At least three well-spread chromosome plates were counted for each individual.

Classical morphometrics

410 individuals (195 diploids and 215 tetraploids) from 42 populations were sampled for morphometric evaluation. 75 diploid and 34 tetraploid plants originated from five mixed-ploidy population (Tab. 2). Ten plants per population were collected in average (ranged from two to 49); a primary aim was to grasp an overall variability across the cytotypes, hence so few plants per population were sampled. The ploidy level of specimens was determined by means of flow cytometry as described above.

Morphological characters were measured on herbarium specimens collected in the field; characters measured on flowers were performed on inflorescences stored in 70% ethanol.

The following 29 quantitative characters were measured: main stem length, number of internodes, mean length of the lower fifth of internodes, number of leaflet pairs of a leaf in the lower third of the stem, number of leaflet pairs of a leaf in the middle of the stem, number of leaflet pairs of a leaf in the upper third of the stem, length of a leaf in the lower third of the stem (without tendril), length of a leaf in the middle of the stem, length of a leaf in the upper third of the stem, mean hairiness of upper surface of leaflets in the lower third of the stem (number of hairs per 1 mm^2 averaged from three leaflets), mean hairiness of upper surface of leaflets in the middle of the stem, mean hairiness of upper surface of leaflets in the upper third of the stem, maximum inflorescence length, length of a flower, length of a calyx, length of upper calyx tips, length of the standard blade, length of the standard claw, distance from standard claw base to the standard claw widest point, width of the standard claw, width in the middle of the standard, width of the standard blade, length of the wing blade, length of the wing claw, width of the wing (two characters, see Fig. 2), length of the keel, length of the keel claw, mean anther length. Delimitation of floral traits is depicted at Fig. 2. In addition, one qualitative character (presence of teeth on stipules in the lower two thirds of the stem) was pursued and four ratios were computed: mean inflorescence/subtending leaf length ratio, standard blade/calyx length ratio, standard blade/claw length ratio, and standard claw/blade width ratio. These characters were selected following initial analysis performed on 20 diploids and 20 tetraploids in order to include characters that might have discriminatory value.

Non parametric Spearman correlation coefficients were computed. At first, principal component analysis (PCA) based on a correlation matrix, and subsequently canonical discriminant analysis (CDA) and non-parametric classificatory discriminant analysis of all individuals were performed on all measured characters. The discriminant function in the classificatory DA was determined by a cross-validation procedure using 21 nearest neighbours. The same analyses were then performed on 14 characters most correlated with the canonical axis selected on the basis of a total canonical structure. In addition, a stepwise discriminant analysis was carried out. Other two datasets analysed in the same way were 14 characters selected from first CDA plus six additional characters selected in stepwise DA, and 16 characters selected in stepwise DA. Moreover, CDA and classificatory DA of individuals coming only from cytotypically pure populations and PCA of population means as objects were performed. Subsequently, a cluster analysis (UPGMA, unweighted pair-group method using arithmetic averages) of population means was performed. Prior to clustering, data were standardised to zero mean and unit standard deviation. A distance matrix was computed using Gower coefficient for mixed data. Mann-Whitney U test was used to test whether the medians of selected characters are different among cytotypes. Chi-square test was performed to test whether there are differences in presence of the teeth on the stipules.

In order to assess an extent of cytotype diversification within mixed and among pure populations, the following procedure was performed. The first 30 principal component scores (explaining 99.8 % of overall variability) from analysis performed on all measured characters from all specimens were used as a data matrix for calculation of Euclidean distances among individuals. Sets of distances between individuals of different ploidy from mixed populations and of distances between individuals of different ploidy from pure populations were extracted and dissimilarity of medians of these two sets was checked using Mann-Whitney U test. The same procedure was done with data from PCA of only characters on generative organs (v19-v37, using 16 principle components explaining 99.8 % of variability) and separately with values of characters measured on the keel (v35 and v36).

Analysis of similarities (ANOSIM) using Euclidean distances calculated form the first 30 principal component scores was employed to test a possible impact of altitude on morphological variability within cytotypes. For this analysis, populations were divided into two groups with the altitude of locality lesser or higher than the median altitude. In addition, permutation test was performed on the same dataset.

All morphometric analyses were done using the SAS 9.1.3 package (SAS Institute 2002-2003), except for the cluster analysis, ANOSIM, permutation test for two multivariate groups,

calculation of Euclidean distances, Mann-Whitney U test and chi-square test which were run using PAST ver. 1.75 (Hammer et al. 2007).

Geometric morphometrics

The shape of flower parts was studied by means of geometric morphometric tools, using a landmark-based methodology. Flowers preserved in 70% ethanol were cut apart into a calyx, standard, wings and keel. Digital photographs were taken under 12.5-fold magnification using a binocular magnifying glass equipped with a camera. A standardized procedure was used; the same flower parts had the same orientation, calyces were unfolded to the plain, standards were folded along the midrib, left wings were used. One flower per plant was used in the analysis. 4-38 individuals per population were analysed, totally 170 individuals from 22 populations, 85 diploids and 85 tetraploids (populations sampled are listed in Tab. 2). Five landmarks were defined on each flower structure except the standard where only three landmarks were defined (Fig. 2). All landmarks were considered to be of Type II; however, the fourth and fifth landmarks of wing are supported as much by histological evidence as by geometric evidence and may be considered Type I landmarks (see Zelditch et al. 2004 for landmark definitions). Additionally, some semilandmarks were used to provide comprehensive coverage of shape of the individual flower parts (Fig. 2).

Landmarks and semilandmarks were digitised in TpsDig ver. 2.10 (Rohlf 2006) and the individual objects were superimposed in TpsRelw ver. 1.45 (Rohlf 2007a) using the generalised Procrustes analysis that standardises the size of the objects and optimises their rotation and translation so that the distances between corresponding landmarks are minimised (Bookstein 1991, Zelditch et al. 2004). Then, using the same software, the relative warps analysis (RWA) with $\alpha = 0$ (=PCA of Procrustes residuals) were performed (Rohlf 1993). Scores of objects on the first 20 axes were used for PCA and classificatory DA in PAST. Unfortunately, TpsRelw does not enable to label individuals of each cytotype, hence PCA of relative warps in PAST was performed in order to show differences between cytotypes. Nevertheless, 20 relative warps described more then 99 % of total variability, so the result of PCA was virtually same as the result of RWA. Further, cluster analysis (UPGMA) of population means was performed in PAST; the distance matrix was computed using Euclidean distances. Scores of objects on the discriminant axis were used as an independent variable in TpsRegr ver. 1.33 (Rohlf 2007b) in order to find how the shape differentiates between cytotypes.

Scores of objects on the first 20 axes were used as data in other analyses performed in PAST. Equality of the means of the two multivariate groups (cytotypes) were tested using permutation with 2000 replicates and the Mahalanobis squared distance measure. Comparison of the extent of divergence between cytotype in mixed versus pure populations was done in the same way as for classically measured morphological characters above.

Crossing experiments

Plants from natural populations were transplanted into pots and grown to flowering in the experimental garden of the Institute of botany, Academy of Sciences of the Czech Republic, Průhonice. The ploidy of each plant was determined using flow cytometry. In total, 54 diploid and 31 tetraploid plants were involved to the crossing experiments during years 2007 and 2008; for the numbers and the origin of plants used in the experiments see Tab. 2. Plants used in the crossing experiments were randomly selected from those that were just flowering at the particular day. Two types of experiments were conducted. First, reciprocal 2x – 4x and 2x – 2x crossings were performed. Alternatively, plants of both cytotypes were pollinated by transferring pollen among flowers within one inflorescence. Each plant served as a maternal and a paternal parent because emasculation was not possible. Pollinated flowers were chosen randomly within the inflorescences and approximately one third of flowers were pollinated at one treatment. Pollination of each inflorescence was repeated at least twice depending on the degree of inflorescence bloom. To pollinate flowers with a brush, a style had to be exposed by putting down a keel. After artificial pollination, inflorescences were prevented from pollination by insects with nylon bags. Ploidy level of the mater seeds was determined in the same way as for adult plants but with one modification – bulked samples of maximally two seeds were prepared. Additionally, 306 seeds from 18 plants sampled in nature from four populations of diploids (no. 232, 234, 247 and 248) were analysed to estimate the rate of origin of triploids and tetraploids through the union of unreduced gametes.

Isozyme analyses

In total, 423 plants from 15 populations were sampled. This comprises 250 diploid individuals (39 individuals from two pure diploid population, 53 plants from two diploid-triploid mixed populations and 158 plants from seven diploid-tetraploid mixed populations), 8 triploids from two above-mentioned populations and 165 tetraploids (109 individuals from five pure tetraploid populations and 56 plants from mixed-ploidy populations) (listed in Tab. 2). Twelve to 46 (26 in average) plants per population were collected.

Live plants were collected in nature, stored in a car refrigerator and processed in the course of 36 hours since collection. Ploidy level was estimated by means of flow cytometry described above. Electrophoresis was performed on crude protein extracts of leaf material. Approximately 60 mg of fresh leaf tissue was ground with Dowex-Cl (1-X8) and homogenized on ice in 0.5 – 0.75 ml Tris-HCl extraction buffer ("viola"): 0.1 M Tris-HCl pH 8.0, 70 mM 2-mercaptoethanol, 26 mM sodium metabisulfite, 11 mM ascorbic acid, 4% (w/v) polyvinylpyrrolidone. Extracts were centrifuged under cooling for 10 min at 15,000 rpm and clear supernatants were stored at -75 °C for up to 2 years until electrophoresis. Isozymes were separated on native-PAGE, 30 µl of each sample was loaded for electrophoresis in a Hoefer vertical electrophoresis unit (Amersham). All enzymes were resolved on the polyacrylamide gels using 8.16% separating gel and 4% stacking gel. The separating gel was made using a buffer of 1.82 M Tris-HCl pH 8.9, and the stacking gel using a buffer of 0.0069 M Tris-HCl pH 6.9. The electrode buffer consisted of 0.02 M Tris and 0.24 M glycine (pH 8.3).

Four enzyme systems were investigated on a small sample of plants in the first step, and since they yielded a clear pattern they were analysed further. They included shikimate dehydrogenase (SHDH, 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 following modifications: For SHDH, 30 mg of shikimic acid, 5 mg of NADP, 6 mg of MTT and 1 mg of PMS were combined and dissolved in 30 ml of 0.1 M Tris-HCl (pH 8.4). 6-PGDH: 30 ml of 0.1 M Tris-HCl (pH 8.4), 10 mg 6-phosphogluconic acid, 5 mg NADP, 30 mg $MgCl_2$, 5 mg MTT and 1 mg PMS. Gels were incubated in the dark at 32°C until bands appeared. The gel stained for LAP was rinsed in buffer [0.2 M Tris-maleate (pH 6)] and incubated in darkness at 35°C for 10 min with 50 mg L-leucyl-ß-naphthylamide.HCl (in 50% acetone) and 60 mg $MgCl_2$ (both dissolved in 30 ml of buffer). Afterwards, a solution of 25 mg Fast Black K salt

in 30 ml of buffer was added and the gel was incubated in dark until bands appeared. Two staining solutions were prepared for AAT (A: 20 ml 0.1 M Tris-HCl (pH 8.4), 240 mg aspartic acid, 40 mg α-ketoglutaric acid and B: 20 ml 0.1 M Tris-HCl (pH 8.4), 50 mg Fast Blue BB salt, 50 mg Fast Violet B, 25 mg pyridoxal-5-phosphate). Solution A was prepared at least 15 min before the application. The gel was rinsed in water and then in Tris-HCl (pH 7.0) buffer. The solutions were then mixed and poured on the gel. The gel was incubated in the dark at 35°C until bands appeared, and then rinsed and fixed with a 1:1:3:5 solution of glycerine, acetic acid, H_2O and methanol. Afterwards, all gels were thoroughly rinsed in distilled water, dried between two cellophane sheets and stored.

The isozymes were labelled *I-II*, *I* being slower, the alleles *a-k*, *a* being the fastest. For all enzymes, banding patterns were examined for relative band intensities that were interpreted as corresponding to genotypes of different allelic dosage. 6-PGDH and AAT were treated as dimeric enzyme systems according to Weeden & Wendel (1989).

Nei's genetic distances (Nei 1972) between 11 diploid and 8 tetraploid populations (tetraploid and triploid individuals of low frequencies were removed from population no. 157, 171, 172, 181, 227 and 230; mixed populations 174, 192 and 232 were divided into two parts with regard to the ploidy level) were calculated based on allelic frequencies using the programme GENDIST from the Phylip 3.62 package (Felsenstein 2005) and were used to construct an UPGMA dendrogram using the NEIGHBOR programme. Nei's distances can be used to compare populations with different ploidy levels, as they rely solely on allele frequencies per population (although those alleles are grouped by two or four within individuals), but care must be taken in the dendrogram in interpreting differences in branch lengths, because for identical genetic and demographic parameters Nei's distances are expected to be smaller in polyploids compared to diploids due to lower genetic drift (Hardy et al. 2000). Correlation between matrices of Nei's genetic distances and geographical distances between populations was tested with Mantel test.

Further, allele presence or absence in 19 above-mentioned populations were scored. Shannon's Index (Bussell, 1999) for each isozyme locus was calculated for each population as

$$H'_j = -\sum p_i \ln p_i ,$$

where p_i is the frequency of the presence of the allele i per individuals in that population. The average diversity over all populations was calculated for each locus as

$$H'_{pop} = \frac{1}{n}\sum H'_j ,$$

where n is number of populations. The species diversity was calculated for each locus as

$$H'_{sp} = -\sum p_s \ln p_s ,$$

where p_s is the frequency of presence the allele s per individuals in the whole sample (250 diploids and 155 tetraploids). H'_j, H'_{pop} and H'_{sp} enable to estimate the partitioning of genetic variation within and between populations for each locus and enable comparison of the levels of variation detected by different isozyme loci. Between-cytotype differences in isozyme variation within and among populations were tested with t test in PAST.

In addition, inbreeding within populations and the level of differentiation among populations were evaluated using F-statistics (Wright 1951) estimated in SPAGeDi 1.2 (Hardy & Vekemans 2003). In this programme, F-statistics are based on allele identity and are types of kinship coefficients. In terms of probabilities of identity by state, they can be defined as $F_{IT} = (Q_0-Q_2)/(1-Q_2)$, $F_{IS} = (Q_0-Q_1)/(1-Q_1)$, and $F_{ST} = (Q_1-Q_2)/(1-Q_2)$, where Q_0, Q_1,Q_2, refer to probabilities of identity of homologous genes within individuals, among individuals within population, and among individuals among populations, respectively. For global F-statistics, Q_2 refers to all populations, whereas for pairwise F_{ST}, Q_2 refers only to the two populations being compared. Equivalently, these statistics can be defined as intra-class correlation coefficients of allelic states for genes within individuals relative to all populations (F_{IT}), genes within individuals relative to a population (F_{IS}), and genes within populations relative to all populations (F_{ST}). For F-statistics, the estimation procedure is based on a nested ANOVA following Weir and Cockerham (1984), where populations are weighted according to their sample size.

No. of pop.		Classical morphometrics			Geometric morphometrics			Crossing experiments			Isozymes			
		2x	4x	Total	2x	4x	Total	2x	4x	Total	2x	3x	4x	Total
35	CZ, Boží Dar		9	9		5	5							
37	CZ, Žlutice, Vladař								1	1			14	14
38	CZ, Železná Ruda, Špičák													
42	CZ, Horská Kvilda, Zhůří								1	1				
44	CZ, Nezdice na Šumavě, Stružno								1	1				
57	CZ, Milý		6	6		5	5		3	3				
62	CZ, Nová Pec		15	15										
67	CZ, Pernek		11	11										
68	CZ, Pasečná, Jasánky		9	9										
69	CZ, Pasečná, Jasánky		22	22										
70	CZ, Slatina		8	8		5	5							
72	CZ, Kovářov		10	10		5	5							
74	CZ, Přední Výtoň, Spáleniště		15	15		5	5							
77	CZ, Český Krumlov, Nové Dobrkovice		14	14					1	1				
82	CZ, Zliv		8	8		5	5		1	1				
84	CZ, Rožmberk nad Vltavou		10	10										
93	CZ, Horusice		17	17		5	5							
99	CZ, Kostelec nad Černými lesy												20	20
107	CZ, Košetice								1	1				
109	CZ, Zbraslavice		5	5		5	5							
124	CZ, Fryšava								2	2				
131	CZ, Čermná nad Orlicí, Číčová		15	15		5	5							
139	CZ, Česká Třebová												25	25
153	CZ, Šternberk								1	1				
154	CZ, Karlova Studánka								3	3				
157	CZ, Radějov	12	2	14	6	2	8				25		3	28
169	CZ, Hrubá Vrbka							3		3				
171	CZ, Machová	35	14	49	19	19	38	19	4	23	23		4	27
172	CZ, Jazevčí	10		10				1		1	30		1	31
174	CZ, Strání, Velká Javořina	20	14	34	14	14	28	3	1	4	22		24	46
175	CZ, Březová	31		31				2		2	27			27
181	CZ, Pulčín							4		4	31		2	33
187	CZ, Halenkov, Černé údolí												30	30
188	CZ, Hlučín, Jasénky												20	20
190	CZ, Měrkovice		4	4		4	4		2	2				
192	CZ, Horní Suchá	6	1	7	6	1	7	7	9	16	9		9	18
200	SK, Sološnica		3	3										
205	SK, Bukovec	4		4										
206	SK, Číčov	5		5	5		5							
213	SK, Vršatské Podhradie	3		3										
214	SK, Chotín	3		3										
224	SK, Vrátna valley	6		6	5		5							
227	SK, Vlkolínec	5		5				4		4	28	3		31
228	SK, Vlkolínec										12			12
230	SK, Lúčky	4		4				5		5	25	5		30
231	SK, Detva	7		7	5		5							

232	SK, Oravská Jasenica	2	3	5				5		5	18		13	31
233	SK, Bešeňová	2		2										
234	SK, Klin							1		1				
235	SK, Demänovská Ice Cave	3		3										
236	SK, Demänovská valley	5		5	5		5							
246	SK, Dlhá Ves	5		5										
247	SK, Lesnica gap	6		6	5		5							
249	SK, Borka	3		3	5		5							
253	SK, Čižatice	7		7										
256	SK, Ladomirová	4		4	5		5							
257	SK, Bystrá	4		4										
261	SK, Zemplínské Hámre	3		3	5		5							
Total		195	215	410	85	85	170	54	31	85	250	8	165	423

Tab. 2 – continued from the previous page. Numbers of plants used in the morphometric analyses, analysis of isozymes and in the crossing experiments. For the accurate location of the populations see Appendix 1.

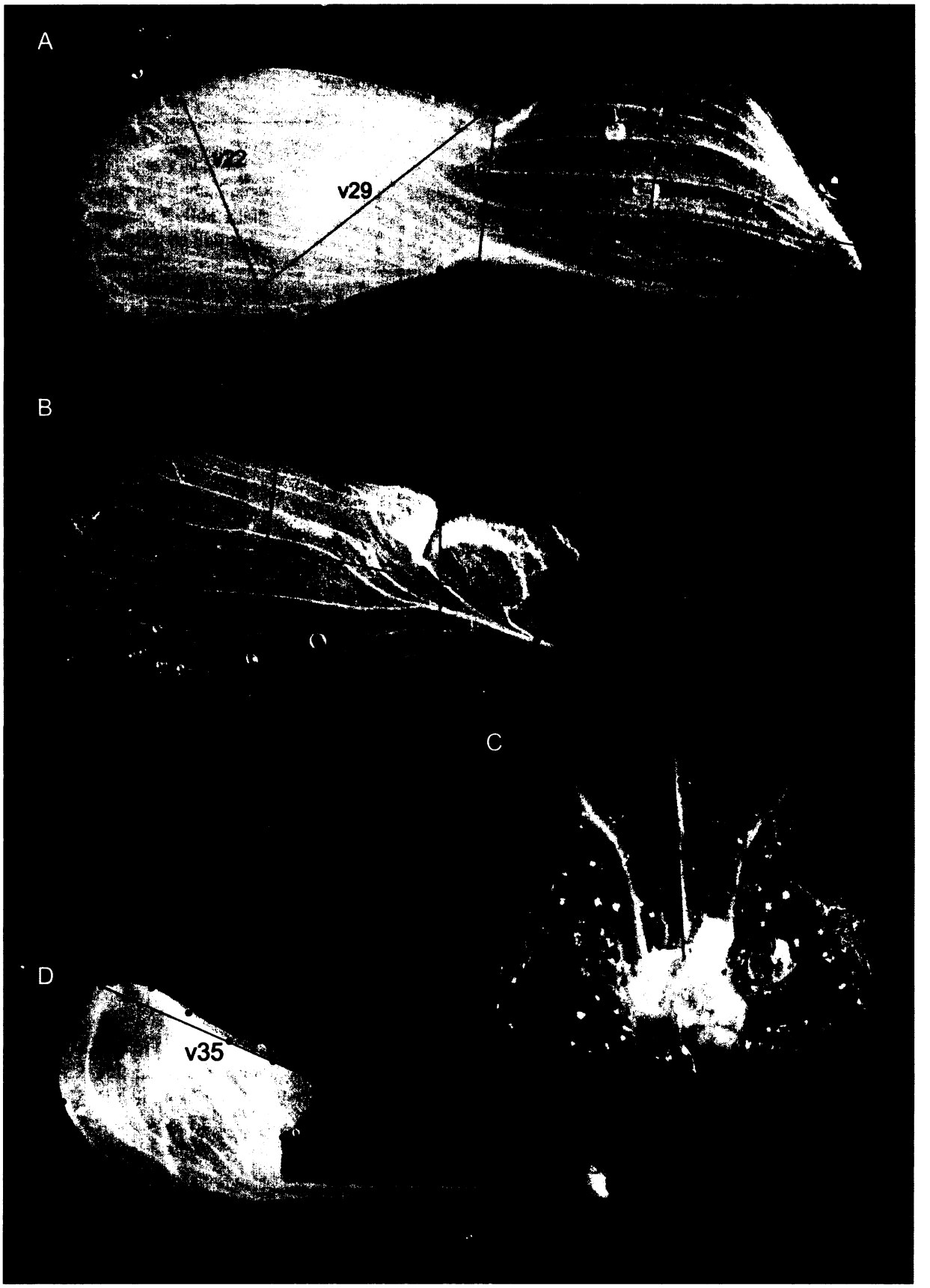


Fig. 2. Position of landmarks and delimitation of characters measured on floral parts: A, standard; B, wing; C, calyx; D, keel. 1-5 landmarks in B-D, and 1-3 landmarks in A, respectively; all other red points are semilandmarks. A key to morphological characters is in Tab. 5. Individual floral organs are depicted at different scales.

Results

Flow cytometry and chromosome counts

Flow cytometry (FCM) analyses yielded mostly high-resolution histograms with average sample CV of 2.58 % (range 1.25-3.5 %) and average standard CV of 2.24 (range 1.2-3.49) (Fig. 3). Three distinct groups of fluorescence intensities were obtained (Tab. 3). Mean fluorescence intensity per monoploid genome was virtually the same in all three cytotypes.

Karyological data confirmed the FCM results and all counted DNA diploids and DNA tetraploids had 14 and 28 somatic chromosomes, respectively (Fig. 4).

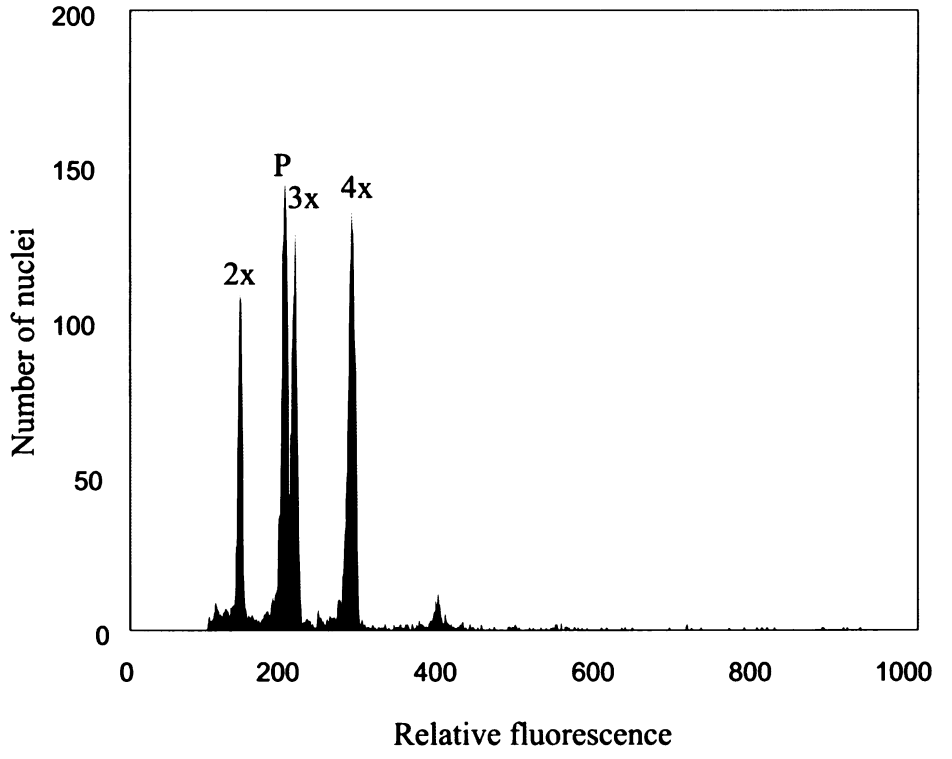


Fig. 3. Flow cytometric fluorescence histogram of three ploidy levels (2x, 3x and 4x) of *V. cracca* together with an internal reference standard (*Pisum sativum* 'Ctirad'; P). Nuclei of all plants were isolated, stained with DAPI and analysed simultaneously.

Ploidy level	Mean fluorescence intensity per cytotype ± SD	Min. relative fluorescence intensity per cytotype	Max. relative fluorescence intensity per cytotype	Mean fluorescence per monoploid genome	Mean numer of plants in sample
2x	0.710 ± 0.008	0.686	0.750	0.355	2.8
3x	1.071 ± 0.005	1.062	1.076	0.357	1
4x	1.415 ± 0.017	1.345	1.456	0.354	2.9

Tab. 3. Summary of relative fluorescence intensities for cytotypes of *V. cracca* based on bulk sample measurements with DAPI staining using *Pisum sativum* cv. Ctirad (2C = 8.84 pg) as a unit value.

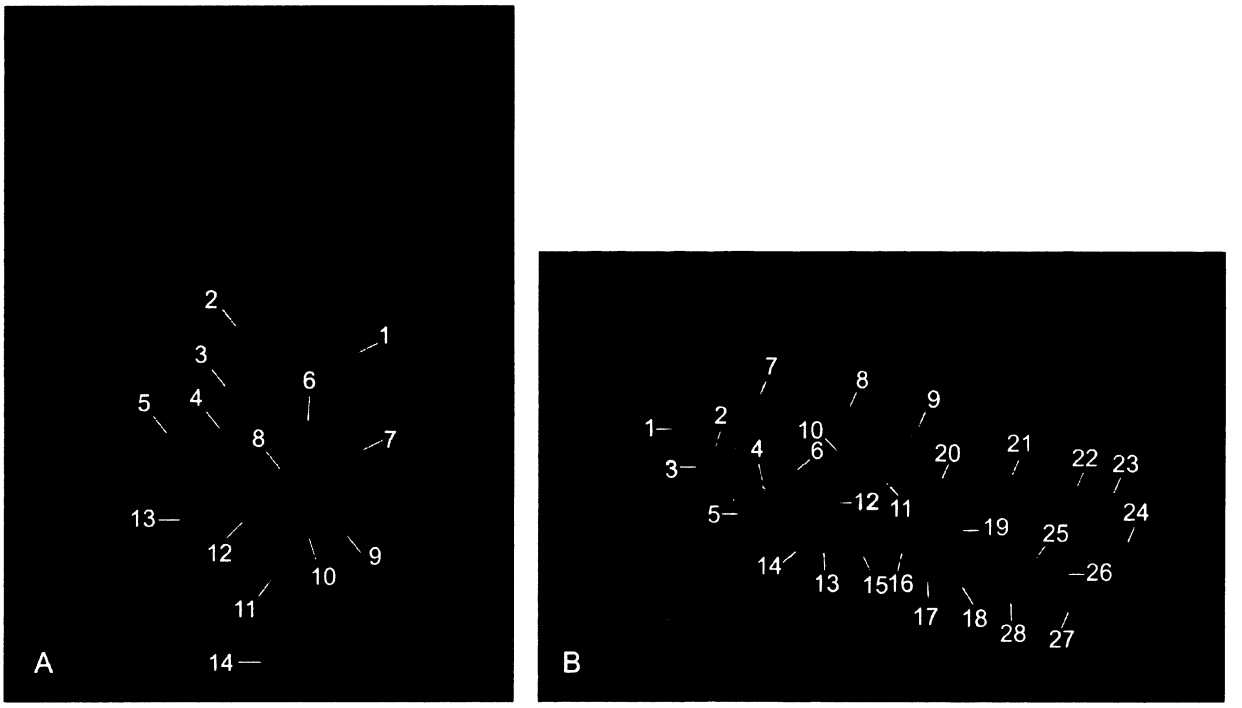


Fig. 4. Mitotic metaphase chromosomes of A) diploid (2n = 14) and B) tetraploid (2n = 28) *V. cracca*.

Distribution of cytotypes

In a total, 6 613 plants from 261 populations were sampled to estimate ploidy level. 180 populations (68.96 %) comprised 4 077 tetraploid plants (61.65 %), and 61 populations (23.37 %) contained 1 789 diploids (27.05 %). 18 populations (6.9 %) consisted of both diploids (446 individuals) and tetraploids (229 individuals). In two diploid populations (0.77 %) consisting of 72 sampled plants (1.09 %), eight triploids were found (0.1 % of all specimens).

Although diploids seemed to be the major cytotype in most mixed-ploidy populations (Fig. 5), the deviation from a uniform distribution was insignificant for both diploids (χ^2_{2x} = 8.11, d. f.$_{2x}$ = 4, P_{2x} = 0.09) and tetraploids (χ^2_{4x} = 9.22, d. f.$_{4x}$ = 4, P_{4x} = 0.06). In other words, both cytotypes were very common, intermediate, or very rare across the mixed-ploidy populations to an indistinguishable extent.

Geographic distribution of cytotypes is non-random; tetraploid populations occur mostly in the west of the investigated area, i.e. in the Czech Republic and in the north of Austria, whereas diploid populations were found especially in the east, i.e. in Slovakia (Fig. 6A). The contact zone with mixed-ploidy populations follows the Czech-Slovak border and continues towards the northwest of Slovakia. One tetraploid population was recorded in the midwest (no. 221, Hliník nad Hronom) and one also in the southeast (no. 254, Slanská Huta) of the Slovakia. They are accompanied with mixed-ploidy populations in their proximity (no. 225, Trnie; no. 251, Jasov). On the other hand, transition from diploids to tetraploids in the west is quite abrupt, and no diploids were found westwards of the contact zone with the exception of one mixed-ploidy population in the northeast of the Czech Republic (no. 192, Horní Suchá). Triploids were recorded within diploid populations (no. 227, Vlkolínec; no. 230, Lúčky) in the neighbourhood of tetraploids (no. 229, Vlkolínec) in the northwest of Slovakia. Absence of any diploids and triploids in the southwest of the Czech Republic is the most evident change of recent distribution in comparison to the historical records (Fig. 6B).

The cytotype distribution at a fine spatial scale in the mixed population no. 189 (Horní Suchá, northeast of the Czech Republic) also significantly deviated from randomness (P = 0.009). Both diploids and tetraploids clustered preferentially with plants of the same ploidy (Fig. 7).

Distribution of cytotypes along the altitudinal gradient is shown at Fig. 8. Although tetraploids tend to occur in higher altitudes and had significantly higher mean (P < 0.01) when compared to diploids (Tab. 4), both cytotypes occupy a wide range of altitudes. No discontinuity in the distribution of diploids along the altitudinal gradient was detected (Fig. 8).

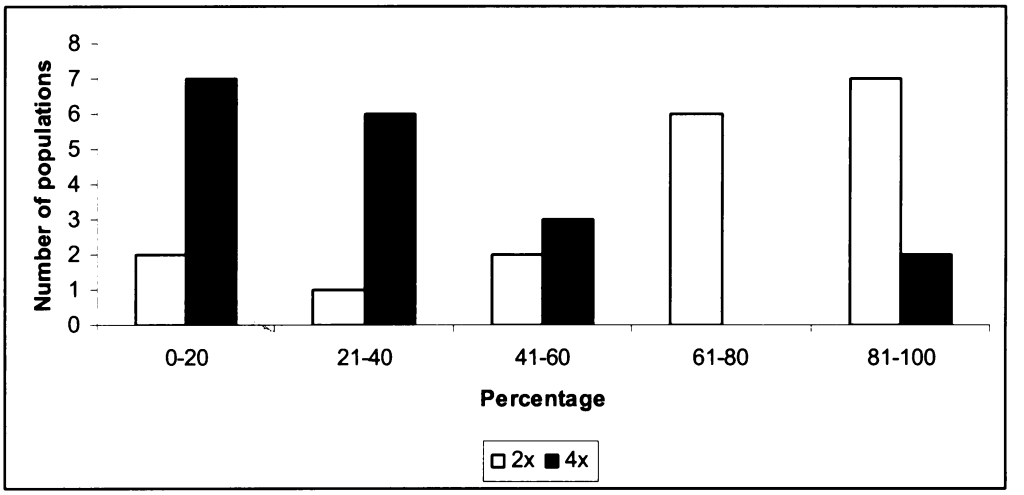
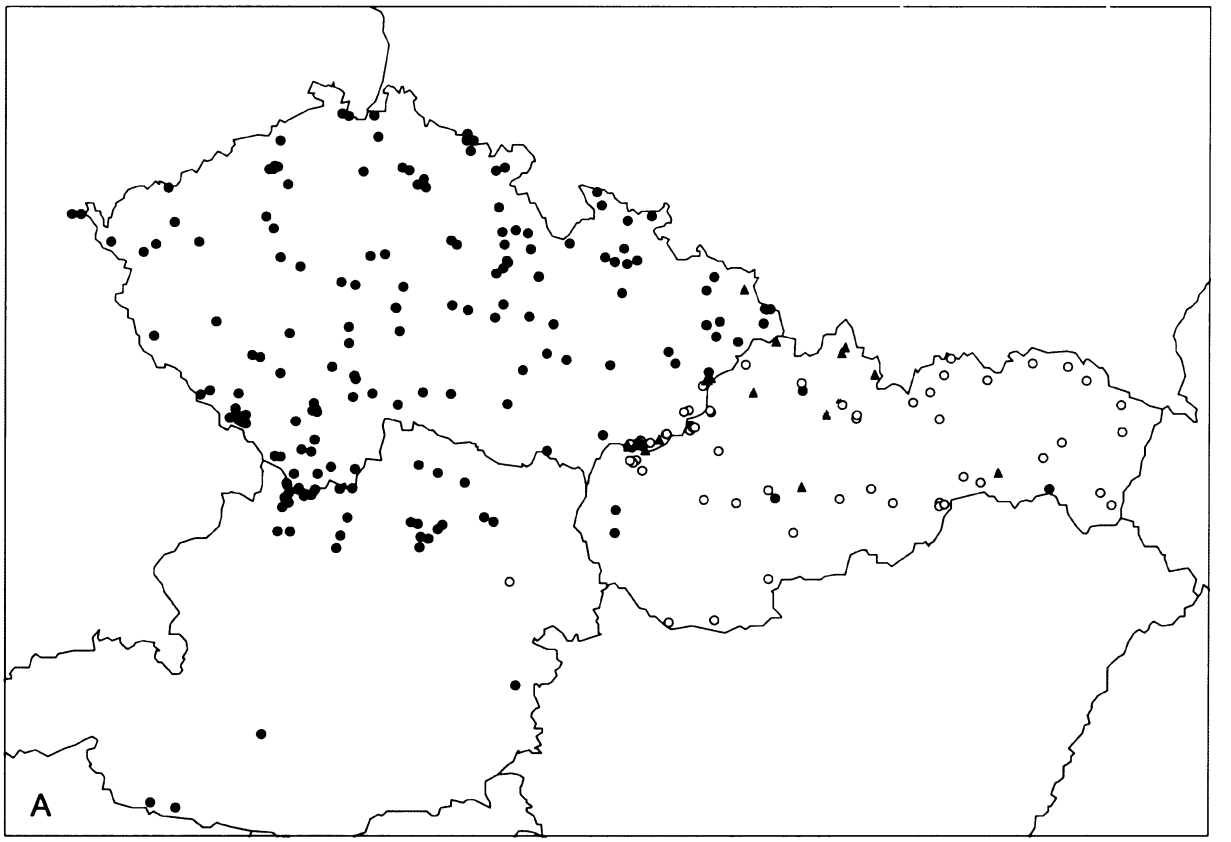


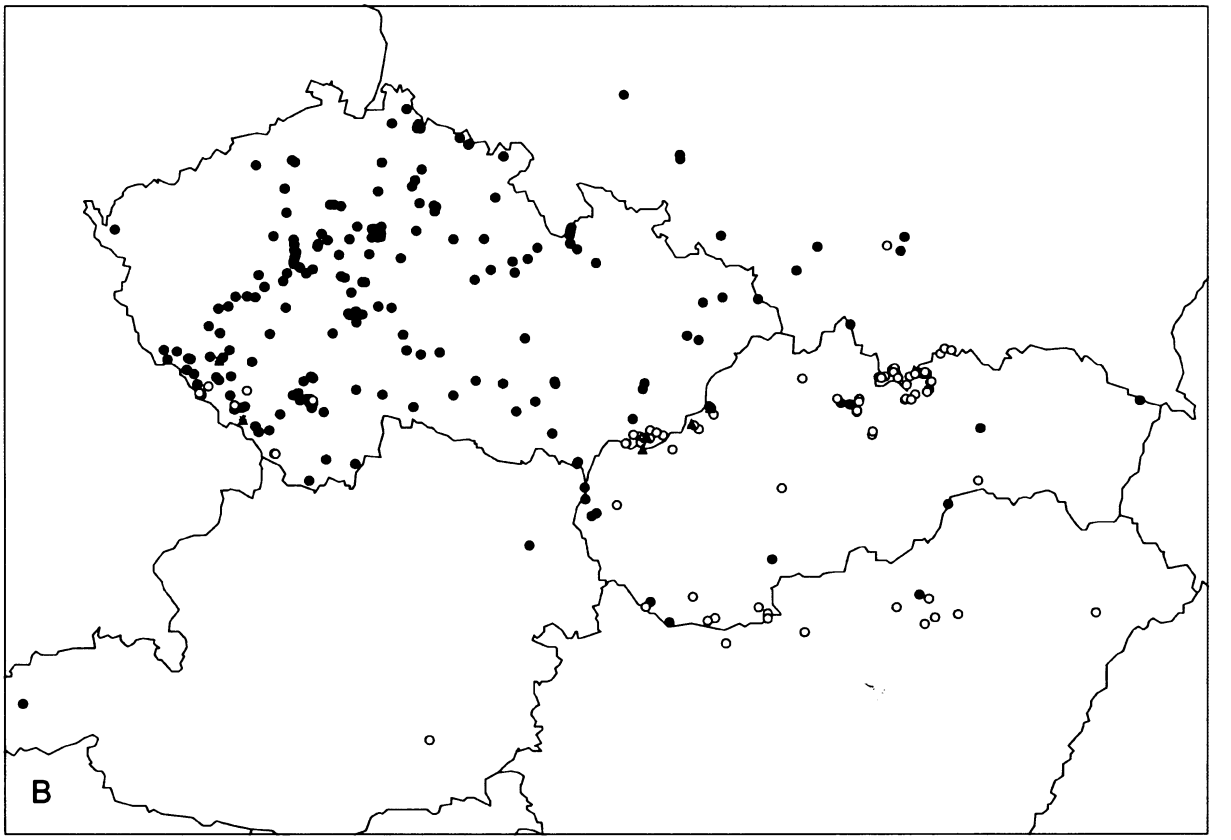
Fig. 5. Distribution of 18 ploidy-mixed populations of *V. cracca* as a function of the frequency of diploids (open bars) and tetraploids (solid bars).

	Mean altitude ± SD (m a. s. l.)	Max. altitude (m a. s. l.)	Min. altitude (m a. s. l.)
2x	441 ± 220	1147	99
4x	524 ± 245	1620	178

Tab. 4. Summary of altitudinal distribution of *V. cracca* cytotypes. The means are significantly different at $P < 0.01$.

Fig. 6. – next page. Distribution of cytotypes of *V. cracca* in the investigated area. A, recent distribution; B, distribution reconstructed on the basis of earlier published chromosome counts (Chrtková-Žertová 1973a; Dvořák et al. 1977; Činčura 1963, 1981; Hallonová 1982). Open circles, diploids; black circles, tetraploids; red triangles, mixed-ploidy populations (2x + 4x); blue asterisks, triploids.


A


B

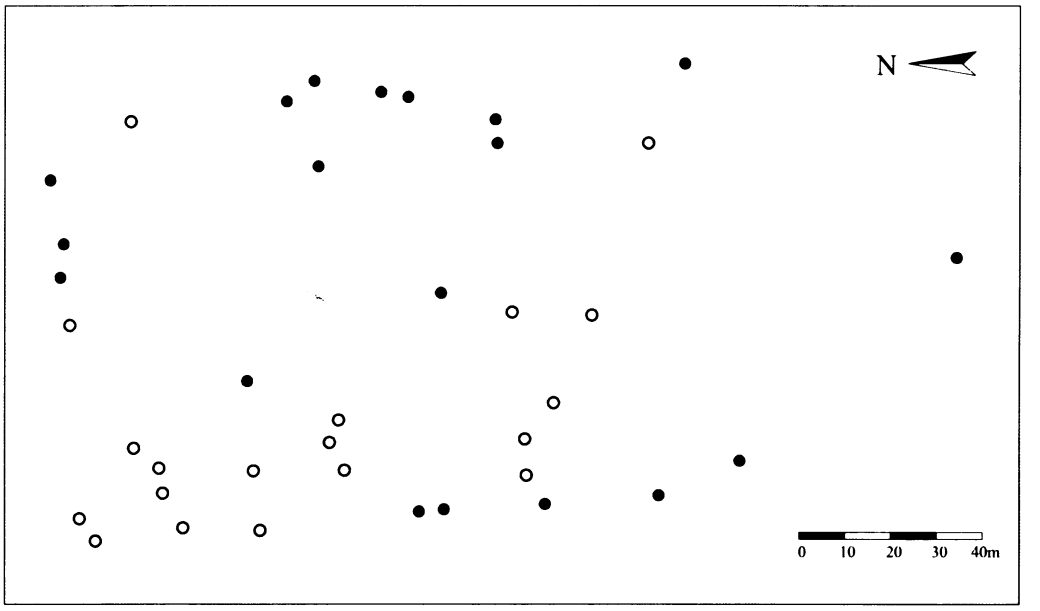


Fig. 7. Fine-scale cytotype distribution in the mixed-ploidy population in Horní Suchá (no. 192). Open circles, diploids; black circles, tetraploids.

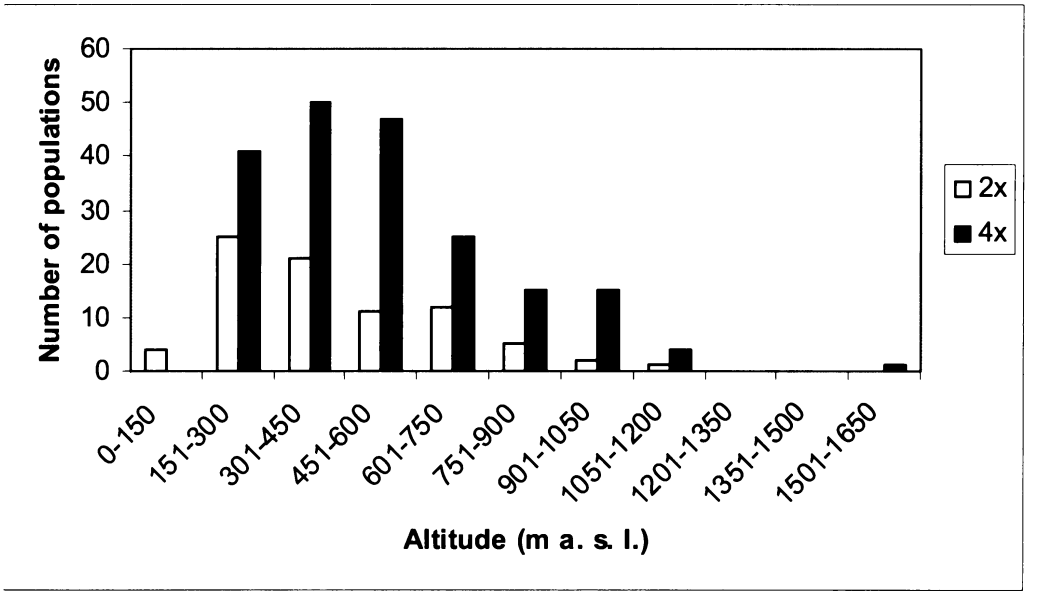


Fig. 8. Distribution of populations of *V. cracca* along the altitudinal gradient. Open bars, population of diploids (n = 81); solid bars, tetraploid populations (n = 198).

Classical morphometrics

PCA of all individuals performed on all measured characters is shown at Fig. 9. The first two axes explain 37.19 % of overall variability. Individuals of a different ploidy level are quite intermingled in the space of these new variables; nevertheless, diploids are more correlated with negative values of the first two axes while tetraploids have more positive coordinates. Characters evaluated on the flowers were those that contribute most to the spreading of individuals along the first axis; second axis is most correlated with vegetative characters as number of leaflet pairs, length of leaves and hairiness of leaves (see eigenvectors in Tab. 5). The third axis explains only a small part of overall variability (8.82 %, not shown); standard blade/claw length ratio contributes mainly to the distribution along this axis. Cytotypes are not completely separated also along the discriminant axis (Fig. 10), 53.4 % of individuals of a different cytotype overlap. Nonetheless, means of individual cytotypes were significantly different ($P < 0.0001$), and only 12 % of the specimens were misclassified in the classificatory discriminant analysis. Approximately one half of incorrectly classified individuals were from mixed-ploidy populations; within pure populations, classification failed mainly in tetraploid populations no. 67, 69, 72, 93 and 131. The size of the standard claw, the length of the keel and the width of the wing are characters mostly contributing to the separation of the cytotypes (see total canonical structure in Tab. 5). The length of the leaf in the middle of the stem is the best vegetative trait discriminating the two cytotypes.

Analyses performed on a reduced number of characters gave similar results (data not shown). Only classificatory DA of 14 best characters selected in CDA plus six characters selected in stepwise DA resulted in a somewhat lesser number of misclassified specimens (10.8 %), but the dataset did not show a better separation of the cytotypes in CDA.

Discriminant analyses of individuals originated only from cytotypically pure populations showed the best separation of diploids and tetraploids (35.5% overlap; $P < 0.0001$; 8.3 % of misclassified individuals) (Fig. 11). However, they were still morphologically very similar (Fig. 12). Characters most contributing to the discrimination were the same as in the case of PCA of all individuals.

Individuals from mixed-ploidy populations were more similar to individuals of the same ploidy level from pure populations than to their counterparts of different cytotype within mixed-ploidy populations (Fig. 13). PCA and a cluster analysis performed on population means gave virtually the same results (Fig. 14, 15). Cytotypes exhibited an apparent separation along the first principal component. Nevertheless, diploids from the mixed-ploidy

population no. 192 and that from the pure population no. 235 were more similar to tetraploids from pure populations than to other diploids (Fig. 14). Separation along the second component is not so distinctive; only tetraploids from the mixed-ploidy population no. 157 had somewhat isolated position. Diploid population no. 235 formed a cluster together with some purely tetraploid populations and tetraploids from the mixed-ploidy population no. 192; this cluster formed a branch sister to all other populations (Fig. 15). Tetraploids from the mixed-ploidy population no. 157 were placed as the next basalmost branch. Populations of different cytotypes clustered separately at the next level with the exception of the populations no. 93 and 192. The tetraploid population no. 93 clustered within diploid populations, while the diploid part of the mixed-ploidy population no. 192 clustered together with tetraploid populations. Individuals of different ploidy level from the same mixed-ploidy population never clustered together.

In CDA, floral traits were those that showed the biggest differences between cytotypes, whereas characters on vegetative organs contributed only slightly to the discrimination (Tab. 5). Tetraploids are generally bigger (Fig. 16); particularly, they have a larger standard claw, a longer keel, and wider wings and longer the whole flowers when compared to diploids. Moreover, they are slightly taller, have longer internodes in the lower part of the stem and longer leaves in the middle of the stem (Fig. 17). On the other hand, diploids are more hairy, usually have teeth on the stipules, and have a slightly longer standard blade compared to the claw and a longer inflorescence when compared to the subtending leaf (Fig. 17). A list of morphological characters exhibiting significant differences between *V. cracca* diploids and tetraploids and potentially useful for routine morphology-based cytotype identification is provided in Tab. 6.

In order to assess a possible impact of altitude on the morphology of *V. cracca*, populations were separately for diploid and tetraploid divided into two groups according to altitude of the locality and the two groups were compared using analysis of similarities (ANOSIM). Whereas diploids exhibited significant differences in morphology between the two groups ($R = 0.02945$, $P = 0.0017$), for tetraploids the differences were insignificant ($R = 0.02113$, $P = 0.1403$). However, permutation tests employed as an alternative to ANOSIM indicated significant differences between the low-altitude and the high-altitude groups for both diploids and tetraploids ($Md_{2x} = 0.1049$, $Md_{4x} = 0.09321$, $P < 0.0005$ for both).

To compare the extent of morphological disparity between cytotypes in pure populations and between cytotypes in mixed-ploidy populations, Euclidean distances were calculated from scores on the first 30 principal components from PCA on all characters and all individuals

(see above). Median values of distances among diploids and tetraploids in cytotypically pure populations were bigger than among diploids and tetraploids from mixed populations (Tab. 7). The same trend was recorded in analyses restricted to characters on generative organs only and to one particular character on the keel (v36). The second character measured on the keel (v35) was the only for which the difference in medians was insignificant (Tab. 7).

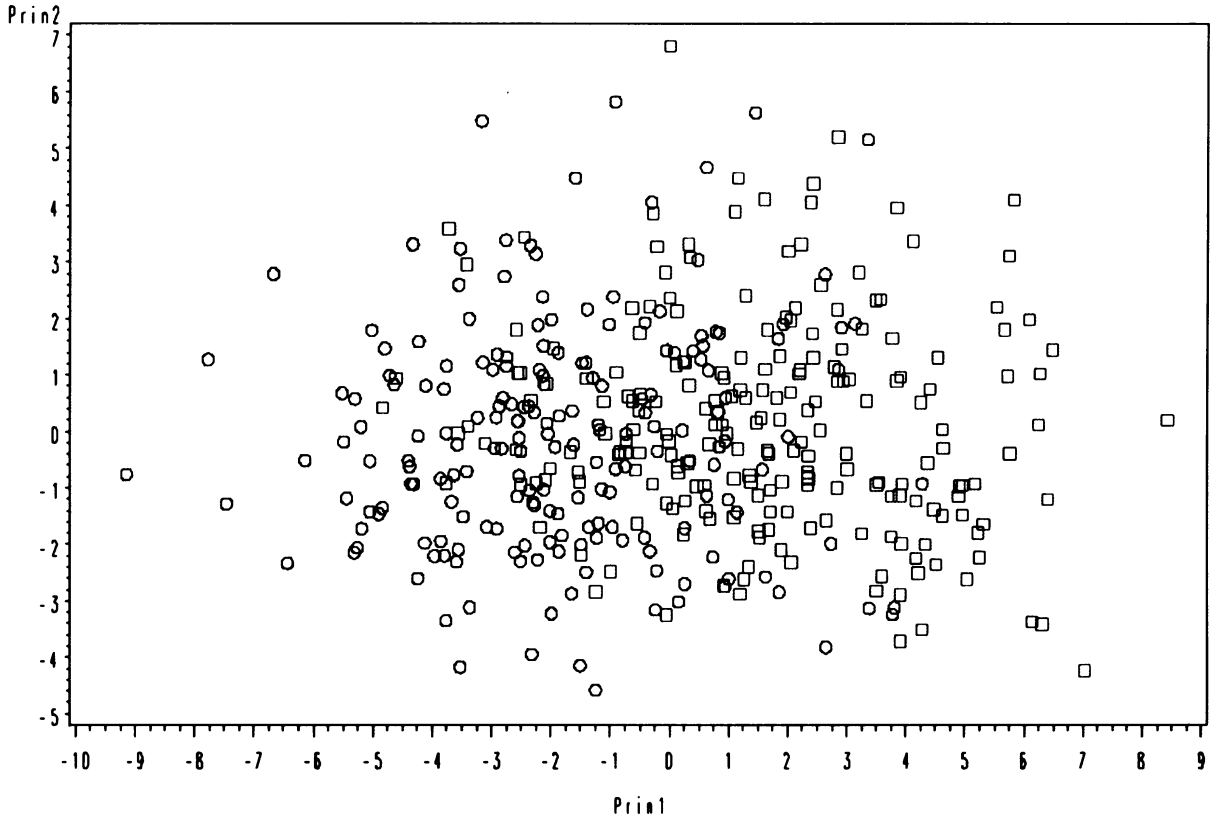


Fig. 9. PCA of all individuals of *V. cracca* based on 34 morphological characters (listed in Tab. 5). Blue circles, diploids; red squares, tetraploids. The first two axes explain 37.19 % of total variability.

No.	Character	Axis 1	Axis 2	Axis 3	CDA
v4	stem length (mm)	0.100	0.336	0.042	0.292
v5	number of internodes	0.007	0.286	0.096	0.029
v6	mean length of the lower fifth of internodes (mm)	0.088	-0.016	-0.048	0.278
v7	number of leaflet pairs of a leaf in the lower third of the stem	-0.019	0.287	0.020	-0.137
v8	number of leaflet pairs of a leaf in the middle of the stem	0.028	0.362	0.060	0.107
v9	number of leaflet pairs of a leaf in the upper third of the stem	-0.023	0.218	0.042	-0.016
v10	length of a leaf in the lower third of the stem (mm)	0.034	0.317	0.001	0.096
v11	length of a leaf in the middle of the stem (mm)	0.129	0.337	0.034	0.442
v12	length of a leaf in the upper third of the stem (mm)	0.019	0.074	0.023	-0.016
v13	mean hairiness of upper surface of leaflets in the lower third of the stem	-0.102	0.254	0.021	-0.224
v14	mean hairiness of upper surface of leaflets in the middle of the stem	-0.099	0.218	-0.035	-0.180
v15	mean hairiness of upper surface of leaflets in the upper third of the stem	-0.067	0.094	-0.079	-0.020
v16	presence of teeth on stipules in the lower two thirds of the stem	-0.081	0.128	0.112	-0.273
v17	mean inflorescence/subtending leaf length ratio	-0.003	-0.042	0.067	-0.219
v18	maximum inflorescence length (mm)	0.106	0.166	0.093	0.054
v19	length of a flower (mm)	0.301	-0.083	-0.033	0.497
v20	length of a calyx (mm)	0.174	0.201	-0.071	0.440
v21	length of upper calyx tips (mm)	0.051	0.183	0.063	0.041
v22	length of the standard blade (mm)	0.241	-0.028	0.309	0.394
v23	length of the standard claw (mm)	0.260	0.012	-0.257	0.708
v24	distance from standard claw base to the standard claw widest point (mm)	0.133	-0.035	-0.123	0.651
v25	standard blade/calyx length ratio	0.039	-0.204	0.317	-0.076
v26	standard blade/claw length ratio	-0.026	-0.032	0.494	-0.284
v27	width of the standard claw (mm)	0.276	0.026	-0.086	0.619
v28	width in the middle of the standard (mm)	0.259	0.069	0.130	0.518
v29	width of the standard blade (mm)	0.275	-0.042	0.212	0.473
v30	standard claw/blade width ratio	-0.027	0.080	-0.367	0.106
v31	length of the wing blade (mm)	0.247	-0.053	-0.244	0.614
v32	length of the wing claw (mm)	0.260	-0.082	0.161	0.251
v33	width of the wing (mm)	0.264	-0.045	0.174	0.454
v34	width of the wing (mm)	0.257	0.017	0.075	0.649
v35	length of the keel (mm)	0.284	-0.042	-0.152	0.656
v36	length of the keel claw (mm)	0.251	-0.047	-0.256	0.567
v37	mean anther length (mm)	0.150	0.048	-0.020	0.490

Tab. 5. Eigenvectors and the total canonical structure expressing correlation of characters with principal components (axis 1-3) and with the canonical axis (CDA) in a morphometric analysis of all individuals of *V. cracca*.

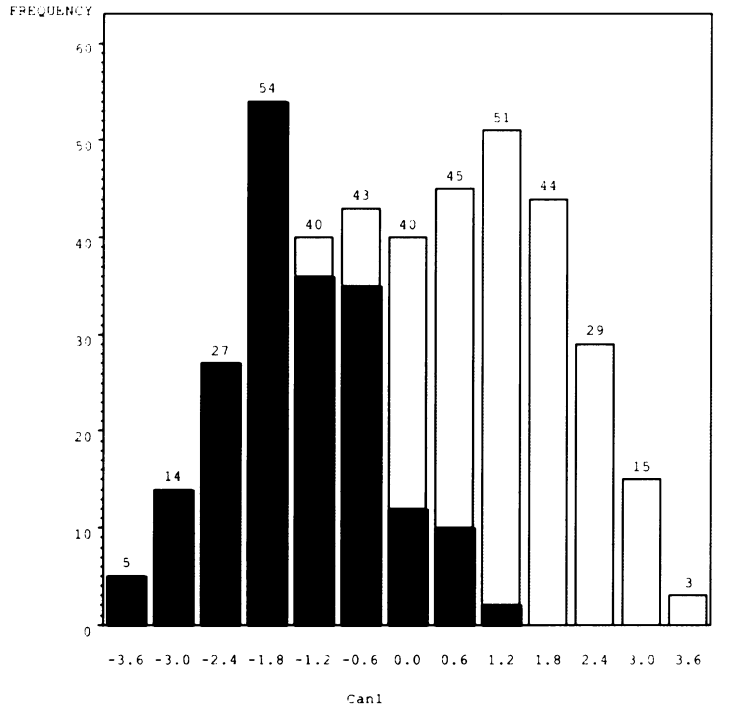


Fig. 10. Histogram of canonical discriminant analysis performed on all individuals of *V. cracca* based on 34 morphological characters. Solid bars, diploids; empty bars, tetraploids. 53.4 % of individuals of different cytotype overlap; P (same means) < 0.0001.

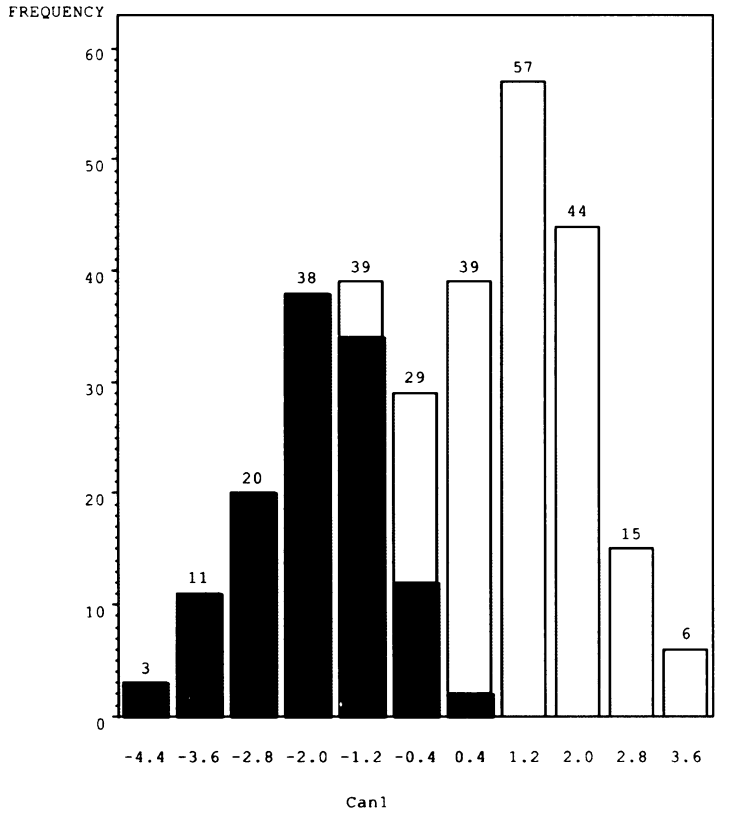


Fig. 11. Histogram of CDA performed on individuals of *V. cracca* originated only from cytotypically pure populations based on 34 morphological characters. Overlap = 35.5 %; P (same means) < 0.0001.

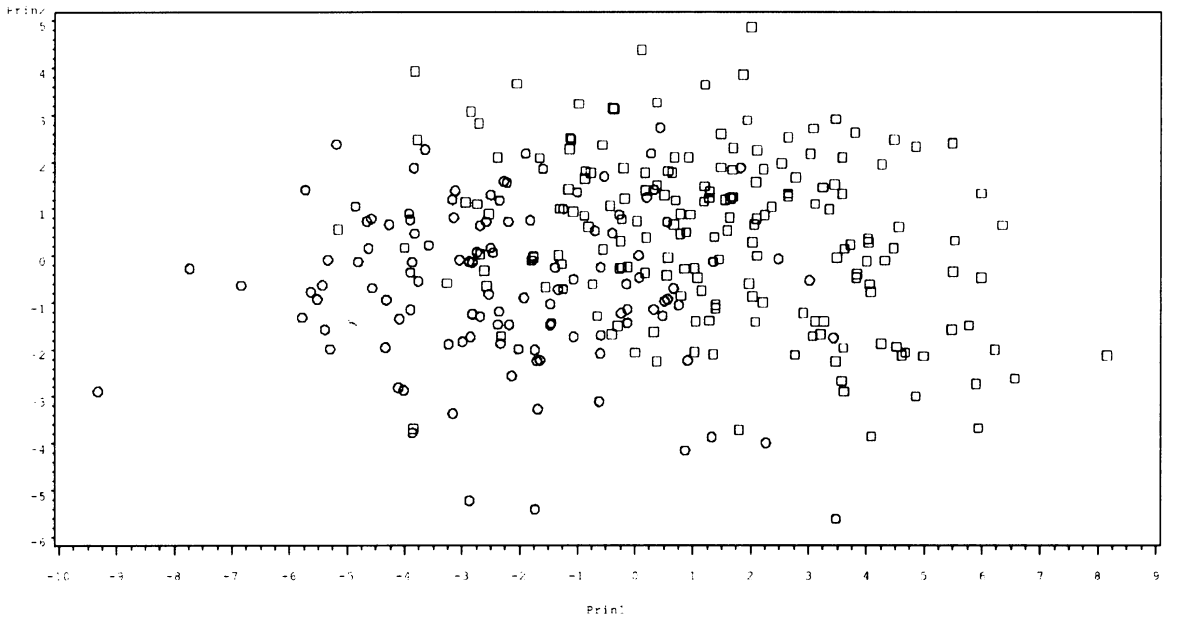


Fig. 12. PCA of individuals from cytotypically pure populations based on 34 morphological characters. Blue circles, diploids; red squares, tetraploids. The first two axes explain 35.72 % of total variability.

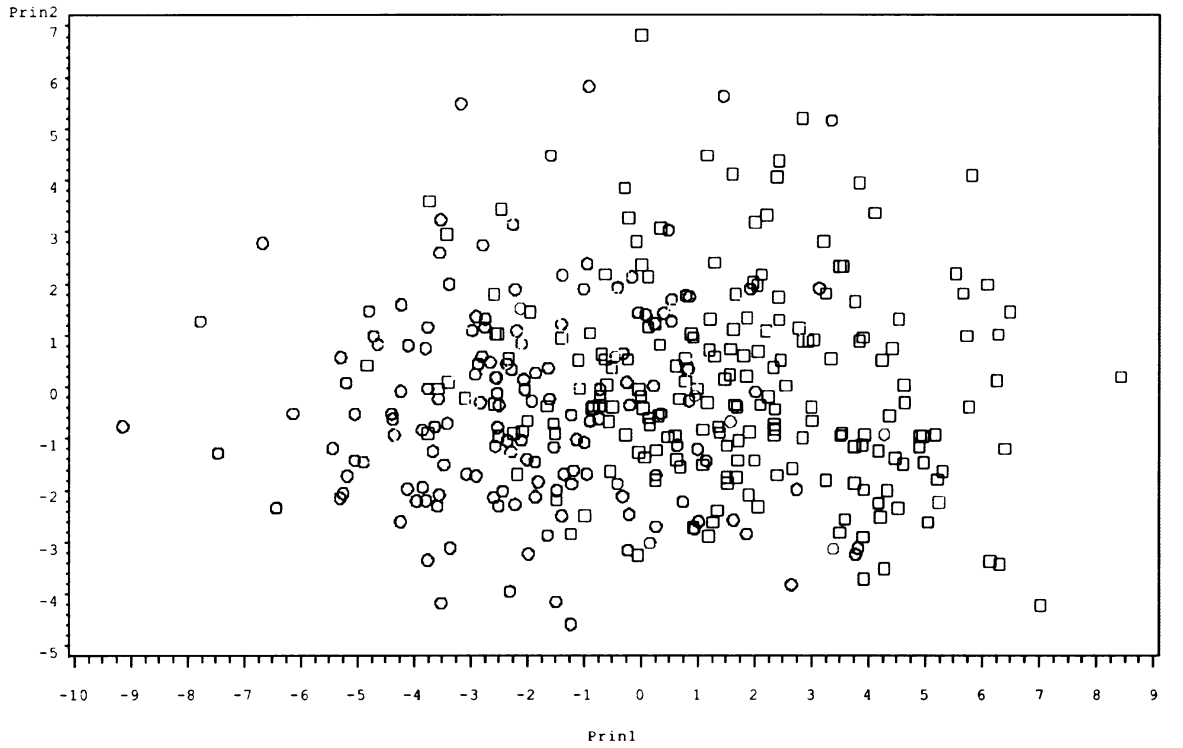


Fig. 13. PCA of all individuals of *V. cracca* based on 34 morphological characters (the same analysis as presented at Fig. 9 with separately highlighted individuals from mixed-ploidy populations). Circles, diploids; squares, tetraploids; blue and red, individuals from cytotypically pure populations; brown, the mixed population no. 157; yellow, the mixed population no. 171; green, the mixed population no. 174; violet, the mixed population no. 192; cyan, the mixed population no. 232. The first two axes explain 37.19 % of total variability.

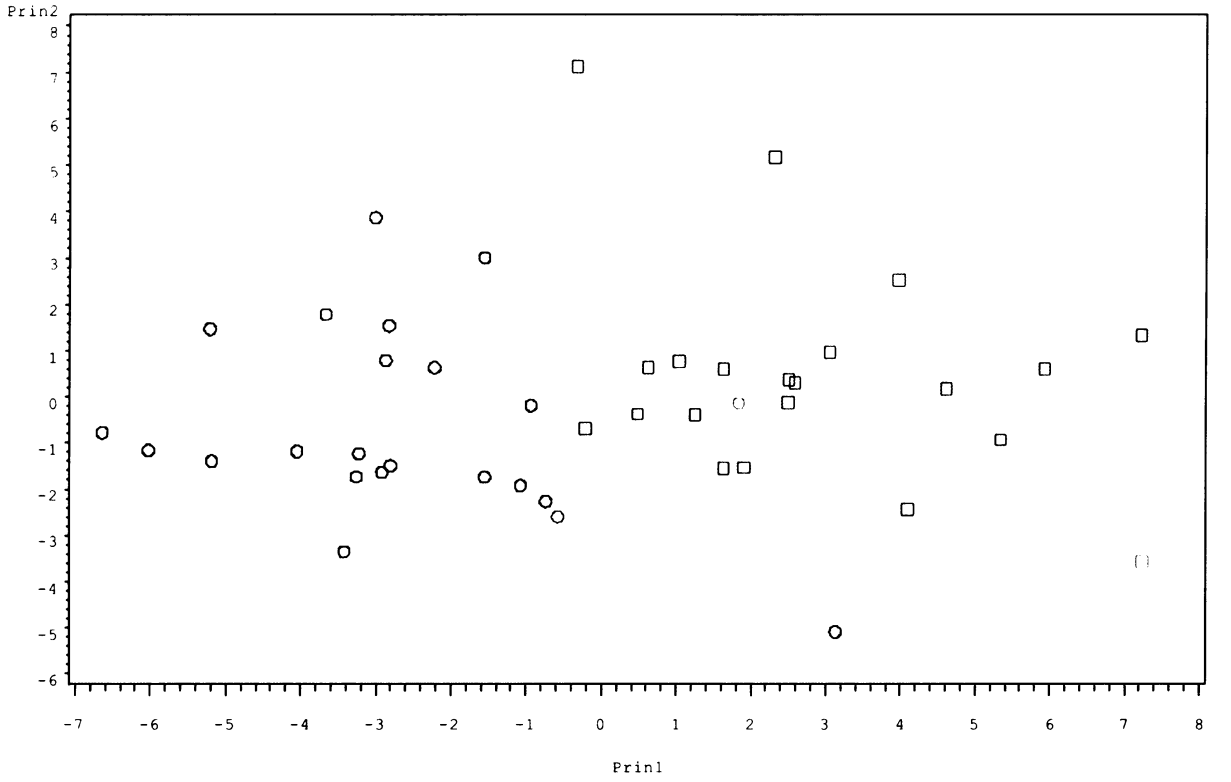


Fig. 14. PCA of population means based on 34 morphological characters. Circles, diploids; squares, tetraploids; blue and red, cytotypically pure populations; brown, the mixed population no. 157; yellow, the mixed population no. 171; green, the mixed population no. 174; violet, the mixed population no. 192; cyan, the mixed population no. 232. The first two axes explain 50.52 % of total variability.

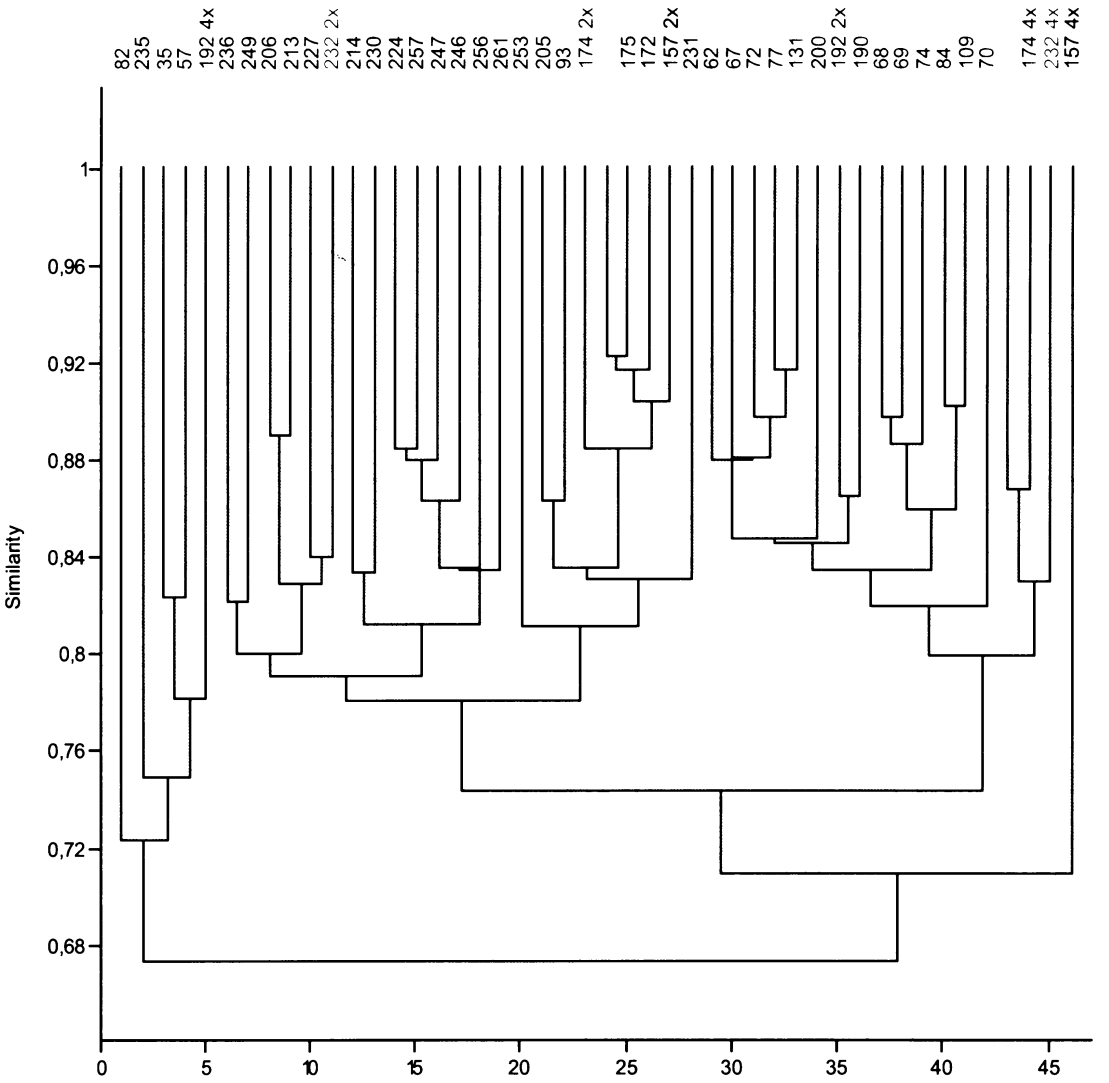


Fig. 15. UPGMA of population means based on 34 morphological characters. Blue, diploids from cytotypically pure populations; red, tetraploids from cytotypically pure populations; brown, the mixed population no. 157; yellow, the mixed population no. 171; green, the mixed population no. 174; violet, the mixed population no. 192; cyan, the mixed population no. 232.

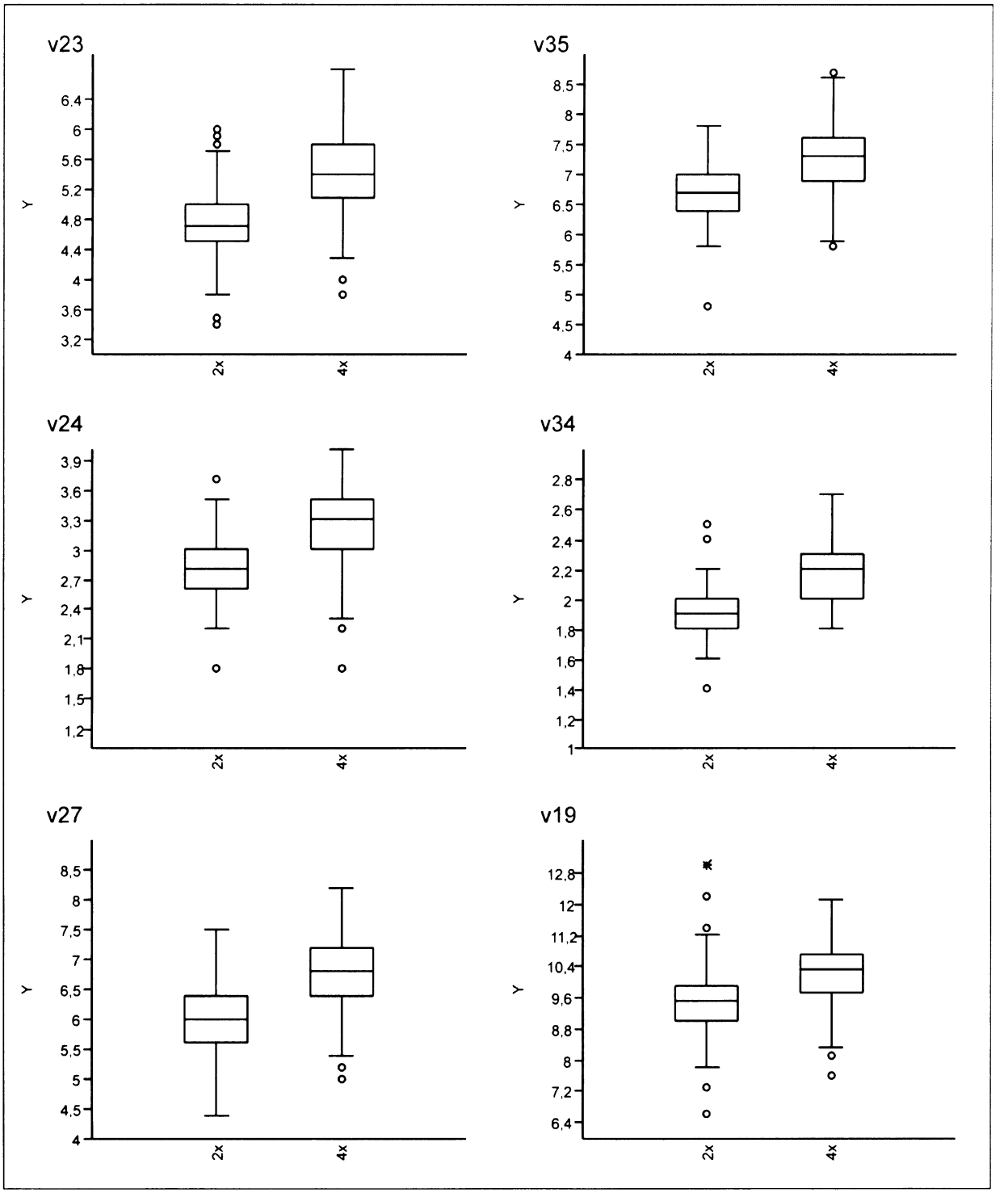


Fig. 16. Variability in morphological characters that selected from those that contribute most to discrimination between diploids and tetraploids in CDA (see Tab. 5). v23, length of the standard claw; v35, length of the keel; v24, distance from the base of standard claw to its widest point; v34, width of the wing; v27, width of the standard claw; v19, length of the flower. Rectangles define 25 and 75 percentiles; horizontal lines correspond to medians; whiskers represent values up to 1.5 times of inter-quartile range; circles are distant observation.

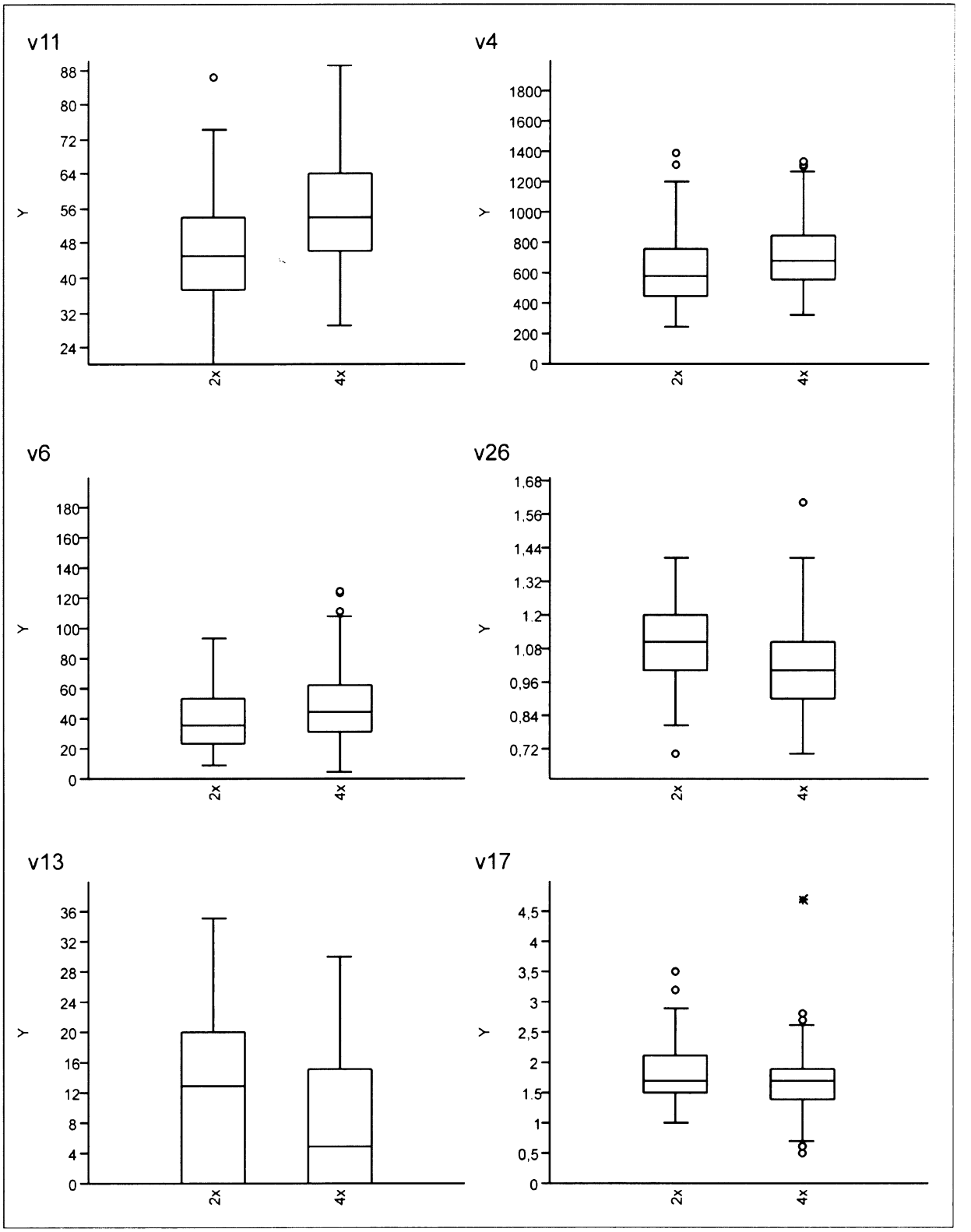


Fig. 17. Variability in six additional morphological characters of diploids and tetraploids (discussed in detail in the text): v11, length of the leaf in the middle of the stem; v4, length of the stem; v6, mean length of the lower fifth of internodes; v26, standard blade/claw length ratio; v13, mean hairiness of the upper surface of leaflets in the lower third of the stem; v17, mean inflorescence/subtending leaf length ratio. Rectangles define 25 and 75 percentiles; horizontal lines correspond to medians; whiskers represent values up to 1.5 times of inter-quartile range; circles are distant observation.

No.	Character	2x	4x
v4	stem length (mm)	(338-) 581 (-1000)	(424-) 680 (-1163)
v6	mean length of the lower fifth of internodes (mm)	(13-) 36 (-69)	(15-) 44 (-91)
v11	length of a leaf in the middle of the stem (mm)	(30-) 45 (-68)	(36-) 54 (-75)
v13	mean hairiness of upper surface of leaflets in the lower third of the stem	(0-) 13 (-30)	(0-) 5 (-25)
v16	presence of the teeth on stipules in the lower two thirds of the stem	often	rarely
v17	mean inflorescence/subtending leaf length ratio	(1.2-) 1.7 (-2.6)	(1-) 1.7 (-2.4)
v19	length of a flower (mm)	(8.4-) 9.5 (-10.7)	(8.7-) 10.3 (-11.5)
v23	length of the standard claw (mm)	(4-) 4.7 (-5.4)	(4.6-) 5.4 (-6.3)
v24	distance from standard claw base to the standard claw widest point (mm)	(2.3-) 2.8 (-3.4)	(2.5-) 3.3 (-3.8)
v26	standard blade/claw length ratio	(0.9-) 1.1 (-1.3)	(0.8-) 1 (-1.3)
v27	width of the standard claw (mm)	(5.2-) 6 (-7)	(5.8-) 6.8 (-7.8)
v34	width of the wing (mm)	(1.6-) 1.9 (-2.2)	(1.9-) 2.2 (-2.5)
v35	length of the keel (mm)	(5.9-) 6.7 (-7.4)	(6.5-) 7.3 (-8.1)

Tab. 6. Selected morphological characters distinguishing cytotypes of *V. cracca*. For quantitative characters, medians with 5 and 95 percentiles in parenthesis are given. Medians are significantly different between cytotypes at $P < 0.001$ (Mann-Whitney U test) in all characters except v13 ($P < 0.01$).

	All characters	v19-v37	v35	v36
Mann-Whitney U	7.8E06	7.7E06	8.5E06	7.0E06
Median $_{mixed}$	1.4125[a]	1.4100[a]	0.6	0.3[a]
Median $_{pure}$	1.4771[a]	1.5123[a]	0.6	0.5[a]

Tab. 7. Differences of medians of between-cytotype Euclidean distances in mixed and pure populations of *V. cracca* calculated for distinct sets of measured morphological characters. [a] $P < 0.001$; $n_{mixed} = 806$; $n_{pure} = 21\ 720$.

Geometric morphometrics

Relative warp analysis of the keel gave the best results among all floral structures in terms of separating diploids and tetraploids. The major trends in changing the shape along the first and the second relative warp (RW), which explained 58.6 % of total variation, are apparent from Fig. 18. Individual cytotypes did not form separated group in the ordination plot. Individuals of different ploidy level from the same locality did not group together; they were rather scattered over the plot. A cluster analysis data from the keel also did not show

separation of cytotypes or clustering of diploids and tetraploids from the same mixed-ploidy populations (not shown). Ordination and cluster plots of other floral structures are not shown because they brought no meaningful results.

Discriminant analysis performed on the keel data correctly classified 88.82 % of objects. Means of individual cytotypes were significantly different (Tab. 8, Mahalanobis distance). The wing and the standard showed a somewhat worse differentiation between cytotypes. The discrimination was successful in 78.82 % of specimens in the analysis of the wing, and in 70.59 % in the analysis of the standard. Calyx proved to be inappropriate to distinguish between cytotypes (Tab. 8); hence, other analyses were not performed on this floral part. Median of between-cytotype distances based on the shape of the keel and the wing was significantly bigger in mixed populations compared with distances between individuals of different ploidy from pure populations (Tab. 8).

Despite the fact that differences in the shape of the keel were significant, it is almost impossible to distinguish between the two cytotypes if we display the most frequent shapes (Fig. 19B, C). Hence, shapes correlated with extreme values of the discriminant axis are presented in order to show the trends in the shape changes (Fig. 19D, E). Diploids tend to have the enlarged part of the keel more flattened and to have the angle between the enlarged part and the claw more obtuse. Additional changes take place at the end of the claw, which tends to be narrower and more deeply indentated in tetraploids. In the case of the wing and the standard, the most frequent shapes of both cytotypes are virtually the same (Fig. 20B, 21B). However, the blade and the claw of the wing tend to be more clenched in tetraploids (Fig. 20D). In diploids, the blade of the standard is narrower, so the claw tends to be more marked (Fig. 21C).

Floral organ	Percents of correctly classified objects	Mahalanobis squared distance	Median of between-cytotype distances	
			within mixed populations	among pure populations
Keel	88.82	0.1801***	0.016547[a]	0.0160955[a]
Standard	70.59	0.09998***	0.014653	0.0141535
Wing	78.82	0.1197***	0.018193[b]	0.0201805[b]
Calyx	67.65	0.06753	-	-

Tab. 8. Summary of statistics calculated in analyses of shape differences between cytotypes of *V. cracca*. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; [a] difference significant at $P < 0.01$; [b] difference significant at $P < 0.001$

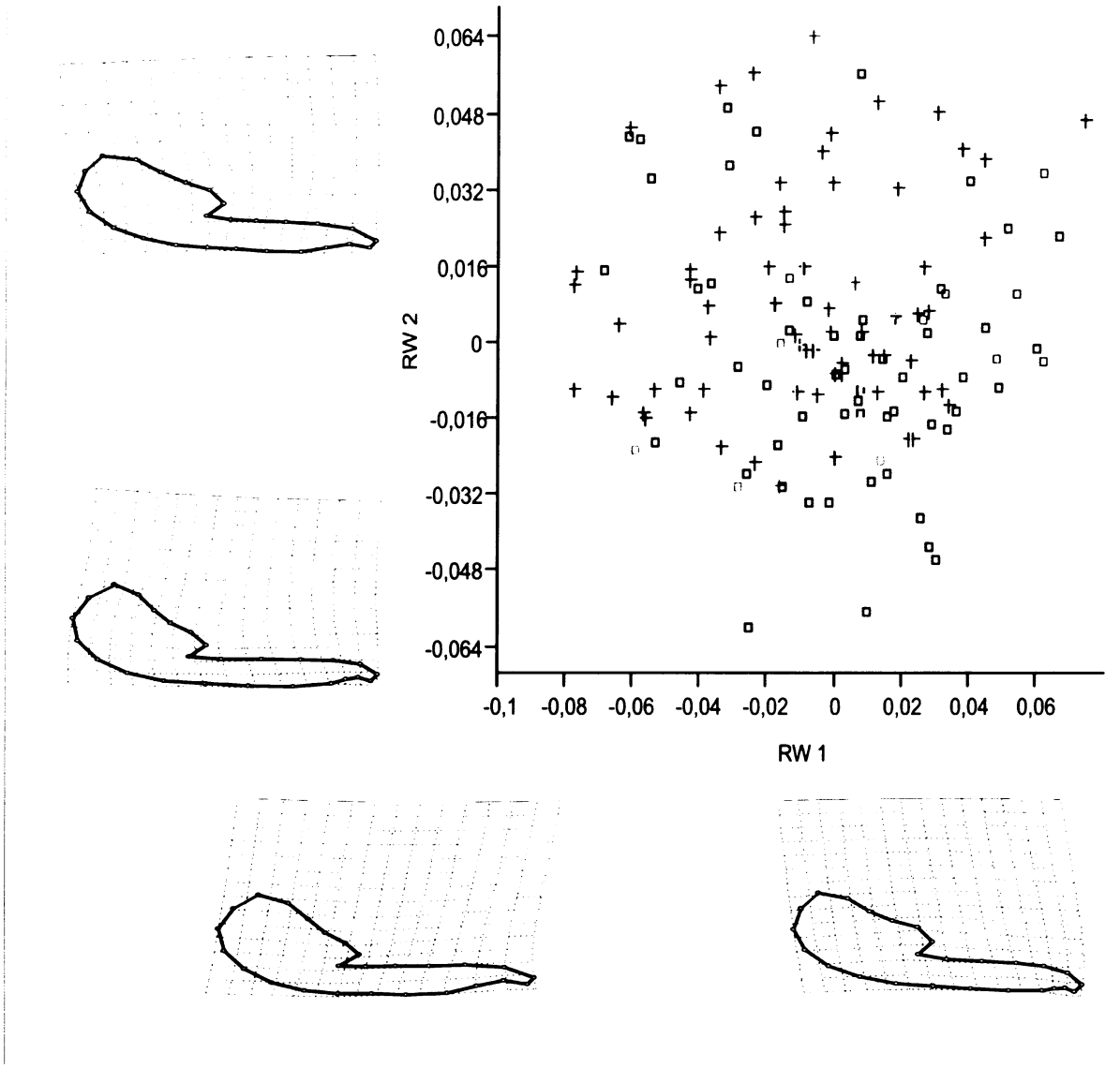


Fig. 18. RW1 × RW2 ordination diagram that accounts for 58.6 % of the total variation in the shape of the keel. Crosses, tetraploids; squares, diploids; red and blue, cytotypically pure populations; brown, individuals from the mixed population no. 157; yellow, individuals from the mixed population no. 171; green, individuals from the mixed population no. 174; violet, individuals from the mixed population no. 192. The changes in the shape at the negative and positive extremes of the RW1 and RW2 axes are illustrated as thin-plate splines.

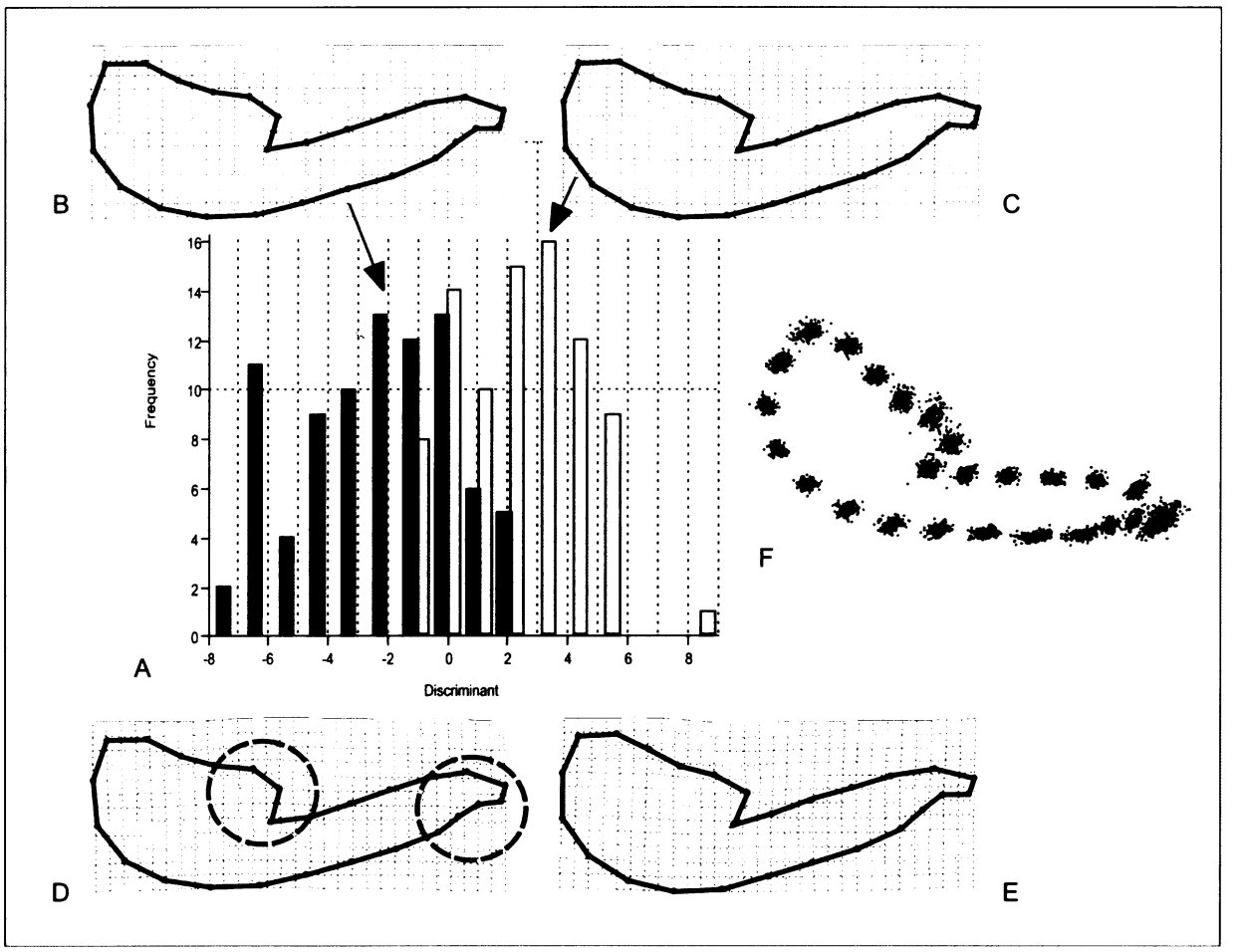


Fig. 19. Differences between cytotypes of *V. cracca* in the shape of the keel. A, discriminant analysis; grey, tetraploids; black, diploids. 88.82 % of objects were correctly classified. B, C, keel shape of a typical diploid, and a tetraploid, respectively. D, E, keel shape correlated with extreme negative and positive value, respectively. Circles indicate zones of the biggest changes. F, consensus shape of the keel.

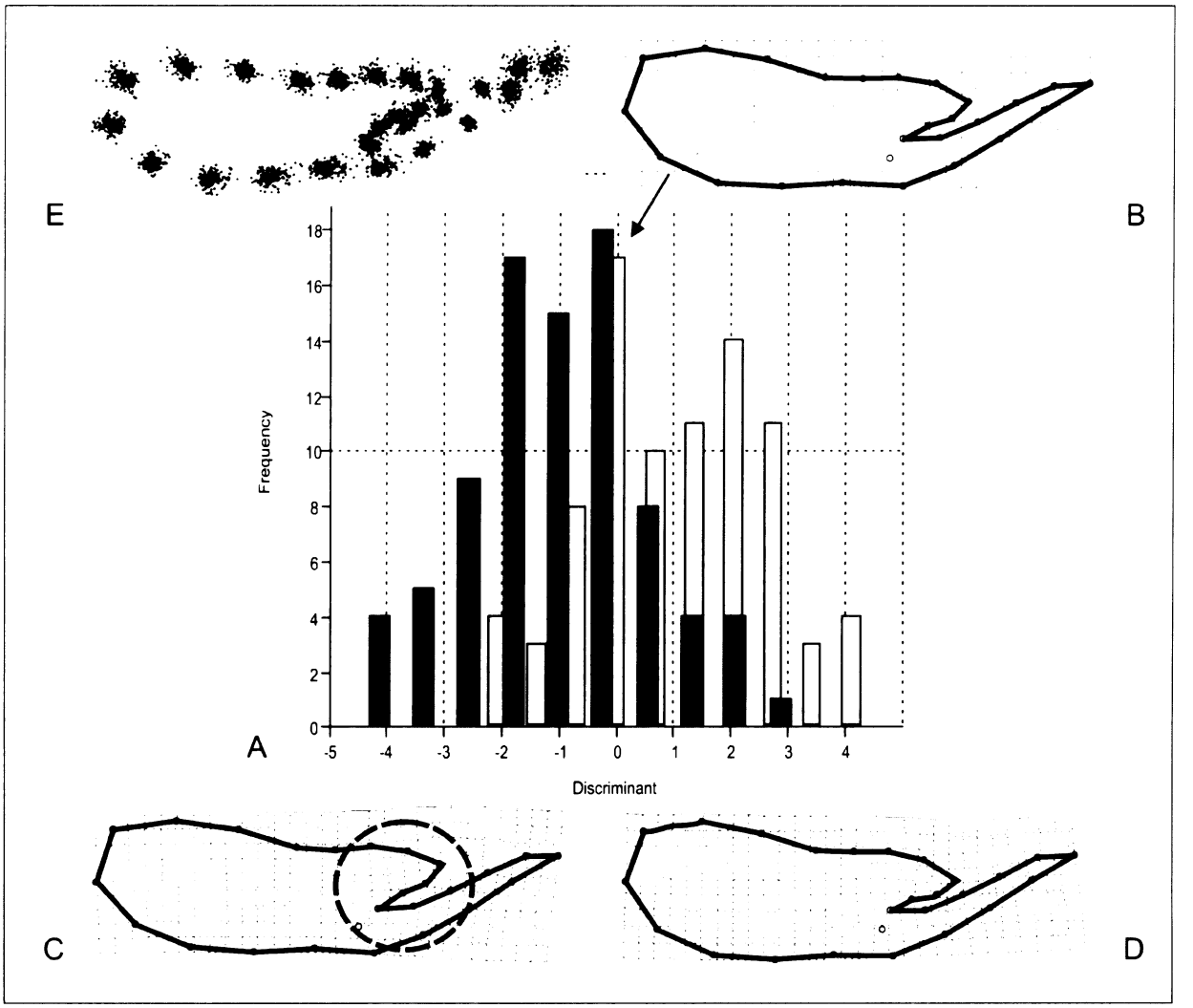


Fig. 20. Differences between cytotypes of *V. cracca* in the shape of the wing. A, discriminant analysis; grey, tetraploids; black, diploids. 78.82 % of objects were correctly classified. B, keel shape of a typical diploid, and a tetraploid, respectively. C, D, keel shape correlated with extreme negative and positive value, respectively. The circle indicates a zone of the biggest changes. E, consensus shape of a keel.

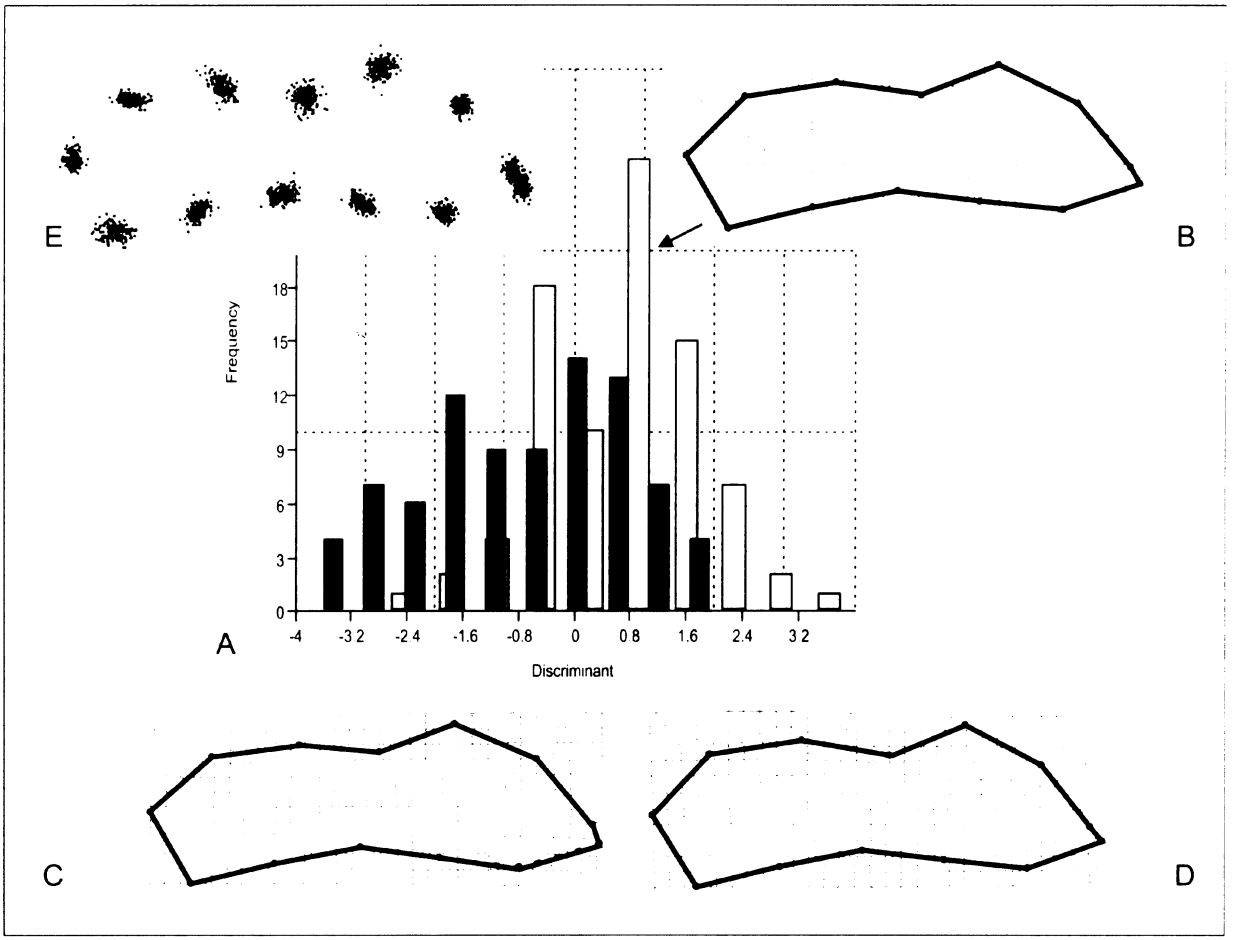


Fig. 21. Differences between cytotypes of *V. cracca* in the shape of the standard. A, discriminant analysis; grey, tetraploids; black, diploids. 70.59 % of objects were correctly classified. B, standard shape of a typical tetraploid. C, D, standard shape correlated with an extreme negative value of the discriminant axis, and with an extreme positive value, respectively. E, consensus shape of the standard.

Crossing experiments

A summary of matings that were carried out and of the resulting progeny is shown in Tab. 9. Among the seeds resulting from 87 reciprocal intercytotype crossings, no triploid was detected. The majority of the progeny was of the same ploidy level as the respective mother plant and could be ascribed to selfing. In addition, one tetraploid seed formed on a diploid plant and two diploid seeds formed on a tetraploid maternal parent were recorded. All matings in which only diploid parents participated gave also only diploid seeds. All additional seeds collected in natural diploid populations were also diploids.

Treatment	Number of inflorescences treated	Number of developed pods	Number of seeds	Number of seeds analysed by FCM	Ploidy level of seeds		
					2x	3x	4x
Intercytotype crossing:							
Mother 2x	87	29	78	48	47	0	1
Mother 4x	87	34	130	74	2	0	72
Intracytotype crossing:							
2x	15	6	28	13	13	0	0
Selfing:							
2x	37	8	26	13	13	0	0
Total	226	97	262	148	75	0	73

Tab. 9. Summary of crossing experiments performed.

Isozyme analyses

Enzyme electrophoresis resolved 6 putative loci. Only one locus was observed for 6-PGDH and SHDH, while two loci could be distinguished for each LAP and AAT. All loci were polymorphic. Banding patterns for LAP II and SHDH were as expected for a monomeric enzyme system with one or two bands for diploids and one to three bands for tetraploids (Fig. 22, 23). 6-PGDH and AAT I exhibited banding patterns expected for a dimeric enzyme, i.e. three-banded phenotype for when two alleles were expressed and six-banded phenotype when three alleles were expressed, with heterodimeric bands resolved between the two respective homodimeric bands (Fig. 24, 25). Banding patterns for LAP I were complicated due to formation of secondary bands by alleles *a-d* (Fig. 26). In addition, the slower form of the allele *a* co-migrated with the faster form of the allele *b*; the same was true for the slower *b* and the faster *c* forms and for the slower *c* and *d* forms. Because of the secondary bands, diploids showed up to four-banded patterns and tetraploids up to six-banded patterns for this locus (Fig. 26). The most complex patterns were expressed in the locus AAT II. There were only three alleles, but each producing one secondary band. Since this enzyme is dimeric, the dimers formed by subunits of the same allele but each representing a different mobility form were resolved as extra bands with an intermediate position. As a result, homozygotes showed three-banded phenotypes (Fig. 27). Moreover, individual bands on gels frequently merged into a single band because of a small difference between alleles, and the faintest bands could not be distinguished. So, the Fig. 27 is an idealised scheme inferred from the actual data rather then observed directly. Nonetheless, homozygous patterns and the position of the richest bands were sufficient guidelines to determine the allelic configuration of heterozygotes.

A total of 41 distinct alleles were observed. Of these, 37 were shared by diploid and tetraploid cytotypes (Tab. 10). Two alleles were found in diploids only: LAP II-*i* (pop. no.

174,175, 181) and SHDH-*k* (pop. no. 174), and two alleles were found in tetraploids only: 6-PGDH-*h* (pop. no. 187, 188) and AAT I-*a* (pop. no. 37, 174). All the cytotype-specific alleles with exception of AAT I-*a* in the population no. 37 were always rare (population frequency < 0.05). Triploids did not possess any unique alleles and they shared alleles with sympatric diploid counterparts. However, these alleles were common also in tetraploids.

In tetraploids, both balanced and unbalanced heterozygotes were observed. In all loci but LAP I, the unbalanced heterozygotes were more frequent (Tab. 11). Complex heterozygotes with three alleles were also detected in all loci and in LAP I locus even individuals with four alleles were observed.

An UPGMA dendrogram based on Nei's genetic distances (Fig. 28) showed that populations were not consistently segregated according to a ploidy level. There are several groups of both diploids and tetraploids at different levels of branching and diploids are in some cases more similar to tetraploids than to other diploids. However, sympatric populations of different cytotypes (i.e. cytotype subpopulations of mixed-ploidy populations) never formed sister branches. The two triploid populations, when included to the analysis, were placed as two separate branches at the base of the dendrogram, far from their respective sympatric diploids (data not shown). Genetic distances between diploids and tetraploids in mixed populations were not smaller than the distances between allopatric cytotypes (Tab. 12). Correlation between genetic and geographic distances was significant (P = 0.0005) only in the test with both ploidy levels included, but the resulting model explained only a small part of total variation in the data and the correlation was not strong (R^2 = 13.22, r = 0.364). When the cytotypes were tested separately, they did not exhibit a statistically significant correlation between genetic and geographic distances (data not shown).

Analysis of diversity with Shannon's index revealed that most isozyme variation was partitioned within, rather than between populations (Tab. 13). Average G'_{ST} was significantly lower (P < 0.05) for diploids (0.218) than for tetraploids (0.316). The locus LAP I showed a reverse trend when compared to other loci, since the among-populations component was bigger for diploids than for tetraploids. Mean inbreeding coefficient, F_{IS}, was 0.1503 for diploids and 0.0829 for tetraploids. These values were significantly different from zero (P < 0.05), but not significantly different from each other. Mean F_{ST} was 0.0583 in diploid populations and 0.0778 in tetraploids, indicating a low but significant (P < 0.05 and P < 0.001, respectively) level of genetic differentiation between populations, but the differences in mean F_{ST} between cytotypes were again insignificant (Tab.14).

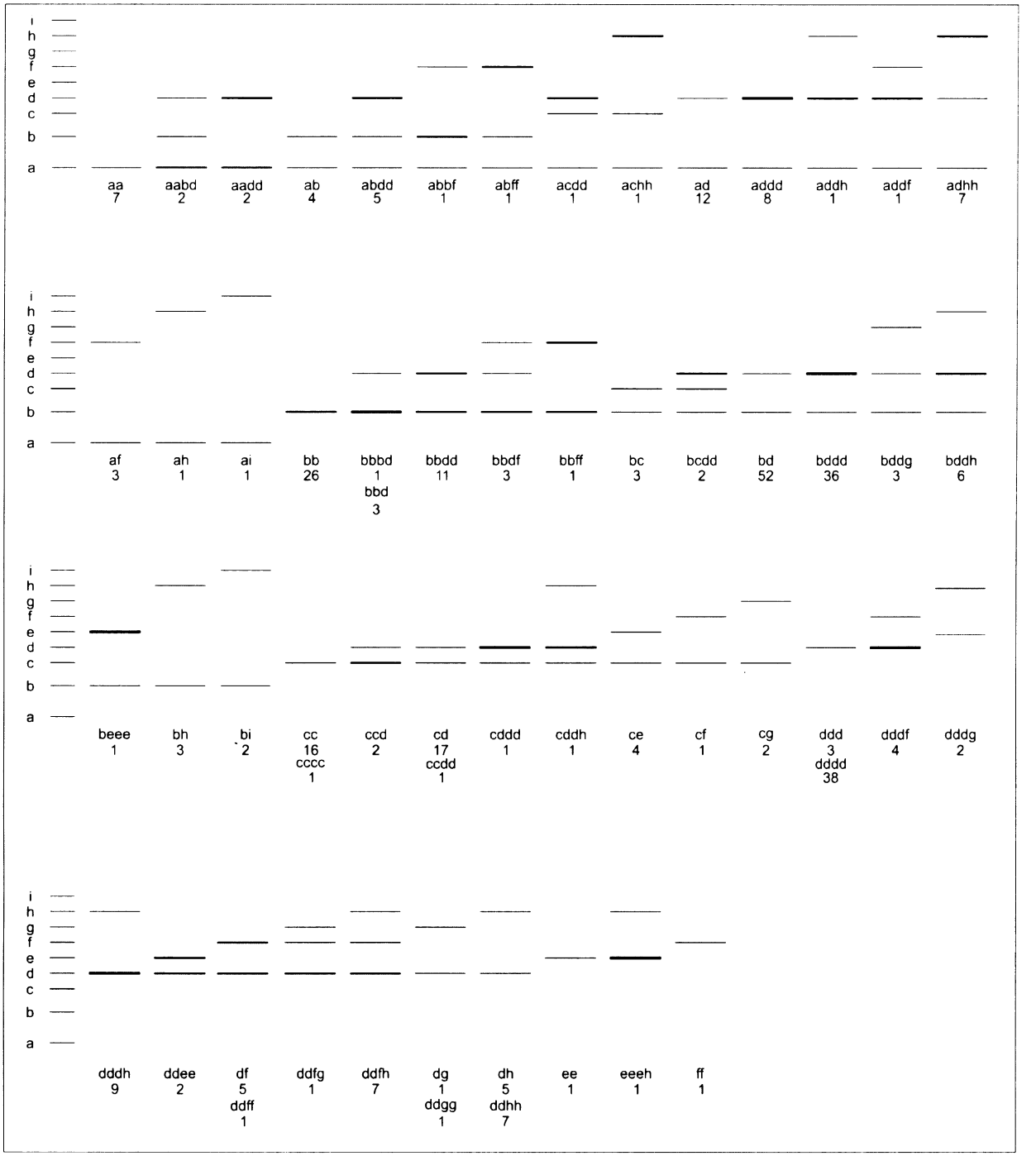


Fig. 22. Schematic representation of observed LAP II phenotypes in diploid and tetraploid *V. cracca* plants. Banding patterns were examined for relative band intensities that were interpreted as corresponding to genotypes of different allelic dosage. The number of plants exhibiting the respective pattern is indicated below each phenotype.

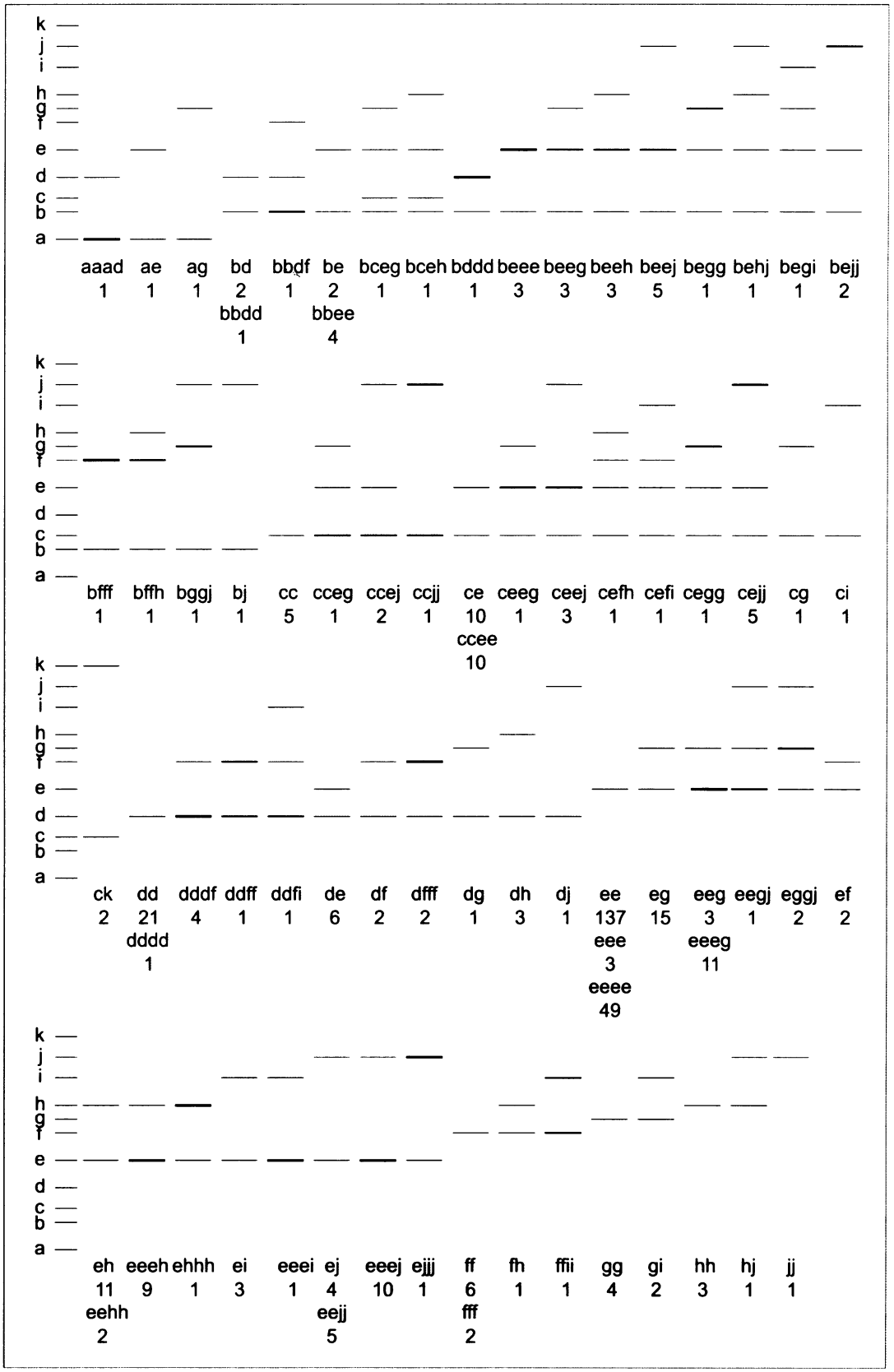


Fig. 23. Schematic representation of observed SHDH phenotypes in diploid and tetraploid *V. cracca* plants. Banding patterns were examined for relative band intensities that were interpreted as corresponding to genotypes of different allelic dosage. The number of plants exhibiting the respective pattern is indicated below each phenotype.

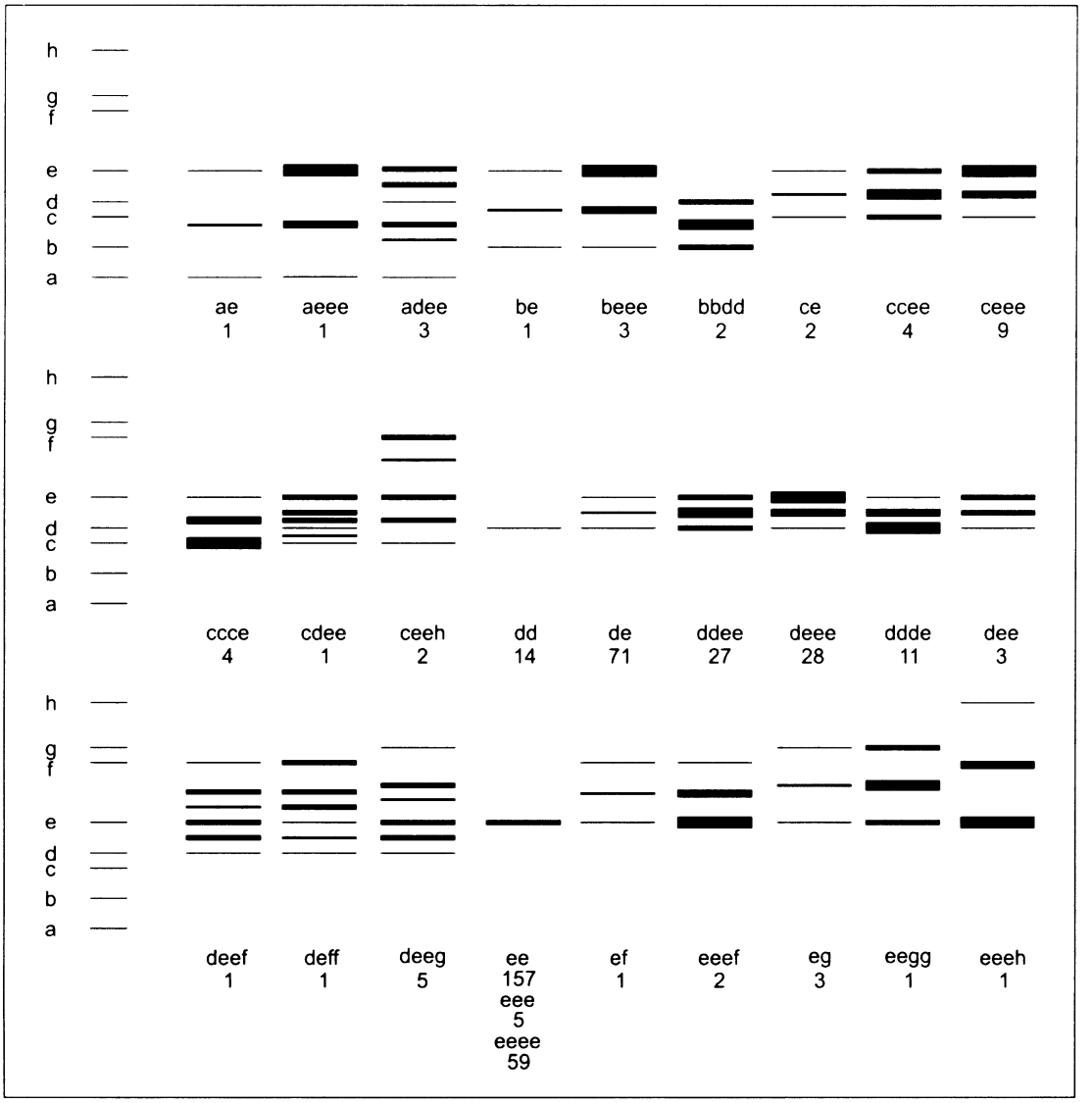


Fig. 24. Schematic representation of observed 6-PGDH phenotypes in diploid and tetraploid *V. cracca* plants. Banding patterns were examined for relative band intensities that were interpreted as corresponding to genotypes of different allelic dosage. The number of plants exhibiting the respective pattern is indicated below each phenotype.

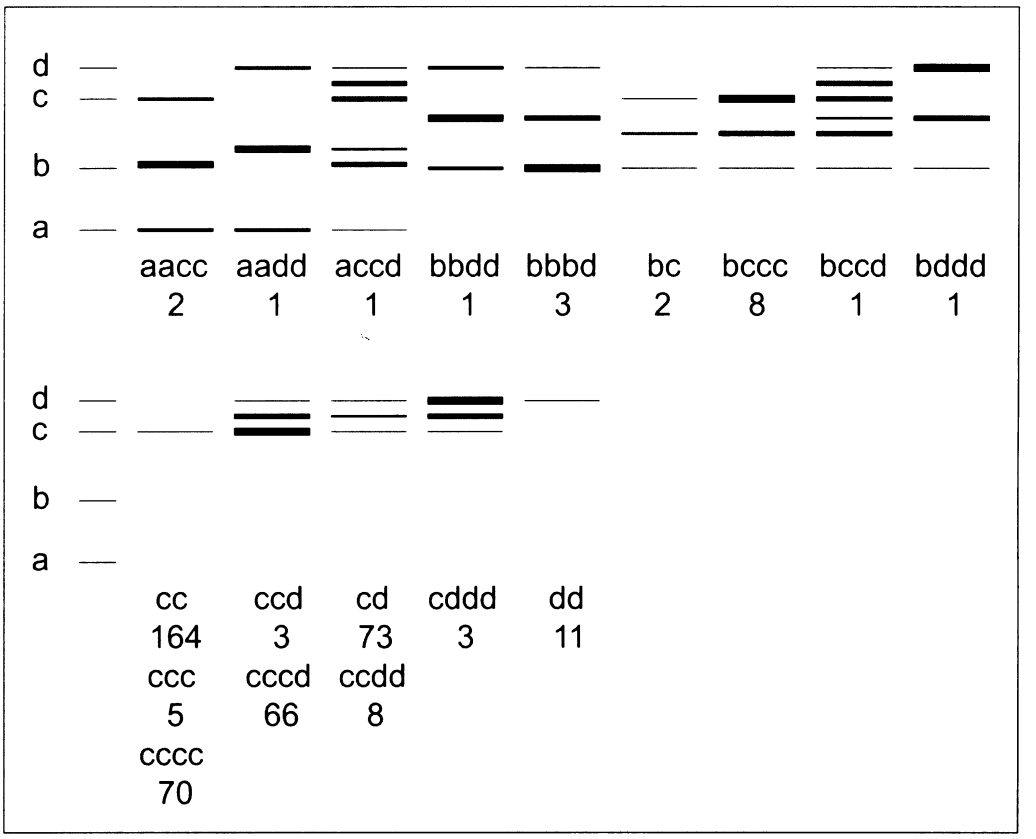


Fig. 25. Schematic representation of observed AAT I phenotypes in diploid and tetraploid *V. cracca* plants. Banding patterns were examined for relative band intensities that were interpreted as corresponding to genotypes of different allelic dosage. The number of plants exhibiting the respective pattern is indicated below each phenotype.

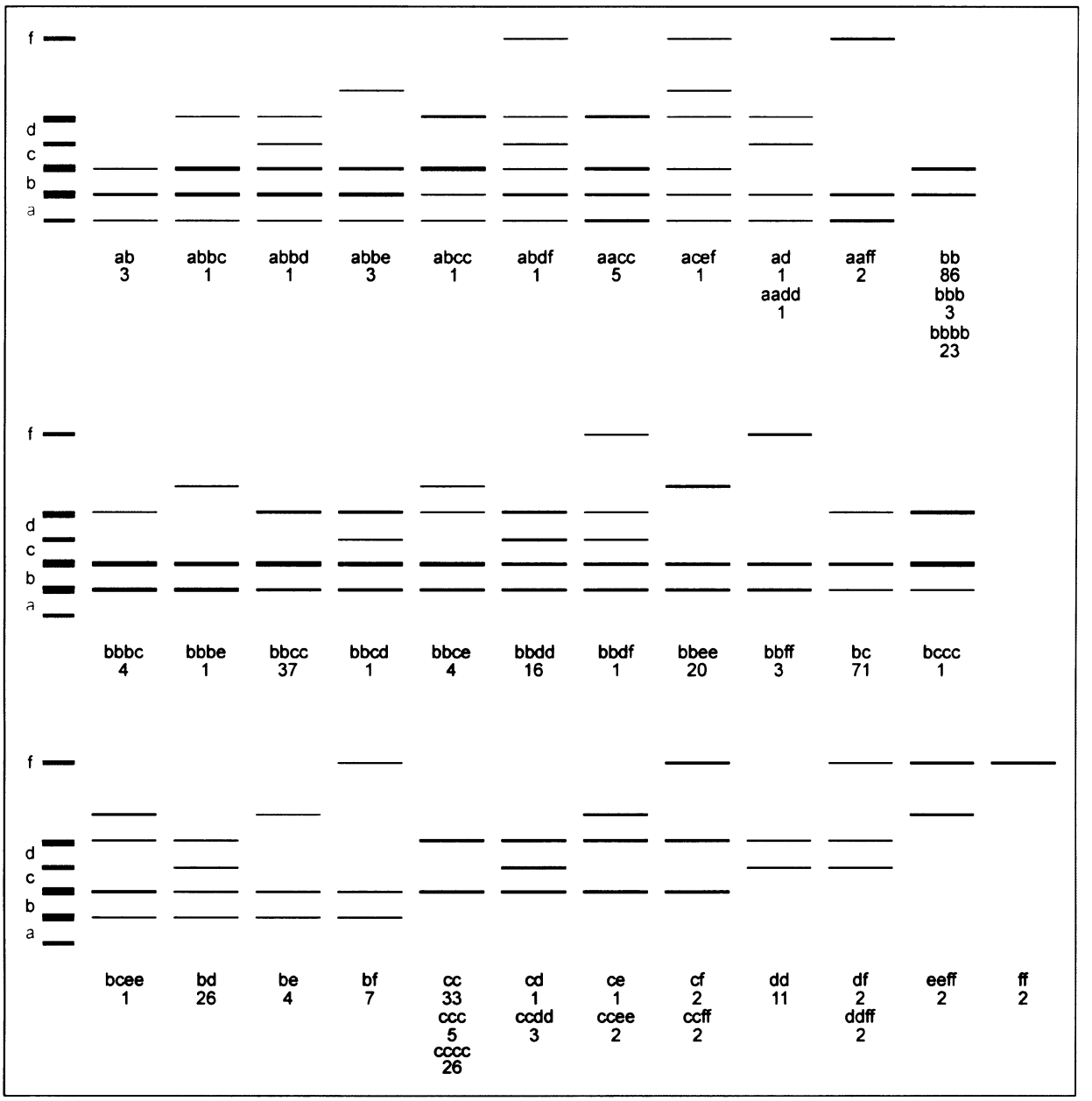


Fig. 26. Schematic representation of observed LAP I phenotypes in diploid and tetraploid *V. cracca* plants. Banding patterns were examined for relative band intensities that were interpreted as corresponding to genotypes of different allelic dosage. The number of plants exhibiting the respective pattern is indicated below each phenotype.

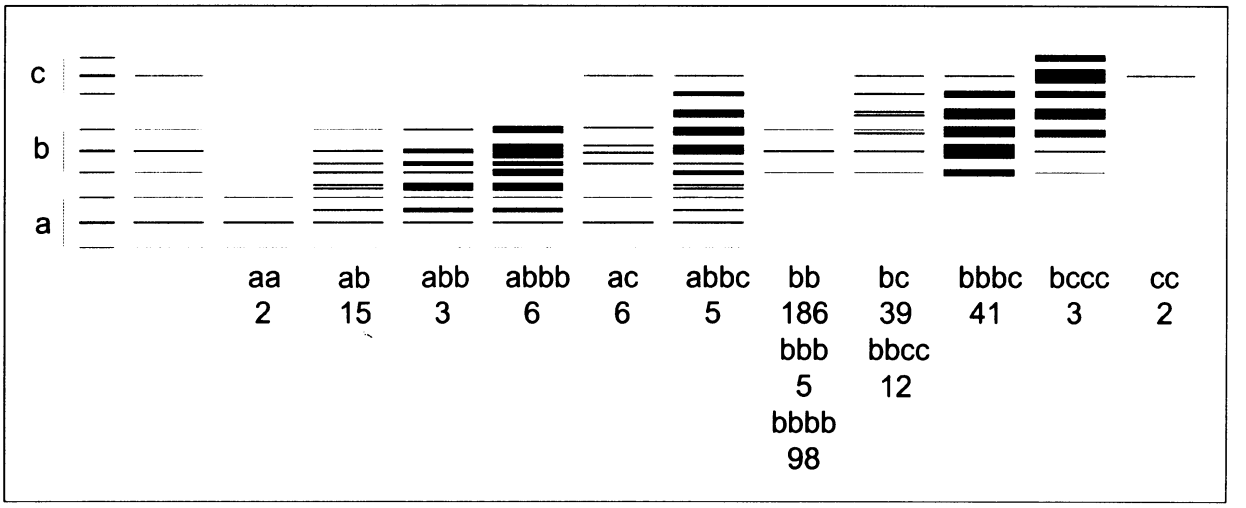


Fig. 27. Schematic representation of observed AAT II phenotypes in diploid and tetraploid *V. cracca* plants. Banding patterns were examined for relative band intensities that were interpreted as corresponding to genotypes of different allelic dosage. The number of plants exhibiting the respective pattern is indicated below each phenotype.

Locus	Total no. of alleles	No. in 2x	No. in 4x	No. of shared allele
6-PGDH	8	7	8	7
LAP I	6	6	6	6
LAP II	9	9	8	8
SHDH	11	11	10	10
AAT I	4	3	4	3
AAT II	3	3	3	3
Total	41	39	39	37

Tab. 10. Alleles shared by diploids and tetraploids of *V. cracca*.

Locus	Balanced heterozygotes	Unbalanced heterozygotes	Heterozygotes with 3 alleles	Heterozygotes with 4 alleles
6-PGDH	34	59	13	0
LAP I	95	6	13	2
LAP II	26	63	37	0
SHDH	29	45	40	0
AAT I	12	81	2	0
AAT II	12	50	5	0
Average (%)	34.7 (21)	50.7 (30.7)	18.3 (11.1)	0.3 (0.2)

Tab. 11. Number of individual types of observed heterozygotes in 165 tetraploids of *V. cracca*.

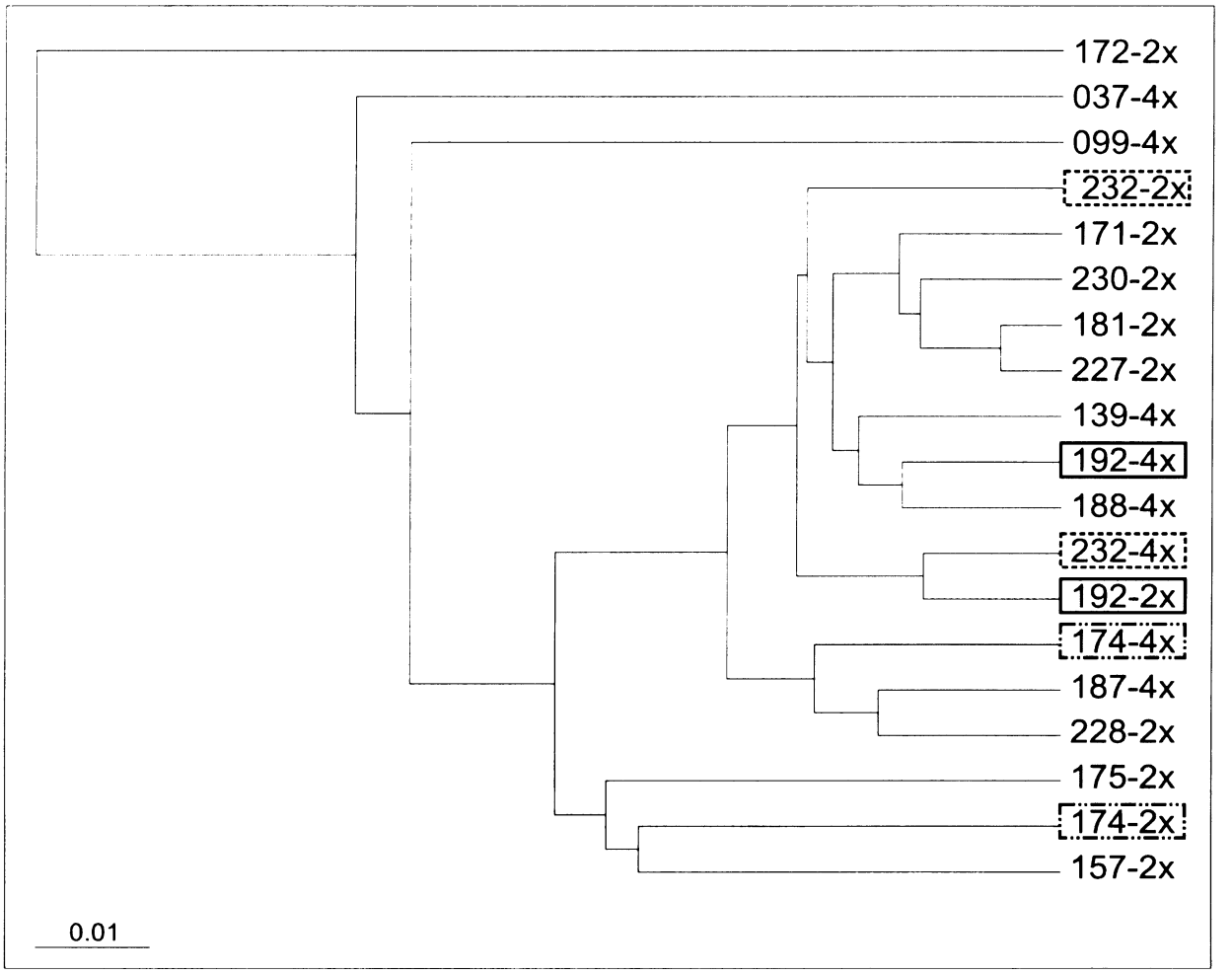


Fig. 28. UPGMA dendrogram calculated from among-population Nei's genetic distances based on population frequencies of individual alleles (2x, diploids; 4x, tetraploids; frames, mixed populations).

	174-4x	187-4x	232-4x	099-4x	192-4x	139-4x	037-4x	188-4x
171-2x	0.073	0.033	0.064	0.091	0.048	0.072	0.120	0.048
172-2x	0.189	0.158	0.206	0.234	0.203	0.243	0.234	0.197
175-2x	0.081	0.053	0.115	0.110	0.071	0.098	0.195	0.085
181-2x	0.077	0.026	0.045	0.092	0.038	0.046	0.091	0.038
174-2x	**0.143**	0.079	0.140	0.123	0.109	0.100	0.190	0.108
157-2x	0.065	0.051	0.104	0.108	0.110	0.129	0.153	0.110
227-2x	0.070	0.029	0.051	0.091	0.028	0.028	0.110	0.036
228-2x	0.048	0.032	0.049	0.112	0.038	0.057	0.106	0.055
232-2x	0.062	0.052	**0.051**	0.147	0.049	0.048	0.089	0.041
230-2x	0.112	0.049	0.040	0.147	0.042	0.031	0.132	0.030
192-2x	0.070	0.035	0.024	0.119	**0.048**	0.079	0.124	0.038

Tab. 12. Comparison of Nei's genetic distances between cytotypes in sympatric populations (bold) and in allopatric populations.

Locus	2x				4x			
	H'pop	*H*'sp	*H*'pop/*H*'sp	(*H*'sp-*H*'pop)/*H*'sp	*H*'pop	*H*'sp	*H*'pop/*H*'sp	(*H*'sp-*H*'pop)/*H*'sp
6-PGDH	0.474	0.579	0.818	0.182	0.765	1.133	0.675	0.325
LAP I	0.806	1.148	0.702	0.298	1.145	1.567	0.731	0.269
LAP II	1.139	1.688	0.675	0.325	1.056	1.635	0.646	0.354
SHDH	1.175	1.594	0.737	0.263	1.365	2.186	0.624	0.376
AAT II	0.497	0.573	0.867	0.133	0.408	0.544	0.750	0.250
AAT I	0.401	0.448	0.894	0.106	0.467	0.691	0.675	0.325
Average			0.782	0.218*			0.684	0.316*

Tab. 13. Partitioning of the genetic diversity of six isozyme loci into within-populations (*H*'pop/*H*'sp) and among-populations [G'_{ST} = (*H*'sp-*H*'pop)/*H*'sp] components of 11 diploid and 8 tetraploid populations of *V. cracca*. Averages idicated by * are significantly different at $P < 0.05$.

Locus	2x			4x		
	F_{IT}	F_{IS}	F_{ST}	F_{IT}	F_{IS}	F_{ST}
All loci	0.234****	0.1727****	0.074****	0.1786****	0.1065****	0.0808****
6-PGDH	0.0832	0.0542	0.0307*	0.1636****	0.0691*	0.1016****
LAP I	0.2045****	0.1493**	0.0649****	0.3046****	0.2361****	0.0897****
LAP II	0.2828****	0.1643***	0.1418****	0.0497*	0.0162	0.0341****
SHDH	0.4728****	0.4213****	0.089****	0.2461****	0.1607****	0.1018****
AAT II	0.086	0.0854	0.0007	0.0596	0.0086	0.0515****
AAT I	0.0494	0.0273	0.0227	0.0939**	0.0068	0.0878****
Mean	0.19645*	0.1503*	0.0583*	0.152917**	0.082917*	0.07775***

Tab. 14. Estimates of *F*-statistics for all loci for the diploid and tetraploid populations of *V. cracca*. Values different from 0 at **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. Data for this table were kindly provided by B. Mandák.

Discussion

The almost identical mean fluorescence per monoploid genome in diploid and tetraploid *V. cracca* plants suggests an autopolyploid origin of the latter cytotype, in agreement with previous hypotheses about the origin of tetraploids (Rousi 1961, 1962, 1973; Chrtková-Žertová 1973a; Dvořák et al. 1977). My allozyme data give additional support for the autotetraploid origin. The small number and rarity of alleles unique to each ploidy level detected in this study tells against the involvement of an additional, genetically different ancestor of the tetraploids. Moreover, the nature of the polyploidisation event can be distinguished by its impact on the type and frequency of heterozygotes encountered in the population. 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. By contrast, allotetraploids are expected to display fixed heterozygosity, as alleles at a given locus on homologous chromosomes segregate independently (Ramsey & Schemske 2002). No evidence of fixed heterozygosity was found in any of the loci examined in *V. cracca*, although in LAP I unbalanced heterozygotes were markedly less frequent than balanced heterozygotes. Nevertheless, unbalanced heterozygotes may occur also in populations of allotetraploids with disomic inheritance provided that their diploid ancestors were themselves heterozygotes. So, the allozyme evidence for autotetraploidy based on population samples should be treated as indirect only. Actually, tetrasomic inheritance can be decisively inferred only from allozyme analyses of progeny raised from experimental crossings between appropriately chosen parents of known genotypes. For example, crossing between a parent with four different alleles and a homozygous parent would result in progeny of four genotypes under disomic inheritance, whereas under tetrasomic inheritance we expect six genotypes or even more in the presence of double reduction (Hardy et al. 2002). Hence, to definitely prove the autotetraploid origin of *V. cracca*, such experiments are to be done in the future.

The cytotypes of *V. cracca* showed segregation at a large geographical scale (Fig. 6A and 7) with a narrow contact zone following Western Carpathians. As it was suggested in other studies of polyploids (Hardy et al. 2000; Mandáková & Münzbergová 2006; Van Dijk & Bakx-Schotman 1997; Baack 2004), this pattern is in accordance with a secondary contact zone hypothesis, when cytotypes of different ploidy level originated in allopatry and then

were spreading up to meeting one another. This hypothesis is supported by low frequencies of mixed-ploidy populations (6.9 %) and a low rate of cytotype intermingling. If tetraploids originated recently from diploids, frequency of tetraploids should be low in the mixed populations. Although I observed an apparent excess of mixed-ploidy populations dominated by diploids (Fig. 5), the deviation from a uniform distribution for both diploids and tetraploids was statistically insignificant. In addition, it is known that arising of tetraploids from diploids through the union of two unreduced gametes is very rare (Ramsey & Schemske 1998) and indeed, I did not record any tetraploid progeny within seeds collected in populations of diploids. The probability of the origin of tetraploids *de novo* increases if viable and fertile triploids arise in the first step and serve as a triploid bridge to the formation of tetraploids (Husband 2004). However, in despite of the comprehensive sampling, only eight triploids out of a total of 6 613 plants were detected. It is evident from these observations that the rate of production of unreduced gametes is either very low in *V. cracca* or a strong selection pressure against triploid zygotes is exerted there. Intercytotype hybrids can also enhance the origin of tetraploids (Peckert & Chrtek 2006). However, no triploid seed formed in my experimental intercytotype matings. Strong prezygotic or postzygotic barriers evidently prevent mating between *V. cracca* cytotypes.

If there are no differences in the fitness or in the colonising ability among cytotypes, coexistence of two cytotypes in a contact zone is frequency-dependent. When inter-cytotype hybrids are nonviable or sterile, the minority cytotype suffers by ineffective matings with counterparts of another ploidy level and decreases in frequency up to exclusion (Levin 1975). Crossing experiments with *V. cracca* show that inter-cytotype matings are indeed not compatible. In these experiments, the progeny had always the same ploidy as the mother plant and hence apparently came from selfing. It is possible that pollen tubes do not germinate on a stigma of a ploidy level different from that of pollen donor or triploid zygotes do arise but abort.

Theoretical models have demonstrated that selfing can ameliorate the minority cytotype disadvantage (Levin 1975; Rodríguez 1996) and some studies even suggested reinforcement of the selfing rate in hybrid zones compared to allopatric populations (Petit et al. 1997). If selfing was a dominant mode of reproduction in *V. cracca*, we would expect a negligible impact of the triploid block and hence a high rate of cytotype intermingling in the contact zone limited only by dispersal abilities. However, the contact zone recorded in the present study is rather narrow and no diploids invade westwards into the range of tetraploids. Interestingly, diploids recorded in the past in the southwest of the Czech Republic have not

been found any more, suggesting that they might have gone extinct, perhaps due to the minority cytotype exclusion. On the other hand, tetraploids are found far in the east surrounded by diploids. It is possible that tetraploids are in some respect superior, which allow them to coexist in minority with diploids. Competitive superiority of autotetraploids associated with higher tiller weight, heavier seeds, faster leaf production and earlier flowering was described for example in *Dactylis glomerata* (Maceira et al. 1993). Possible competitive superiority together with at least partial autogamy, iteroparity and vegetative reproduction might be the reason for the successful invading of diploids by tetraploids. A comprehensive study of life histories of both cytotypes will be necessary to corroborate this scenario.

As was documented above, triploid production via inter-ploidy crosses does not contribute to gene flow between cytotypes of *V. cracca*. Inter-ploidy gene flow may also occur through unreduced gametes from diploids. Although rare, triploids arising probably from union of reduced and unreduced gametes produced by diploids were recorded in nature. It remains to be tested if these triploids are fertile and can successfully backcross with diploids. Interestingly, I encountered a single case of a tetraploid seed that arose on a diploid plant in one of my crossing experiments. This finding is in agreement with Rousi's (1973) consideration that tetraploids arising from unreduced egg cells in 2x – 4x crosses may provide a mechanism for introgression of genes from diploids into tetraploids. However, as mentioned above, this is perhaps a very rare event. Another pathway of inter-cytotype gene flow could be the production of diploid progeny by tetraploids through the apomictic development of egg cells. This situation may have occurred for one seedling in *Centaurea jacea* (Hardy et al. 2001). I also recorded in my experiments with *V. cracca* two diploid seeds matured on tetraploid plants, but polyhaploids are expected to be very improbable in this case. This confusing result may have been reached perhaps due to a mistake during the seeds collecting.

Although *V. cracca* proved self-compatible in my pollination experiments, there is a device that warrants outcrossing. Anthers open before anthesis and pollen is deposited within the tip of the keel around the upper part of the style in Fabaceae (Endress 1994). When a pollinator is landing on the keel, it is moved downwards so the stigma and the anthers are exposed from its tip. Pollen is then brushed out of the keel tip by the hairy tip of the style. However, pollen surrounding the stigma cannot germinate unless the flower at anthesis is visited by a pollinator, because the stigma is covered by a membrane that is disrupted only by the tripping process (Endress 1994).

The pattern of isozyme variability also suggests that *V. cracca* is only a facultatively selfing species. It shows that the rate of inbreeding (F_{IS}) in natural populations is significant

but rather small in *V. cracca* and that there is an insignificant difference between both cytotype in this aspect. Values of the fixation index (F_{ST}) indicate a small genetic differentiation among populations of individual cytotypes. Values of the G_{ST} coefficient of gene differentiation among populations are a bit higher than F_{ST}, but still support relatively high gene flow among populations within each cytotype. The value of G_{ST} calculated for diploids corresponds with a value described for short-lived outbreed perennials (Hamrick & Godt 1996). Thus, considering all indices together we can conclude that Zhang & Mosjidis (1998) and Rousi (1973), who maintained that *V. cracca* is autogamous, as well as Jaaska (2005), who claimed the reverse (i.e. allogamous), were right. The significantly higher mean G_{ST} calculated for tetraploids might be due to higher rates of self-fertilisation. Data on angiosperm polyploids support the hypothesis that polyploids have, on average, higher rates of self-fertilisation than their diploid relatives (Barringer 2007). This is generally explained by the suggestion that autopolyploids may exhibit lesser inbreeding depression than diploids, owing to the presence of multiple gene copies and the associated reduction in the rate of homozygote formation.

The relatively high gene flow between populations within cytotypes is in accordance with the finding that there is insignificant correlation between geographical and genetic distances within individual ploidy level. Although the correlation between genetic and geographic distances in a model with both cytotypes included together proved significant, it explained only 13.22 % of total variability. Hence, it is not surprising that no clear geographical pattern is observed in the UPGMA dendrogram constructed from genetic distances of individual populations (Fig. 28). In addition, the genetic distance data do not support a notion of tetraploids arising recently from diploids, because tetraploids from mixed-ploidy populations never branch sister to sympatric diploid populations in the UPGMA dendrogram. Triploids, which are assumed to originate from unreduced gametes of diploids, are expected to cluster with the respective sympatric diploid populations, but this was not seen in the UPGMA analysis. However, the number of triploids, for which isozyme data could be obtained (three and five, respectively, for the two separate populations), was low due to their rarity, so the calculation of their genetic distances may suffer from a large sampling error.

According to my classical and geometric morphometric analyses, diploids and tetraploids of *V. cracca* are slightly, but significantly, different with respect to size and shape of some organs. Characters that Chrtková-Žertová (1973a) claimed as discriminating between the cytotypes (the number of leaflet pairs, the length of the inflorescence and mean

inflorescence/subtending leaf length ratio) have proved inappropriate in the present study, although the latter character did show small differences between the cytotypes (Tab. 7, Fig. 17F). My analyses revealed that the most expressed differences between the cytotypes were in floral traits. Tetraploids of *V. cracca* tended to have a bigger standard claw, a longer keel and wider wings. This is not surprising, because polyploids inherently have bigger cells and usually also bigger whole organs (Segraves & Thompson 1999, Stebbins 1971).

More interesting are the differences between cytotypes in the shape of floral structures. Besides the size, the shape undoubtedly has an impact on plant-animal interactions, which was demonstrated, for instance, in a beautiful geometric morphometric study by Gómez et al. (2006). Unfortunately, plant biologists have so far been rarely using geometric morphometrics to study morphological differences among polyploids. This lack of trust to this method results especially from the fact that sometimes there is a problem with determining homologous points as potential landmarks (Jensen 2003). However, this problem disappears at the intraspecific level. In the present study, geometric morphometric tools proved instrumental to detect between-cytotype differences in the shape of the standard, wing and the keel. The standard is the main optical display organ and the keel, together with the wings, forms a landing platform for pollinating insects in the genus *Vicia* (Endress 1994). The inner surface of the wings forms two little pleats that adnate to the analogous hollows in the keel tip. This pattern may be species-specific and may serve as a prepollination isolating mechanism (Endress 1994). Henceforth, even fine changes in the shape of these floral structures might have an impact on the reproductive strategy and success.

Results of a cluster analysis based on morphological characters might be viewed as other evidence supporting the hypothesis of the secondary contact zone, since the individuals from mixed-ploidy populations clustered with other members of their own ploidy level rather than with counterparts of different cytotypes within the same populations (Fig. 15). However, it is possible that polyploidisation *per se* have a so profound systemic impact on the morphology that even independently arisen polyploids would cluster together rather than with their diploid ancestors. Nevertheless, the morphological disparity between sympatrically occurring diploids and tetraploids of *V. cracca* seems lower than that between diploids and tetraploids from pure populations. First, even though individuals from mixed-ploidy populations constitute a minority among all individuals analysed, they form a full half of all individuals misclassified by the classificatory DA. Second, the differences in both "classical" morphological characters and the shape of floral parts (except the keel) between diploids and tetraploids from mixed populations were significantly less pronounced than between diploids

and tetraploids from pure populations when tested via Euclidean distances derived from principal component scores. This indicates that diploids and tetraploids in mixed populations are generally mutually more similar than diploids and tetraploids growing in allopatry. Although this might be interpreted as indicating a specific genetic relationship of diploids and tetraploids in mixed populations, another explanation might as well be that plants of both ploidies in sympatry converge to the same form due to the same ecological conditions they are facing.

Quite interestingly, the keel exhibited an opposite trend, as the differences between its shape in diploids and tetraploids from mixed populations were statistically higher that the differences between diploids and tetraploids from pure populations. This unexpected phenomenon may indicate the operation of disruptive selection on the shape of the keel in plant of different ploidy. Since I demonstrated in crossing experiments the existence of a triploid block between diploids and tetraploids of *V. cracca*, it is conceivable to assume that a selection pressure is exerted *against* mating between plants of a different ploidy level. In other words, a selection pressure *for* assortative mating among individuals of the same ploidy is expected to take place in *V. cracca.* Indeed, it was reported that pollinators may exhibit a preference for flowers of one or another cytotype (Husband & Sabara 2003, Nuismer & Cunningham 2005). Hence, originally slight differences in the floral organs distinguished by the pollinators may be driven by the disruptive selection to diverge apart so that cross-pollination of plants of different ploidy is minimised.

Chrtková-Žertová (1973a) distinguished a mountain and a lowland race within diploids of *V. cracca*. I show in this study that altitude really can have an impact on the morphology of the cytotypes, yet not only in diploids, but also in tetraploids. Furthermore, diploids as well as tetraploids occur in the whole range of altitudes without any discontinuity (Fig. 8). Actually, even when the distribution would be discontinuous, it would be meaningful to discern two morphotypes only if the morphological differences were stable in transplantation experiments. Thus, I think that diploids should be treated as one entity at present.

Conclusions

Results obtained with the use of flow cytometry and an analysis of allozymes bring further support for the assumed autopolyploid origin of the tetraploid *V. cracca* in the area of central Europe. A relatively narrow zone of contact of tetraploids and diploids, which follows the line of the Western Carpathians at the Czech-Slovak border, was delimited by increasing the density of sampling compared to previously known distribution of the cytotypes. Allozyme data suggest a secondary origin for this contact zone, but further studies, probably employing additional molecular markers, are necessary to corroborate this notion. A strong triploid block between the two cytotypes, proved by experimental crosses, probably results in minority cytotype exclusion participating on the maintenance of the narrow tetraploid-diploid contact zone. Triploid block between cytotypes caused probably also the disappearance of diploids from the southwest of the Czech Republic. Triploids resulting from fusion of reduced and unreduced gametes within diploid populations were very rare (0.1 %) and restricted to a small region at the northwest of Slovakia. Gene flow between cytotypes via the formation of tetraploid seeds by diploid mother plants seems to be possible but also extremely rare. Both cytotypes of *V. cracca* seems to have mixed breeding system with prevailing outcrossing. The cytotypes are slightly but significantly different with regard to morphology, especially of flower characters. Tetraploids are generally bigger but besides the quantitative characters there are also some changes in the shape of floral structures correlated with ploidy level. Although there appears to be an impact of the altitude on morphology of both diploids and tetraploids, recognition of the two diploid races described by Chrtková-Žertová (1973a) is probably not justified.

References

Arohonka T. (1982): Chromosome counts of vascular plants of the island Seili in Nauvo, southwestern Finland. Turun Yliopiston Julkaisuja: Sarja A II, Biologia-Geographica 3: 1-12.

Baack E. J. (2004): Cytotype segregation on regional and microgeographic scales in snow buttercups (*Ranunculus adoneus*: Ranunculaceae). American Journal of Botany 91 (11): 1783-1788.

Baksay L. (1954): Chromosomenstudien an den ungarischen *Vicia*-Arten. Annales Historico-Natururales Musei Nationalis Hungarici (ser. nova) 5: 139-148.

Barringer B. C. (2007): Polyploidy and self-fertilization in flowering plants. American Journal of Botany 94 (9): 1527-1533.

Belaeva V. A. & Diplivinky V. N. (1977): Chromosome numbers and taxonomy of some species of Baikal flora III. Botanicheskiy Zhurnal 62: 1132-1142.

Belaeva V. A. & Siplivinky V. N. (1975): Chromosome numbers and taxonomy of some species of Baikal flora I. Botanicheskiy Zhurnal 60: 864-872.

Belaeva V. A. & Siplivinsky V. (1981): In Chromosome number reports LXXIII. Taxon 30: 857-861.

Bennett M. D. (2004): Perspectives on polyploidy in plants – ancient and neo. Biological Journal of the Linnean Society 82: 411-423.

Bever J. D. & Felber F. (1992): The theoretical population genetics of autopolyploidy. In: Futuyma D. & Antonovics J. (eds.): Oxford Surveys in Evolutionary Biology. Oxford. Pp. 185-217.

Bookstein F. L. (1991): Morphometric tools for landmark data: geometry and biology. Cambridge University Press, Cambridge.

Bussell J. D. (1998): The distribution of random amplified polymorphic DNA (RAPD) diversity amongst populations of *Isotoma petraea* (Lobeliaceae). Molecular Ecology 8: 775-789.

Činčura F. (1963): Poznámky k cytológii druhov rodu *Vicia* L. zo slovenských nálezísk II. Acta Facultatis Rerum Naturalium Universitatis Comenianae (Ser. Bot.) 9: 349-388.

Činčura F. (1981): *Vicia cracca* agg. in der Slowakei. Acta Facultatis Rerum Naturalium Universitatis Comenianae (Ser. Bot.) 28: 77-94.

Choi B.-H., Seok D.-I., Endo Y. & Ohashi H. (2006): Phylogenetic significance of stylar features in genus Vicia (Leguminosae): an analysis with molecular phylogeny. Journal of Plant Research 119: 513-523.

Chrtková-Žertová A. (1973a): Cytotaxonomic study of *Vicia cracca* complex 1. Czechoslovak taxa. Folia Geobotanica et Phytotaxonomica 8: 67-93.

Chrtková-Žertová A. (1973b): The cytotaxonomic study of the *Vicia cracca* complex 2. The taxa of the southern part of Northern Europe. Folia Geobotanica et Phytotaxonomica 8: 249-253.

Dvořák F., Dadáková B. & Grüll F. (1977): Studies of the morphology of chromosomes of some selected species. Folia Geobotanica et Phytotaxonomica 12: 343-375.

Endress P. K. (1994): Diveristy & evolutionary biology of tropical flowers. Cambridge University Press. Cambridge (UK).

Felber F. (1991): Establishment of a tetraploid cytotypes in a diploid population: Effect of relative fitness of the cytotypes. Journal of Evolutionary Biology 4: 195-207.

Felber-Girard M., Felber F. & Buttler A. (1996): Habitat differentiation in a narrow hybrid zone between diploid and tetraploid *Anthoxanthum alpinum*. New Phytologist 133: 531-540.

Felsenstein J. (2005): Phylip (Phylogeny Inference Package) version 3.6. Distributed by the author. Department of Genome Science, University of Washington, Seattle.

Gadella Th. W. J. & Kliphuis E. (1963): Chromosome numbers of flowering plants in the Netherlands. Acta Botanica Neerlandica 12: 195-230.

Gadella Th. W. J. & Kliphuis E. (1970): Cytotaxonomic investigations in some Angiosperms collected in the Valley of Aosta and in the National Park „Gran Paradiso". Caryologia 23: 363-379.

Gómez J. M., Perfectti F. & Camacho J. P. M. (2006): Natural selection on *Erysimum mediohispanicum* flower shape: insights into the evolution of zygomorphy. The American naturalist 168 (4): 531-545.

Grant V. (1981): Plant speciation. 2nd ed. Columbia University Press. New York.

Greilhuber J. & Ebert I. (1994): Genome size variation in *Pisum sativum*. Genome 37: 646-655.

Hallonová A. (1982): Floristické pomery horskej skupiny Skalky (Strážovské vrchy). Stredné Slovensko 2: 308-323.

Hammer O., Harper D. A. T. & Ryan P. D. (2001): PAST: Paleontological Statistics Software Package for Education and Data Analysis. Paleontologia Electronica 4: 1-9.

Hamrick J. L. & Godt M. J. W. (1996): Effects of life history traits on genetic diversity in plant species. Philosophical Transactions of the Royal Society of London, Biological Sciences 351: 1291-1298.

Hanelt P. & Mettin D. (1989): Biosystematics of the genus Vicia L. (Leguminosae). Annual Review of Ecology and Systematics 20: 199-223.

Hardy O. J., De Loose M., Vekemans X. & Meerts P. (2001): Allozyme segregation and inter-cytotype reproductive barriers in the polyploid complex *Centaurea jacea*. Heredity 87: 136-145.

Hardy O. J., Vanderhoeven S., De Loose M. & Meerts P. (2000): Ecological, morphological and allozymic differentiation between diploid and tetraploid knapweeds (*Centaurea jacea*) from a contact zone in the Belgian Ardennes. New Phytologist 146: 281-290.

Hardy, O. J. & X. Vekemans (2002). SPAGeDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes* 2: 618-620.

Heywood V.H. & Ball P.W. (1968): Leguminosae. In: Tutin T. G. (ed.) (1968) Flora Europaea. Vol. 2., Rosaceae to Umbelliferae. Cambridge University Press, Cambridge, UK.

Hommel P. W. F. M. & Weiffering J. M. (1979): In IOPB chromosome number reports LXIII. Taxon 28: 277-278.

Husband B. C. (2004): The role of triploid hybrids in the evolutionary dynamics of mixed-ploidy populations. Biological Journal of the Linnean Society 82: 537-546.

Husband B. C. & Sabara H. A. (2003): Reproductive isolation between autotetraploids and their diploid progenitors in fireweed, *Chamerion angustifolium* (*Onagraceae*). New Phytolologist 161: 703-713.

Huziwara Y. & Kondo S. (1963): Studies on the karyotypes of *Vicia*. Botanical Magazine Tokyo 76: 324-331.

ILDIS [International Legume Database & Information Service]. (2005): Version as of November 2005. http://www.ildis.org/

İnceer H. & Hayirlioğlu A. S. (2005): Giemsa C-banded karyotypes of *Vicia cracca* L. subsp. *cracca* and *V. bithynica* L. Turkish Journal of Botany 29: 311-316.

Jaaska V. (2005): Isozyme variation and phylogenetic relationships in *Vicia* subgenus *Cracca* (Fabaceae). Annals of Botany 96: 1085-1096.

Jensen R. J. (2003): The conundrum of morphometrics. Taxon 52: 663-671.

Krasnikov A. A. & Schaulo D. N. (1990): Chromosome numbers in representatives of some families of vascular plants in the flora of the Novosibirsk region II. Botanicheskiy Zhurnal 75: 118-120.

Krasnoborov I. M., Rostovtzeva T. S. & Ligus S. A. (1980): Chromosome numbers of some plant species of south Siberia and the Far East. Botanicheskiy Zhurnal 65: 659-668.

Kuzmanov B. A. (1975): In IOPB chromosome number reports XLIX. Taxon 24: 504.

Ledingham G. F. (1957): Chromosome numbers of some Saskatchewan Leguminosae with particular reference to *Astragalus* and *Oxytropis*. Can. J. Bot. 35: 657-666.

Leht M. (2005): Cladistic and phenetic analyses of relationships in *Vicia* subgenus *Cracca* (Fabaceae) based on morphological data. Taxon 54 (4): 1023-1032.

Levin D. A. (1975): Minority cytotype exclusion in local plant populations. Taxon 24: 35-43.

Lökvist B. & Hultgård U.-M. (1999): Chromosome numbers in south Swedish vascular plants. Opera Botanica 137: 1-42.

Maceira N. O., Jacquard P. & Lumaret R. (1993): Competition between diploid and derivative autotetraploid *Dactylis glomerata* L. from Galicia. Implications for the establishment of novel polyploid populations. New Phytologist 124 (2): 321-328.

Mahy G., Bruederle L. P., Connors B., Van Hofwegen M. & Vorsa N. (2000): Allozyme evidence for genetic autopolyploidy and high genetic diversity in tetraploid cranberry, *Vaccinium oxycoccos* (Ericaceae). American Journal of Botany 87 (12): 1882-1889.

Mandáková T. & Münzbergová Z. (2006): Distribution and ecology of cytotypes of the *Aster amellus* aggregates in the Czech republic. Annals of Botany 98: 846-856.

Měsíček J. & Soják J. (1969): Chromosome counts of some Mongolian plants. Folia Geobotanica et Phytotaxonomica 4: 55-86.

Měsíček J. & Soják J. (1995): Chromosome numbers of Mongolian angiosperms II. Folia Geobotanica et Phytotaxonomica 30: 445-453.

Mulligan G. A. (1961): Chromosome numbers of Canadian weeds III. Canadian Journal of Botany 39: 1057-1066.

Nei M. (1972): Genetic distance between populations. American Naturalist 106: 283-292.

Ness B. D., Soltis D. E. & Soltis P. S. (1989): Autopolyploidy in *Heuchera micrantha* (Saxifragaceae). American Journal of Botany 76 (4): 614-626.

Nikiforova O. D. (1984): Karyological study of the siberian species of the genus *Vicia* L. (Fabaceae). Citologia 26: 1126-1130.

Nikiforova O. D. (1990): Chromosome numbers in some Siberian species of the genera *Vicia* (Fabaceae) and *Beckmania* (Poaceae). Botanicheskiy Zhurnal 75: 121.

Nuismer S. L. & Cunningham B. M. (2005): Selection for phenotypic divergence between diploid and autotetraploid *Heuchera grossulariifol;ia.* Evolution 59: 1928-1935.

Otto F. (1990): DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. In.: Crissman H. A. & Darzynkiewicz Z. (eds), Methods in cell biology: flow cytometry, pp. 105-110, Academic Press, San Diego, CA.

Otto S. P. & Whitton J. (2000): Polyploid incidence and evolution. Annual Review of Genetics 34: 401-437.

Peckert T. & Chrtek J. jun. (2006): Mating interactions between coexisting diploid, triploid and tetraploid cytotypes of *Hieracium echioides* (Asteraceae). Folia Geobotanica 41: 323-334.

Petit C., Lesbros P., Ge X. & Thompson J. D. (1997): Variation in flowering phenology and selfing rate across a contact zone between diploid and tetraploid *Arrhenatherum elatius* (Poaceae). Heredity 79: 31-40.

Petit C., Bretagnolle F. & Felber F. (1999): Evolutionary consequences of diploid-polyploid hybrid zones in wild species. Trends in Ecology and Evolution 14 (8): 306-311.

Podlech P. & Dieterle A. (1969): Chromosomenstudien an afganischen Pflanzen. Candollea 24: 185-243.

Qu L., Hancock J. F. & Waylon J. H. (1998): Evolution in an autopolyploid group displaying predominantly bivalent pairing at meiosis: Genomic similarity of diploid *Vaccinium darrowi* and autotetraploid *V. corymbosum* (Ericaceae). American Journal of Botany 85: 698-703.

Ramsey J. & Schemske D.W. (1998): Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annual Review of Ecology and Systematics 29: 467-501.

Ramsey J. & Schemske D.W. (2002): Neopolyploidy in flowering plants. Annual Review of Ecology and Systematics 33: 589-639.

Rausch J. H. & Morgan M. T. (2005): The effect of self-fertilization, inbreeding depression, and population size on autopolyploid establishment. Evolution 59 (9): 1867-1875.

Rodríguez D. J. (1996): A model for the establishment of polyploidy in plants. The American Naturalist 147 (1): 33-46.

Rohlf F. J. (1993): Relative warp analysis and an example of its application to mosquito wings. In: Marcus L. F., Bello, E. & Garcia-Valdecasas A. (eds.): Contributions to morphometrics. Museo Nacional de Ciencias Naturales, Madrid. Pp. 134-159.

Rohlf F. J. (2006): TpsDig Version 2.10. Department of Ecology and Evolution, State University of New York at Stony Brook, New York.

Rohlf F. J. (2007): TpsRelw Version 1.45. Department of Ecology and Evolution, State University of New York at Stony Brook, New York.

Roti-Michelozzi G. (1984): Biosystematic investigations on North Western Italian populations of the *Vicia cracca* aggregate. Preliminary observations. Webbia 38: 815-827.

Roti-Michelozzi G. (1992): Karyotype variation by whole arm translocation in Italian specimens of the *Vicia cracca* group (Fabaceae). Atti della Societa Toscana di Scienze Naturali Residente in Pisa Memorie Serie B 99: 75-84.

Roti-Michelozzi G. & Allione S. (1987): Numeri chromosomici per la flora Italiana: 1118-1126. Informatore Botanico Italiano 19: 305-313.

Rousi A. (1961): Cytotaxonomical studies on *Vicia cracca* L. and *V. tenuifolia* Roth. I. Chromosome numbers and karyotype evolution. Hereditas 47: 81-110.

Rousi A. (1962): Cytotaxonomical studies on *Vicia cracca* L. and *V. tenuifolia* Roth. II. Meiosis. Hereditas 48: 390-408.

Rousi A. (1973): Cytotaxonomical studies on *Vicia cracca* L. and *V. tenuifolia* Roth. III. The relation between karyotype and morphology. Annales Botanici Fennici 10: 89-96.

Ryka Cz. (1954): Cytological studies in *Vicia cracca* L. and *V. tenuifolia* Roth. Acta Societatis Botanicorum Poloniae 23: 321-333.

Şahin A. & Babaç M. T. (1990): Cytotaxonomic investigations on some *Vicia* L. species in East and Southeast Anatolia I. Turkish Journal of Botany 14: 124-138.

SAS Institute (2002-2003): SAS OnlineDoc®, ver. 9.1.3 (available online). SAS Institute, Cary.

Segraves K. A. & Thompson J. N. (1999): Plant polyploidy and pollination: floral traits and insect visits to diploid and tetraploid *Heuchera grossulariifolia*. Evolution 53 (4): 1114-1127.

Semerenko L. V. (1989): Chromosome numbers in some members of the family Asteraceae, Fabaceae, Orchidaceae and Poaceae from the Berezinsky biosphere reservation (Byelorussia) flora. Botanicheskiy Zhurnal 74: 1671-1673.

Senn H. A. (1938): Chromosome number relationships among the Leguminosae. Biliographia Genetica 12: 175-336.

Sokolovskaya A. P., Probatova N. S. & Rudyka E. G. (1989): Chromosome numbers in some species of the flora of the Soviet Far East from the families Actinidiaceae, Aristolochiaceae, Fabaceae, Ranunculaceae, Saxifragaceae. Botanicheskiy Zhurnal 74: 268-271.

Soltis D. E., Soltis P. S. & Tate J. A. (2003): Advances in the study of polyploidy since *Plant speciation.* New Phytologist 161: 173-191.

Soltis D. E., Soltis P. S., Schemske D. W., Hancock J. F., Thompson J. N., Husband B. C. & Judd W. S. (2007): Autopolyploidy in angiosperms: have we grossly underestimated the number of species? Taxon 56 (1): 13-30.

Stebbins G. L. (1971): Chromosomal evolution in higher plants. Addison-Wesley, London.

Stebbins G. L. (1985): Polyploidy, hybridization, and the invasion of new habitats. Annals of Missouri Botanical Garden 72: 824-832.

Steele K. P. & Wojciechowski M. F. (2003): Phylogenetic analyses of tribes Trifolieae and Vicieae, based on sequences of the plastid gene *mat*K (Papilionideae: Leguminosae). In: Klitgaard B. B. & Bruneau A. (eds.): Advances in Legume Systematics, part 10, Higher Level Systematics. pp. 355-370. Royal Botanic Gardens, Kew.

Tomkins D. J. & Grant W. F. (1978): Morphological and genetic factors influencing the response of weed species to herbicides. Canadian Journal of Botany 56: 1466-1471.

Trewick S. A., Morgan-Richards M., Russell S. J., Henderson S., Rumsey F. J., Pintér I., Barrett J. A., Gibby M. & Vogel J. C. (2002): Polyploidy, phylogeography and Pleistocene refugia of the rockfern *Asplenium ceterach*: evidence from chloroplast DNA. Molecular Ecology 11: 2003-2012.

Tzvelev N. N. (1987): In: Flora Partis Europaeae URSS, Vol. 6. Leningrad. (Rus)

Vallejos C. E. (1983): Enzyme activity staining. In: Tanksley S. D. & Orton T. J. (eds.): Isozyme in plant genetics and breeding, Part A. Elsevier, Amsterdam. Pp. 469-516.

Van de Wouw M., Enneking D., Maxted N. & Robertson L. D. (2001): Genetic resources of Mediterranean *Vicia* species. Chapter 9 in: Maxted N. & Bennett S. J. (eds.): Plant genetic resources of legumes in the Medterranean. Kluwer, Dordrecht. Pp. 132-157

Van Dijk P. & Bakx-Schotman T. (1997): Chloroplast DNA phylogeography and cytotype geography in autopolyploid *Plantago media.* Molecular Ecology 6: 345-352.

Van Dijk P., Hartog M. V. & Van Delden W. (1992): Single cytotype areas in autopolyploid *Plantago media* L. Biological Journal of the Linnean Society 46: 315-331.

Volkova S. A., Boyko E. V. & Gavrilenko I. G. (1999): Chromosome in the representatives of some families of the Primorye territory flora. Botanicheskiy Zhurnal 84: 140-141.

Weeden N. F. & Wendel J. F. (1989): Genetics of plant isozymes. In: Soltis D. S. & Soltis P. S. (eds.): Isozymes in plant biology. Advances in plant sciences series. Vol. 4. Dioscorides Press, Portland (Oregon).

Weir B. S. & C. C. Cockerham, 1984. Estimating F-statistics for the analysis of population structure. Evolution 38: 1358-1370.

Wright S. (1951): Evolution in Mendelian populations. Genetics 16: 97-159.

Yefimov K. F. (1987): Chromosome numbers in some members of the Fabaceae family from central Caucasus. Botanicheskiy Zhurnal 72: 845.

Yefimov K. F. (1988): Karyological study of the species of the genus *Vicia* (Fabaceae) from the central Caucasus. Botanicheskiy Zhurnal 73: 641-651.

Zhang X. & Mosjidis J. A. (1998): Rapid prediction of mating system of *Vicia* species. Crop Science 38: 872-875.

Zelditch M. L., Swiderski D. L., Sheets D. H. & Fink W. L. (2004): Geometric morphometrics for biologists: a primer. Elsevier Academic Press, London.

Appendix 1

Code	Country	Locality	Latitude	Longitude	Altitude	Collectors	Ploidy level 2x	3x	4x	Total number of individuals
1	AT	Carinthia/Hermagor, Maria Luggau - right riverside of the Gail river ca 1 km S from the village church	46.7023	12.7411	1098	DK			19	19
2	AT	Carinthia/Hermagor, Kötschach - left riverside of the Gail river ca 2 km SW from the town centre	46.6685	12.9826	717	DK			20	20
3	AT	Salzburg/Lungau, Tamsweg - grassy roadside by the road in the valley of Mur river ca 2 km S from the southern margin of the town	47.1148	13.8142	1036	DK			19	19
4	AT	Upper Austria, Stroheim - grassy hillside above the local road to Karling by the Schaunberg ruin	48.3415	13.9677	450	MŠ, FK			6	6
5	AT	Upper Austria, Neufelden - grassy hillside above the road to St. Peter a. Wimberg ca 2 km E from the village	48.4846	14.0109	500	MŠ, FK			4	4
6	AT	Upper Austria, Auberg - hillside in the valley of Mühl river between hop-field and local road to Auberg and Neudorf settlements	48.5445	14.0360	510	MŠ, FK			6	6
7	AT	Upper Austria, Sankt Peter a. Wimberg - meadow by the road to Marbach ca 2 km N from the village	48.5138	14.0737	580	MŠ, FK			10	10
8	AT	Upper Austria, Haslach an der Mühl - grassy roadside along the road from Haslach to St. Stefan am Walde ca 3 km from the town	48.5713	14.0739	650	MŠ, FK			2	2
9	AT	Upper Austria, Goldwörth - maedow by the local road to Feldkirchen a. d. Donau ca 2 km NW from the village	48.3394	14.0888	260	MŠ, FK			14	14
10	AT	Upper Austria, Guglwald - meadow by the road to St. Stefan am Walde ca 1 km W from the village	48.5901	14.1668	730	MŠ, FK			8	8
11	AT	Upper Austria, Vorderweissenbach - hillside above the road to the lodge Sternwald ca 0.5 km NE from church in the village	48.5547	14.2224	730	MŠ, FK			12	12
12	AT	Upper Austria, Bad Leonfelden - meadow at the hillside of Sternstein (1122 m a.s.l.) ca 4 km N from the northern margin of the town	48.5592	14.2932	910	MŠ, FK			10	10
13	AT	Upper Austria, Mauthausen - grassy roadside at a bridge embankment over the Danube river	48.2397	14.5333	240	MŠ, FK			11	11
14	AT	Upper Austria, Mairspindt - hillside by the road to Leopoldschlag at the southern margin of the village	48.5975	14.5663	700	MŠ, FK			6	6
15	AT	Upper Austria, Hohensteg - grassy hillside in the valley of Aist river by the road to Schwertberg ca 1.5 km SE from the village	48.3164	14.5746	300	MŠ, FK			5	5
16	AT	Upper Austria, Gutau - grassy ditch by the local road to Weberberg ca 3 km E from the village	48.4233	14.6396	470	MŠ, FK			5	5
17	AT	Lower Austria, Singenreith - grassy hillside above the road from Spitz to Ottenschlag by a turning to Singenreith settlement	48.3954	15.2555	755	PT, FK			8	8

18	AT	Lower Austria, Ötzbach - meadow by the road to Spitz ca 0.5 km N from the village	48.3854	15.3232	494	PT, FK		10	10
19	AT	Lower Austria, Scheideldorf - meadow close to the St. Hubert chapel ca 500 m W from the village	48.7405	15.3300	530	PT, FK		17	17
20	AT	Lower Austria, Emmersdorf - grassy ground in a crossroad of the main road no. 1 and 3 by the NE margin of the village	48.2450	15.3413	212	PT, FK		18	18
21	AT	Lower Austria, Maria Laach am Jauerling/ Zeissing - meadow ca 0.5 km E from the eastern margin of Zeissing settlement	48.3075	15.3515	580	PT, FK		14	14
22	AT	Lower Austria, Aggsbach Dorf - hillside above the road to Gansbach at the eastern margin of the village	48.2972	15.4267	231	PT, FK		8	8
23	AT	Lower Austria, Oberbergern - meadow by the road to Aggsbach Dorf ca 1 km SW from the village	48.3546	15.5137	404	PT, FK		12	12
24	AT	Lower Austria, Brunn a.d. Wild - hillside above the road to Horn at a western margin of the village	48.6947	15.5160	464	PT, FK		13	13
25	AT	Lower Austria, Mauternbach - rocky hillside by the road from Mautern to Unterbergen ca 0.5 km S from the southern margin of Mauternbach village	48.3792	15.5607	265	PT, FK		11	11
26	AT	Lower Austria, Kuhnring - hillside above the local road close to the western margin of the village	48.6342	15.7765	374	PT, FK		10	10
27	AT	Lower Austria, Hippersdorf - grassy hillside above the local road to Ruppersthal ca 0.5 km N from the village	48.4252	15.9625	182	PT, FK		3	3
28	AT	Lower Austria, Gaisruck - meadow under the road to Stetteldorf a. Wagram at the western margin of the Gaisruck village	48.3995	16.0532	197	PT, FK		23	23
29	AT	Lower Austria, Siegenfeld - shrub margin ca 2.5 km E from the village	48.0358	16.2101	376	MŠ	8		8
30	AT	Burgenland/Oberwart, Bernstein - grassy roadside by a crossroad ca 1.5 km NE from the town centre	47.4115	16.2679	340	MŠ, FK		1	1
31	CZ	Cheb - meadow on the hilltop of the Špitálský vrch (517 m) hill at the NW margin of the town	50.0859	12.3548	515	PV		30	30
32	CZ	Kunžvart - Kladská - meadow by the crossroad close to the S margin of the settlement	50.0250	12.6667	838	MŠ		3	3
33	CZ	Poběžovice - meadow by the road from the Poběžovice village to the Drahotín village close to the Nature Reserve Drahotínský les	49.5210	12.7727	482	PT, LL, TU		9	9
34	CZ	Nová ves - meadow underneath of the Dominova skalka rock	50.0714	12.7863	759	PT, LL		26	26
35	CZ	Boží Dar - meadow at the margin of the nature reserve Božídarské rašeliniště 1 km west from the village centre	50.4106	12.9072	985	PT, MŠ		14	14
36	CZ	Karlovy Vary, Andělská Hora - grassy hilltop surrounding the Andělská Hora ruins	50.2049	12.9653	355	PT		19	19
37	CZ	Vladař - hill southeast of Žlutice village, meadow on the top	50.0833	13.2042	514	AE		38	38
38	CZ	Železná Ruda, Špičák - meadow at the bottom of the ski tow to the	49.1650	13.2202	868	PT, LL, TU		26	26

		Špičák (1202 m a.s.l.) hill						
39	CZ	Železná Ruda, Starý Brunst - meadow in the valley of the Křemelná river close to the road from the town Železná Ruda to the Javorná village	49.1913	13.3107	938	PT, LL, TU	33	33
40	CZ	Přeštice - Krasavce - grassy ditch by the road from Krasavce to Snopušovy close to the wayside cross	49.6051	13.3703	379	MŠ	1	1
41	CZ	Modrava - meadow at the eastern margin of the village	49.0244	13.5000	1050	MŠ	9	9
42	CZ	Horská Kvilda, Zhůří - meadow close to the guide-post at the Zhůří settlement	49.0808	13.5577	1149	PT, LL, TU, MŠ	47	47
43	CZ	Kvilda-Pod Políčky - meadow by the turist path to Nevé Hutě ca 1.3 km N from the church in Kvilda village	49.0307	13.5810	1091	PT, LL	30	30
44	CZ	Nezdice na Šumavě, Ostružno - meadow between the Klepačka settlement and the Koksův vrch (807 m a.s.l.) hill	49.1722	13.5861	777	PT, LL, TU	26	26
45	CZ	Kvilda-Vilémov - meadow by a margin of nature reserve Vltavské stráně ca 0.8 km SSE from the church in Kvilda village	49.0124	13.5863	1032	PT, LL	30	30
46	CZ	Valley of Teplá Vltava river - meadow by the road between Borová Lada and Kvilda ca 2 km SE from the church in Kvilda village	49.0076	13.6016	1022	PT, LL	31	31
47	CZ	Valley of Teplá Vltava river - meadow by the road between Borová Lada and Kvilda ca 2.5 km WNW from the chapel in Borová Lada village	48.9998	13.6264	940	PT, LL	124	124
48	CZ	Staré Hutě - meadow close to a forest ca 1 km WSW from the church in Nové Hutě village	49.0327	13.6332	1082	PT, LL	30	30
49	CZ	Valley of Teplá Vltava river - meadow by the road between Borová Lada and Kvilda ca 2 km WNW from the chapel in Borová Lada village	48.9995	13.6339	945	PT, LL	91	91
50	CZ	Svinná Lada - meadow between road and Teplá Vltava river ca 1.2 km WNW from the church in Borová Lada village	48.9953	13.6434	933	PT, LL	90	90
51	CZ	Nové Hutě - meadow by the southern margin of the village ca 90 m W from the church	49.0374	13.6447	1023	PT, LL	30	30
52	CZ	Svinná Láda - meadow under a road ca 700 m NW from church in Borová Lada village	48.9955	13.6510	912	PT, LL	156	156
53	CZ	Nové Hutě - meadow by the road ca 1 km NE from the church	49.0432	13.6563	992	PT, LL	30	30
54	CZ	Borová Lada - grassy ditch close to the southern margin of the village	48.9912	13.6590	893	PT, LL	94	94
55	CZ	Chanovice - grassy ground by the crossroad in the centre of the village	49.4044	13.7172	540	JS	9	9
56	CZ	Lnářský Málkov - grassy roadside between Velká Lípa and Malá Lípa ponds	49.3923	13.7951	490	MŠ	5	5
57	CZ	Milý - fallow by a fieldpath ca 1.2 km W from the village	50.2353	13.8520	381	AE	5	5
58	CZ	Lukov - meadow in the valey of Lukovský potok brook ca 700 m	50.5225	13.8807	453	PT	13	13

		SW from the centre of Lukov village						
59	CZ	Medvědice - meadow by a road ca 800 m N from the centre of Medvědice village	50.5248	13.9224	449	PT	27	27
60	CZ	Nové Strašecí - meadow by a small pond east of the town	50.1659	13.9239	457	AE	36	36
61	CZ	Milešov - meadow by a playground at the nothern margin of Milešov village	50.5430	13.9322	401	PT	16	16
62	CZ	Nová Pec - meadow approx. 1km NW from the village centre	48.7953	13.9394	741	PT, MŠ	34	34
63	CZ	Velemín - fallow by the road from Velemín to Milešov ca 500 m W from the western margin of Velemín village	50.5369	13.9659	324	PT	13	13
64	CZ	Ústí nad Labem - Všebořice, Podhoří, weedery behind the town	50.6928	13.9883	249	AE	41	41
65	CZ	Strakonice, Kbelnice - grassy roadside close to the eastern margin of the Kbelnice village	49.2962	13.9904	452	PT, LL, TU	18	18
66	CZ	Nižbor - Nová Huť, hillside by a wood	49.9908	13.9908	295	AE	33	33
67	CZ	Pernek - meadow at the western margin of the village	48.7926	13.9939	811	PT, MŠ	21	21
68	CZ	Pasečná, Jasánky - meadow by the road from the Přední Zvonková village to the Pasečná village close to the enclave of Jasánky	48.6309	14.0559	768	PT, MŠ	16	16
69	CZ	Pasečná, Jasánky - meadow close to the border of the nature reserve Jasánky	48.6192	14.0587	733	PT, MŠ	40	40
70	CZ	Slatina - meadow by the railway station Slatina pod Hazmburkem ca 2.5 km NNE from the town Libochovice	50.4306	14.0625	178	AE	5	5
71	CZ	Mirovice - Zalužany - grassy roadside by the petrol station at S margin of the village	49.5365	14.0813	463	PT	80	80
72	CZ	Kovářov - meadow on the left waterside of the water basin Lipno close to the village Kovářov	48.6871	14.1283	740	PV	30	30
73	CZ	Lhenice - margin of the orchard ca 1.2 km NW from the village church	49.0046	14.1407	600	MŠ	12	12
74	CZ	Přední Výtoň, Spáleniště - meadow at the road from the Přední Výtoň village to the border checkpoint Guglwald close to the Spáleniště settlement	48.6020	14.1701	754	PT, MŠ	27	27
75	CZ	Karlštejn - grassy roadside in the eastern part of the village ca 300 m from the Plešivec hill (362 m a.s.l.)	49.9358	14.1844	240	PV	1	1
76	CZ	S Bohemia, Šumavsko-Novohradské podhůří, mesophytic grasslands on the N slopes of the hill Svatý kříž, 1 km S off the village Chvalšiny	48.8333	14.2000	584	BK, VČ	34	34
77	CZ	Český Krumlov, Nové Dobrkovice - meadow above the village close to the western margin of the nature reserve of Vyšenské kopce	48.8210	14.2913	555	PT, MŠ	22	22
78	CZ	Plástovice - meadow by the road to Sedlec at the S margin of the village	49.0675	14.3017	397	FK	17	17
79	CZ	Dívčice - meadow at the waterside of the Blatec pond by the road to the village Nákří	49.1136	14.3151	392	PT	4	4

80	CZ	Holubov - grassy ground by monument in the centre of the village	48.8917	14.3250	500	FK	15	15
81	CZ	Studánky - hillside along the road from Studánky to Vyšší Brod close to the N margin of the village	48.5922	14.3268	660	MŠ, FK	5	5
82	CZ	Zliv - meadow in the nature reserve Mokřiny u Vomáčků close to the Pašice village	49.0724	14.3423	386	PT	10	10
83	CZ	Zliv - wet meadow close to the Zlivský rybník pond 1.1 km SW from railway station Zliv	49.0583	14.3500	385	FK	25	25
84	CZ	Rožmberk nad Vltavou - grassy roadside along the road in the Vltava river valley close to the Závratná settlement	48.6873	14.3552	525	PT, MŠ	14	14
85	CZ	Kaplice - grassy ground in proximity to the petrol station by the S margin of the village	48.7293	14.4822	553	PT	13	13
86	CZ	Dobronice u Bechyně - alluvial meadow in the valley of Lužnice river ca 1 km SW from the village	49.3331	14.4926	476	PV, TU	5	5
87	CZ	Chrást nad Sázavou - Podělusy, meadow in the village	49.8422	14.5784	250	AE	37	37
88	CZ	Jiřetín pod Jedlovou - meadow under the ski-lift at the SE hillside of Tolštejn hill (670 m a.s.l.)	50.8560	14.5817	650	PT, DK	25	25
89	CZ	Horní Světlá - meadow by the forest margin close to the Myslivny settlement	50.8404	14.6466	637	PT, DK	11	11
90	CZ	Miličín - field margin close to the W margin of the village by the road to the quarry	49.5735	14.6528	621	PT	18	18
91	CZ	Chotoviny - grassy roadside close to the settlement Lapáček by the road to Řevnov	49.4752	14.6535	482	PT	17	17
92	CZ	Pohoří na Šumavě - meadow ca 0.6 km from the church ruin in the village	48.6010	14.6880	900	MŠ, FK	6	6
93	CZ	Horusice - meadow at the margin of the nature reserve Ruda by the lodge V Rudě	49.1505	14.6928	419	PT	31	31
94	CZ	Klenovice - meadow by the railway close to the Ovčín settlement	49.2786	14.7050	417	PT	23	23
95	CZ	Hojná Voda, Staré Hutě - meadow by the road at the southern margin of the Staré Hutě village	48.7168	14.7112	796	PT, LL	15	15
96	CZ	Žíňánky ú Mrače - grassy hillside above the road from Čerčany to Soběhrdy ca 350 m WNW from church in Žíňany	49.8254	14.7131	350	PT	22	22
97	CZ	Soběslav - grassy right waterside of Čenkovický potok brook ca 100 m from the junction with Lužnice river	49.2583	14.7167	415	PV	18	18
98	CZ	Bělá pod Bezdězem - meadow close to the NW margin of the town	50.5089	14.7875	304	PT, DK	17	17
99	CZ	Kostelec nad Černými lesy - meadow at the northwest border of the town	49.9994	14.8568	369	AE	36	36
100	CZ	Kardašova Řečice - meadow by the road to the Mnich village ca 550 m NW from the railway station Mnich	49.1720	14.8774	461	PT	15	15
101	CZ	Chotyně - hillside eastern of the village	50.8419	14.8896	330	AE	32	32
102	CZ	Druzcov - meadow by a road to Janův Důl village	50.7154	14.9285	442	AE	33	33

103	CZ	Kouřim - grassy field margin close to the Zárybník settlement 1.5 km NE from the Kouřim town centre	50.0110	14.9972	268	PT, LL	16	16
104	CZ	Borovsko - grassy waterside of the water reservoir Švihov under the highway bridge	49.6897	15.1008	400	FK	6	6
105	CZ	Borovsko - open serpentinic pine-wood by the road form Borovsko to Bernartice	49.6873	15.1099	427	PT, DK	7	7
106	CZ	Člunek - meadow above the village Člunek ca 400 m from the hilltop of Lejskův vrch (587 m a.s.l.)	49.1047	15.1265	576	PT	13	13
107	CZ	Košetice - grassy roadside along the road to Křelovice village 1.5 km SSE from the eastern margin of the Košetice village	49.5492	15.1434	533	PT, LL	8	8
108	CZ	Vyskeř - grassy roadside along the road to Hrubá Skála village in the NE margin of the village of Vyskeř	50.5321	15.1641	401	DK	6	6
109	CZ	Zbraslavice - margin of a field southwest of the town	49.8145	15.1787	460	AE	48	48
110	CZ	Troskovice, Trosky - meadow on the SW slope of the hill with the Trosky ruins	50.5153	15.2327	420	DK	5	5
111	CZ	Jičín, Podhradí - meadow at the crossroad between the Podhradí village and the Březina village	50.4289	15.3117	279	DK	6	6
112	CZ	Mrákotín - meadow close to the small pond by the road from Mrákotín to Praskolesy	49.1782	15.3684	577	PT	20	20
113	CZ	Železnice - meadow on the right riverside of the Cidlina river close to the railway station Železnice	50.4620	15.3740	293	DK	17	17
114	CZ	Popovice (Jičín district) - meadow southeast of the town	50.4133	15.3954	261	AE	35	35
115	CZ	Bukovka - grassy waterside of Dolní Jílovka pond ca 1.4 km ESE from the church in the village	50.0930	15.6409	247	PT	19	19
116	CZ	Svojkovice - grassy waterside in the vicinity of U Anděla lodge by the road to Želetava	49.1689	15.6413	637	PT, DK	19	19
117	CZ	Chotěboř - meadow by a road to Rozsochatec village	49.7035	15.6511	521	AE	37	37
118	CZ	Lázně Bohdaneč - meadow between Semtín and Lázně Bohdaneč ca 1.4 km SE from the church in Lázně Bohdaneč town	50.0681	15.6932	221	PT	7	7
119	CZ	Velká Úpa - meadow enclave Končiny, NW from the Janovy boudy	50.6952	15.7866	976	PT	8	8
120	CZ	Malá Úpa - meadow below a ski lift at Žacléřské boudy, above a yellow turistic pathway	50.7350	15.7918	955	PT	6	6
121	CZ	Staré Ransko, Nature reserve Ransko - undergrowth of a serpentine pine-wood on the waterside of Panský potok brook	49.6756	15.8000	570	FK	9	9
122	CZ	Svoboda nad Úpou - meadow on the northern slope of the Kraví vrch (681 m a.s.l.) hill	50.6308	15.8220	647	PT, LL	31	31
123	CZ	Horní Albeřice - sunny slope above the quarry Stará Celnice	50.6945	15.8492	769	PT	57	57
124	CZ	Fryšava - meadow below the road app. 800 m east from the edge of the village	49.6297	16.0625	701	PT	23	23
125	CZ	Rtyně v Podkrkonoší - hillside by the municipal waste dump area	50.5146	16.0680	398	AE	38	38

126	CZ	Střemošice - grassy ground by the chapel at a margin of the nature reserve Střemošická stráň	49.8949	16.0724	442	PT	37	37
127	CZ	České Meziříčí - nature reserve Zbytka 0.6 km west from the railway station Pohoří	50.2917	16.0972	256	FrKr	16	16
128	CZ	Rašovice - meadow by the southeastern margin of the village ca 0.5 km SSE from the agricultural area	50.1451	16.1316	285	PT, AE, TU	23	23
129	CZ	Vysoké Mýto, Knířov - meadow by the pond ca 200 m WNW from the church	49.9261	16.1415	301	PT	8	8
130	CZ	Pustá Rybná - alluvial meadow on the left waterside of Pavlásecký potok brook ca 350 m ENE from the cemetery chapel	49.7094	16.1421	667	PT	6	6
131	CZ	Čermná nad Orlicí, Číčová - grassy roadside at the western margin of the Číčová village	50.0693	16.1527	274	PT	20	20
132	CZ	Stárkov - meadow on the slope ca 100 m S from the village church	50.5323	16.1549	434	PT, LL	12	12
133	CZ	Vysoké Mýto, Oklikov - meadow by the pond in local part Oklikov ca 650 m ENE from Bučkův kopec hill (315 m a.s.l.)	49.9705	16.1759	267	PT	24	24
134	CZ	Mohelno - grassy edge along a field path app. 950 m ENE from a dam of the water reservoir Mohelno	49.1070	16.1846	256	PT	33	33
135	CZ	Vysoké Mýto, U Vinic - wet meadow under a colony of gardens	49.9614	16.1899	292	PT	38	38
136	CZ	Rychnov nad Kněžnou, Rychnovská Lhotka - meadow at the northern end of the Rychnovská Lhotka village	50.1563	16.2621	332	PT	20	20
137	CZ	Deblín - grassy water-side by the small pond ca 600 m SSW from village Deblín	49.3142	16.3328	489	PT	14	14
138	CZ	Slatina nad Zdobnicí - meadow at a hillside above Zdobnice river ca 100 m S from railway station Slatina nad Zdobnicí	50.1358	16.3785	387	PT, AE, TU	16	16
139	CZ	Česká Třebová - meadow in local part of the town Ke Křivolíku in the west end of the town	49.9010	16.4230	425	AE	25	25
140	CZ	Svojanov - Manova Lhota - alluvial meadow in Třetínka river valley ca 350 m WSW from the village Manova Lhota	49.6364	16.3947	473	PT	5	5
141	CZ	Žampach - meadow at the eastern margin of the village	50.0398	16.4077	481	PT	20	20
142	CZ	Černá Hora - meadow by the road between Žernovník and Černá Hora ca 400 m NW from the hilltop Ješetiny (424 m a.s.l.)	49.4119	16.5652	344	PT	15	15
143	CZ	Mikulov, Březí - grassy hillside by the road to Dolní Dunajovice ca 0.5 km NE from the viilage	48.8256	16.5709	214	FK	6	6
144	CZ	Letovice - Svárov - meadow by the road between Svárov and Malá Roudka ca 900 m E from the village Svárov	49.5916	16.6316	482	PT	16	16
145	CZ	Ostrov u Macochy - meadow by the entrance to the Balcarka cave ca 0.8 km SE from the village	49.3752	16.7573	450	MŠ	7	7
146	CZ	Králíky, Dolní Hedeč - grassy roadside along the road to the baroque monastery	50.0750	16.7860	774	PT, LL	15	15
147	CZ	Javorník - grassy side ditch along the road from the village Bernatice	50.3847	17.0484	262	PT, LL	3	3

		to the town Javorník							
148	CZ	Žulová - meadow along the road to the Vápenná village in the southern margin of the village	50.3039	17.0964	392	PT, LL		25	25
149	CZ	Dubňany - meadow close to the cemetery in the eastern margin of the village	48.9215	17.1101	277	PT, LL		16	16
150	CZ	Sobotín-Rudoltice - meadow by the road no. 11 ca 1.8 km ENE from the chapel in Rudoltice village	49.9914	17.1280	548	PT, AE, TU		66	66
151	CZ	Víceměřice - hillside above a railway ca 0.35 km WSW from the church in Víceměřice village	49.3439	17.1786	222	PT, AE, TU		25	25
152	CZ	Stará Ves u Rýmařova - pasture by the road no. 11 at the western margin of the village ca 1.5 km W from the church	49.9634	17.2217	655	PT, AE, TU		32	32
153	CZ	Šternberk - meadow at SW slope of the Ostrá Hora (612 m a.s.l.) hill, 5.5 km north from the centre of the town	49.7776	17.2917	564	PT, LL		16	16
154	CZ	Karlova Studánka - meadow on the stream-banks of the Bělokamenný brook by the road from the Karlova Studánka village to the Malá Morávka village	50.0445	17.3109	720	PT, LL		17	17
155	CZ	Malá Štáhle u Rýmařova - grassy roadside ca 200 m E from the village chapel	49.9518	17.3413	606	PT, AE, TU		20	20
156	CZ	Heřmanovice - meadow by the forrest between the village Heřmanovice and the Rejvíz settlement	50.2121	17.3445	722	PT, LL		39	39
157	CZ	Radějov - old orchard between a road and the small river Radějovka app. 250 m to SE of the village edge	48.8537	17.3534	244	PT	1	5	6
158	CZ	Radějov -lower part of the meadow above the small river Radějovka app. 350 m to SSE of the village edge	48.8525	17.3539	262	PT	5	2	7
159	CZ	Radějov -middle part of the meadow above the small river Radějovka app. 550 m to SSE of the village edge	48.8508	17.3541	288	PT	27		27
160	CZ	Radějov - ditch above a road from Radějov to Dolní Mlýn app. 400 m to ESE of the edge of Radějov	48.8536	17.3556	289	PT		3	3
161	CZ	Dolní Mlýn - meadow enclave between a road and a forest app. 350 m to NE of the gamekeeper's lodge not far from Radějov	48.8557	17.3755	275	PT	16		16
162	CZ	Nature reserve Čertoryje - meadow near the Kněždubský háj grove in the northern part of the protected area	48.8643	17.4156	372	PT, LL		40	40
163	CZ	Nature reserve Čertoryje - meadow in the central part of the protected area	48.8602	17.4214	396	PT, LL	2	45	47
164	CZ	Bruntál, Uhlířský vrch - meadow in the proximity of a church on the hilltop of Uhlířský vrch (672 m a. s. l.)	49.9727	17.4395	670	PT, AE, TU		51	51
165	CZ	Malá Vrbka - grassy ground in the center of the village	48.8700	17.4589	276	HC	9		9
166	CZ	Hrubé padělky - loučka nad rybníkem na Malanském potoce cca 1.5 km SSZ od S okraje obce Hrubá Vrbka	48.8869	17.4666	232	PT	45	3	48
167	CZ	Hrubá Vrbka - meadow in the proximity of a farmsteading on the	48.8672	17.4712	274	HC	9		9

		west margin of the village by the road to Malá Vrbka							
168	CZ	Hrubá Vrbka - grassy roadside on the west margin of the village by the road to Malá Vrbka	48.8683	17.4742	270	HC	9	2	11
169	CZ	Hrubá Vrbka - old orchard by the road from the Hrubá Vrbka village to the Lipov village	48.8873	17.4757	277	PT, LL	50		50
170	CZ	Kuželov - small meadow along a road app. 200 m to SSE of the SE village edge	48.8553	17.4938	351	PT	28		28
171	CZ	Machová - meadow between the nature reserve Machová and the railway app. 600 m to NNE of the railway station Vrbovce (SK)	48.8295	17.5200	368	PT	62	31	93
172	CZ	Jazevčí - middle part of the nature reserve in the slope above a goup of trees app. 1.2 km to SSW of Suchovské Mlýny settlement	48.8744	17.5695	359	PT	40		40
173	CZ	Pitárné - meadow by the road above the river Mušlov ca 1.5 km WSW from the village church	50.2389	17.5813	337	PT, AE, TU		17	17
174	CZ	Strání, Velká Javořina - meadow near the Kamenná Bouda chalet by the turist path to Velká Javořina (970 m) hill	48.8921	17.6553	642	HC, PT, AE, LL	44	21	65
175	CZ	Březová - meadow approx. 1.2 km west from the centre of the village	48.9264	17.7217	568	PT, AE, LL	20		20
176	CZ	Březová, nature reserve Kalábová - mosaic of xerophytic, mesophytic and wet grasslands and boggy springs, N of the village Březová	48.9282	17.7309	500	BK, VČ	26		26
177	CZ	Osíčko - meadow by the southern margin of the village ca 0.25 km E from the railway station Osíčko	49.4235	17.7465	415	PT, AE, TU		23	23
178	CZ	Troják u Hošťálkové - meadow by the settlement Troják ca 4 km W from the church in Hošťálková village	49.3546	17.8129	551	PT, AE, TU		33	33
179	CZ	Rokytnice - meadow close to the forest margin 1.5 km SW from the village centre	49.0601	17.8984	400		54		54
180	CZ	Jestřabí - meadow in the valley of the river Rokytenka close to the western margin of the village	49.0708	17.9450	332	PT, AE, LL	28		28
181	CZ	Pulčín - meadow in the southern margin of the village	49.2169	18.0806	633	PT, AE, LL	45		45
182	CZ	Štramberk, Kotouč - meadow at the S hillside of the hill Kotouč (495 m a.s.l.) ca 0.7 km SSW from the church in the town Štramberk	49.5865	18.1144	404	PT, AE, TU		56	56
183	CZ	Klimkovice - Hýlov, meadow by a road at the eastern border of the village	49.7930	18.1165	280	AE		31	31
184	CZ	Štramberk, Kotouč - grassy plateau on the Kotouč hilltop (495 m a.s.l.) ca 0.8 km S from the church in the town Štramberk	49.5848	18.1188	475	PT, AE, TU		37	37
185	CZ	Zděchov - meadow in a slope above the left bank of the brook in NW edge of the village	49.2655	18. 0787	439	AE, ME	23	7	30
186	CZ	Lužná - meadow between a road and the river Senica in the SE edge of the village	49.2396	18.0236	367	AE, ME	25	5	30
187	CZ	Halenkov-Černé údolí - meadow by the settlement U Kuželů ca 1.5 km SSW from the Halenkov church	49.3029	18.1384	509	PT, AE, TU		23	23

188	CZ	Hlučín - Jasénky, meadow by the river Opava	49.8743	18.1901	222	AE		38	38
189	CZ	Trojanovice - grassy hillside by the western margin of the village ca 2.4 km W from the village chapel	49.5163	18.2090	462	PT, AE, TU		30	30
190	CZ	Měrkovice - meadow at the northeastern border of the village	49.6056	18.2466	389	AE		31	31
191	CZ	Staré Hamry - meadow by a road at the western margin of the water reservoir Šance ca 1.7 km NW from the church in Staré Hamry village	49.4834	18.4224	503	PT, AE, TU		17	17
192	CZ	Horní Suchá - meadow by the cemetary	49.7960	18.4823	277	AE	17	22	39
193	CZ	Ostrý - mountain about 5 km southwest of Bystřice nad Olší village, meadow on a hillslope	49.5964	18.6709	825	AE		33	33
194	CZ	Třinec - Sosna, margin of a forest	49.6818	18.6822	363	AE		31	31
195	CZ	Třinec - Jahodná, margin of a forest	49.6785	18.6975	400	AE		27	27
196	CZ	Malý Ostrý - mountain about 4 km northeast of Vendryně village, meadow under the top	49.6809	18.7376	533	AE		26	26
197	DE	Bavaria, Woja - aluvial meadow close to the serpentinic quarry ca 750 m SSW from the village Woja	50.2531	11.9719	500	MŠ		10	10
198	DE	Rehau - meadow in the valley of Höllbach brooke in the vicinity of the town Rehau	50.2500	12.0611	540	MŠ		13	13
199	SK	Pezinok - Hrubá dolina, bush around the lay-by at a road to Pernek village	48.3309	17.2293	273	AE, ME		13	13
200	SK	Sološnica - ditch beside a road at the northeast border of the village	48.4687	17.2359	215	AE, ME		21	21
201	SK	Tvarožná Lhota - grassy roadside by the road to Horní Mlýn settlement ca 1.3 km SE from the centre of the village	48.8683	17.3718	288	RS, JS	16		16
202	SK	Hilltop Havran (541 m a.s.l.) - meadow on the hilltop ca 2.5 km NE from the centre of Částkov village	48.7677	17.3723	541	RS, JS	12		12
203	SK	Galašovci settlement - meadow by the road ca 2.5 km N from the centre of Soboliště villige	48.7548	17.4025	268	RS, JS	18		18
204	SK	Medzny Mlyn settlement - meadow in the valley of Teplica river by the road between Vrbovce and Soboliště	48.7709	17.4302	260	RS, JS	10		10
205	SK	Bukovec - road edge northwest of the village	48.7070	17.4928	390	AE, ME	31		31
206	SK	Číčov - ditch beside a road to Klúčovec village	47.7910	17.7524	106	AE, ME	33		33
207	SK	Horná Súča - Dúbrava, meadow beside a brook	48.9498	17.9566	376	AE, ME	39		39
208	SK	Horná Súča - fallow land by the road ca 250m NE from the margin of the Dolná Závrská village	48.9756	17.9657	365	PT, LL	48		48
209	SK	Horná Súča - bush beside Súčanka brook	48.9655	17.9863	273	AE, ME	6		6
210	SK	Dolná Súča - road edge between Horná and Dolná Súča villages	48.9680	18.0021	261	AE, ME	11		11
211	SK	Urmince - bushes by the road at the southern border of the village	48.5309	18.0939	222	AE, ME	9		9
212	SK	Vršatské Podhradie - hillside under Vršatské hradné outlier	49.0691	18.1531	678	AE, ME	15		15
213	SK	Vršatské Podhradie - meadow southeast of the village	49.0592	18.1587	552	AE, ME	33		33
214	SK	Chotín - ditch beside a road to the town Komárno	47.8066	18.1992	109	AE, ME	45		45

215	SK	Krásná Ves - meadow by a road at the southern border of the village	48.8238	18.2353	228	AE, ME	22			22
216	SK	Skýcov - bush by a road northwest of the village	48.5108	18.4075	452	AE, ME	38			38
217	SK	Makov - Javorník saddle, meadow by a hotel	49.3477	18.4998	754	AE, ME	38			38
218	SK	Súlovské rocks - bush beside a road between villages Súlov and Jablonové	49.1761	18.5733	321	AE, ME	49		3	52
219	SK	Prochotský brook valley - bush beside a road between Horná Ždaňa and Prochot villages	48.5884	18.7191	419	AE, ME	20			20
220	SK	Perec river valley - ditch beside a road between Sikenica and Šalov villages	48.0543	18.7201	162	AE, ME	24			24
221	SK	Hliník nad Hronom - grassy roadside ca 1.2 km E from railway station	48.5389	18.7861	236	FK			3	3
222	SK	Svrčinovec - bush beside a road to the state border	49.4849	18.7901	407	AE, ME	8		9	17
223	SK	Štiavnica river valley - meadow by a road to Královce - Krnišov village	48.3320	18.9620	300	AE, ME	15			15
224	SK	Vrátna valley, meadow by the crossroad to Štefanová	49.2366	19.0403	606	AE, ME	29			29
225	SK	Trnie - meadow northeast of the village	48.6069	19.0424	448	AE, ME	17		9	26
226	SK	Malá Fatra - grassy roadside by the road to Velký Kriváň Mt. under the Chleb Mt. hilltop	49.1889	19.0528	1620	MŠ			6	6
227	SK	Vlkolínec, settling SW of Ružomberok, meadow by the entry to the open-air museum	49.0381	19.2769	692	AE, ME	33	3		36
228	SK	Vlkolínec, settling SW of Ružomberok, meadow by the pathway to the open-air museum	49.0340	19.2720	593	AE, ME	12			12
229	SK	Vlkolínec - meadow by the pathway to the nature reserve Krkavá skala close to the E margin of the village	49.0403	19.2831	740	BK, VČ	37		44	81
230	SK	Lúčky - hillside at the western village margine	49.1235	19.3991	645	AE, ME	32	4		36
231	SK	Detva - meadow by a road at the SE border of the town	48.5351	19.4122	413	AE, ME	50			50
232	SK	Oravská Jasenica, meadow at the northern village margine by the NW side of a road to Oravské Veselé	49.4138	19.4287	688	AE, ME	31		9	40
233	SK	Bešeňová -grassy hillside by the Bešeňovské travertiny nature reserve	49.1042	19.4378	565	AE, ME	31			31
234	SK	Klin, field margine at the NW border of the village	49.4508	19.4680	743	AE, ME	36		5	41
235	SK	Demänovská Ice Cave - forest edge by a parking	49.0192	19.5764	775	AE, ME	35			35
236	SK	Demänovská valley - meadow by the central parking	49.0411	19.5796	702	AE, ME	42			42
237	SK	Kokava nad Rimavicou - Kokava - Háj, bush beside a road	48.5972	19.7210	798	AE, ME	35			35
238	SK	Oravice - meadow close to the Bobrovecká dolina valley embouchure	49.2833	19.7500	865	MŠ	2		3	5
239	SK	Rimavská Baňa - ditch beside a road west from the village	48.5103	19.9327	263	AE, ME	13			13
240	SK	Vyšné Hágy spa - stands surrounding a railway	49.1181	20.1255	1147	AE, ME	16			16
241	SK	Tatranské Matliare - meadow in the former leisure centre at the northern settling margine	49.1787	20.2911	927	AE, ME	23			23

242	SK	Primovce - meadow by the nature reserve Primovské skaly at the eastern margin of the village	49.0157	20.3823	579	AE	51		51
243	SK	Čoltovo - meadow at the E margin of the village	48.4919	20.3825	231	FK	7		7
244	SK	Bohúňovo - meadow by the N margin of the village close to the church	48.5147	20.3856	201	FK	1		1
245	SK	Magurské gap - grassy slope beside a road	49.2819	20.4299	990	AE, ME	40		40
246	SK	Dlhá Ves - fallow by a road to Plešivec village	48.5030	20.4334	349	AE, ME	25		25
247	SK	Lesnica gap - meadow by a buffet	49.3830	20.4935	759	AE, ME	38		38
248	SK	Čipkov vrch mountain - meadow enclave by a road from Smolnik village to Krásnohorské Podhradie village	48.6705	20.6196	611	AE, ME	35		35
249	SK	Borka - meadow by a chapel at the border of the national park	48.6335	20.7819	665	AE, ME	46		46
250	SK	Plaveč - Podzámok, meadow by a road	49.2526	20.8485	594	AE, ME	35		35
251	SK	Jasov - Počkaj, meadow by the river Bodva	48.6922	20.9530	255	AE, ME	33	4	37
252	SK	Bardejov - Dlhá Lúka, meadow by a road	49.3534	21.2895	321	AE, ME	32		32
253	SK	Čižatice - fallow by a road to Rozhanovce village	48.7836	21.3923	267	AE, ME	46		46
254	SK	Slanská Huta - medow close to the forest ca 1 km S from the village	48.5973	21.4536	500	AE		64	64
255	SK	Juskova Voľa - pasture at the edge of the village	48.8757	21.5728	258	AE, ME	20		20
256	SK	Ladomirová - grassy hillside at the NE border of the village	49.3336	21.6302	346	AE, ME	40		40
257	SK	Bystrá - pasture at the NW border of the village	49.2516	21.8114	393	AE, ME	40		40
258	SK	Nature reserve Raškovský Luh - alluvial meadow by the Laborec river ca 3 km W from the centre of Vojany village	48.5719	21.9500	99	AE	63		63
259	SK	Nature reserve Latorica - alluvial meadow on the right riverside of Latorica river ca 4.5 km NE from the centre of Leles village	48.4990	22.0549	102	AE	73		73
260	SK	Hostovice - fieldpath edge south of the village	49.1020	22.1473	471	AE, ME	19		19
261	SK	Zemplínské Hámre - meadow by a quarry	48.9397	22.1593	482	AE, ME	49		49

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