Hybridization between polyploids *Cardamine enneaphyllos* and *C. glanduligera* in the West Carpathians: evidence from morphology, pollen fertility and PCR-RFLP patterns

Hybridizácia medzi polyploidmi *Cardamine enneaphyllos* a *C. glanduligera* v Západných Karpatoch: morfológia, fertilita peľu a PCR-RFLP analýzy

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Lihová J., Kochjarová J. & Marhold K. (2007): Hybridization between polyploids *Cardamine enneaphyllos* and *C. glanduligera* in the West Carpathians: evidence from morphology, pollen fertility and PCR-RFLP patterns. – Preslia 79: 101–125.

In the present study hybridization between the decaploid *Cardamine enneaphyllos* and hexaploid *C. glanduligera* (both previously assigned to *Dentaria*) was examined. The study area was located in the West Carpathians in Slovakia, where the distribution ranges of the putative parental species overlap, and they occur sympatrically. The putative hybrid *C.* ×*paxiana* was studied in many localities in terms of its morphological variation, pollen fertility and PCR-RFLP patterns. Prior to analyses hybrid individuals were tentatively determined based on three morphological characters reported as diagnostic: flower colour, presence of glands on leaves and length of rhizome internodes. Such tentative hybrid identification was confirmed by strongly decreased pollen fertility and an additive restriction pattern in the nuclear ITS region. The possible sources of the substantial morphological variation of hybrids, revealed by morphometric analyses, are discussed. Based on the results of the PCR-RFLP analysis of cpDNA, bidirectional hybridization occurred, although *C. enneaphyllos* was usually the maternal parent. Geographic distribution and sterility of hybrid individuals suggest that they are repeatedly generated from crosses between the parental species, and represent F_1 or early generation hybrids maintained by vegetative reproduction.

Keywords: *Brassicaceae*, *Cruciferae*, *Dentaria,* morphometrics, nrDNA ITS sequences, *trnL* intron, Slovakia

Introduction

The genus *Cardamine* L. is one of the largest genera in the family *Brassicaceae*, comprising at least 200 species distributed worldwide. Thirteen sections were recognized in the traditional infrageneric classification concept proposed by Schulz (1903). This sectional classification based on a few morphological features, however, apparently does not reflect the phylogenetic relationships within the genus. Monophyly of several sections was rejected by recent molecular studies (Franzke et al. 1998, Sweeney & Price 2000, Bleeker et al. 2002) and a comprehensive genus-level phylogenetic study is under progress (T. A. Carlsen et al., unpublished). Reconstruction of the evolutionary history of a genus like *Cardamine*, which is strongly affected by reticulate evolution and polyploidization, is definitely not straightforward (Lihová & Marhold 2006). Much effort will be needed in future

to reach a robust and well resolved genus phylogeny and eventually to propose a natural infrageneric subdivision.

A group of about 20 species distributed disjunctly in E and W North America, and Eurasia were traditionally separated in a distinct genus *Dentaria* L. or as a group(s) within *Cardamine*. It comprises woodland perennials with high levels of polyploidy and pronounced vegetative reproduction; several distinguishing morphological characters were also recognized (Sweeney & Price 2000). Several authors, both in Eurasia and North America, treated *Cardamine* and *Dentaria* as two distinct genera or subgenera (e.g. Detling 1936, Spooner 1984, Kotov 1979, Slavík 1992, Jones & Akeroyd 1993), while some American authors (Al-Shehbaz 1988, Rollins 1993) did not recognize *Dentaria* at any taxonomic level and included all species within *Cardamine.* In the taxonomic concept of Schulz (1903), a separate section *Dentaria* (L.) O. E. Schulz was delimited, but some species originally described or combined under the genus *Dentaria* were placed into three other sections, implying that there is no consistent delimitation of *Dentaria* species in earlier literature. In the study of Sweeney & Price (2000) chloroplast sequence data were used to examine phylogenetic positions of selected *Dentaria* and *Cardamine* species. The results clearly showed that *Dentaria* is polyphyletic with at least three separate origins corresponding to the three main biogeographic groups, and that the morphological characters used for delimitation are not reliable on a worldwide basis. So far only four European species of *Dentaria* have been analysed (Franzke et al. 1998, Sweeney & Price 2000) and it remains to be determined whether European representatives form a monophyletic group.

In Europe, nine species traditionally treated within *Dentaria* are currently recognized (Jalas & Suominen 1994, Cesca & Peruzzi 2002). All of them are polyploids of hexa- or higher ploidy levels of unknown ancient origins, and morphologically can be characterized by subterranean rhizomes and petiolate cotyledons (Jones & Akeroyd 1993). The present study focused on two of them: decaploid yellow-flowered *C. enneaphyllos* (L.) Crantz (≡ *D. enneaphyllos* L.) with 2n = 80, and hexaploid purple-flowered *C. glanduligera* O. Schwarz (≡ *D. glandulosa* Waldst. & Kit.) with 2n = 48. The distribution area of *C. enneaphyllos* is central Europe, the Apennine Peninsula and W Balkans, while that of *C. glanduligera* is the whole Carpathian range and adjacent areas (Jalas & Suominen 1994; see also Fig. 1). Thus, their distribution ranges overlap in the West Carpathians (Slovakia, S Poland, E Moravia). Both species show similar ecological preferences and are found growing mainly in shady sites in beech, fir-beech and spruce forests in the colline to the subalpine belts (Kochjarová et al. 1999, Marhold & Kochjarová 2002). The hybrid between these two polyploid species was described by Schulz (1903) as *C.* ×*paxiana* O. E. Schulz, based on a specimen from Poland (Silesia). Since then hybrid individuals have been repeatedly reported mainly from the localities where both parental species occur sympatrically, in several mountain ranges within the West Carpathians in Slovakia (Kochjarová et al. 1999, Marhold & Kochjarová 2002), more rarely from NE Moravia (Slavík 1992), and occasionally from Silesia and the Tatry Mts in Poland (Schulz 1903, Pawłowski 1956). The colour of petals, morphology of rhizomes and presence of glands on leaves are considered to be the most important diagnostic characters for distinguishing hybrid individuals, since they exhibit intermediate appearance in respect of parental species. In addition, assumed hybrids are reported to be completely sterile with no siliquae and seed formation. Apparently their propagation and persistence is achieved by rhizome growth and fragmentation (Kochjarová et al. 1999).

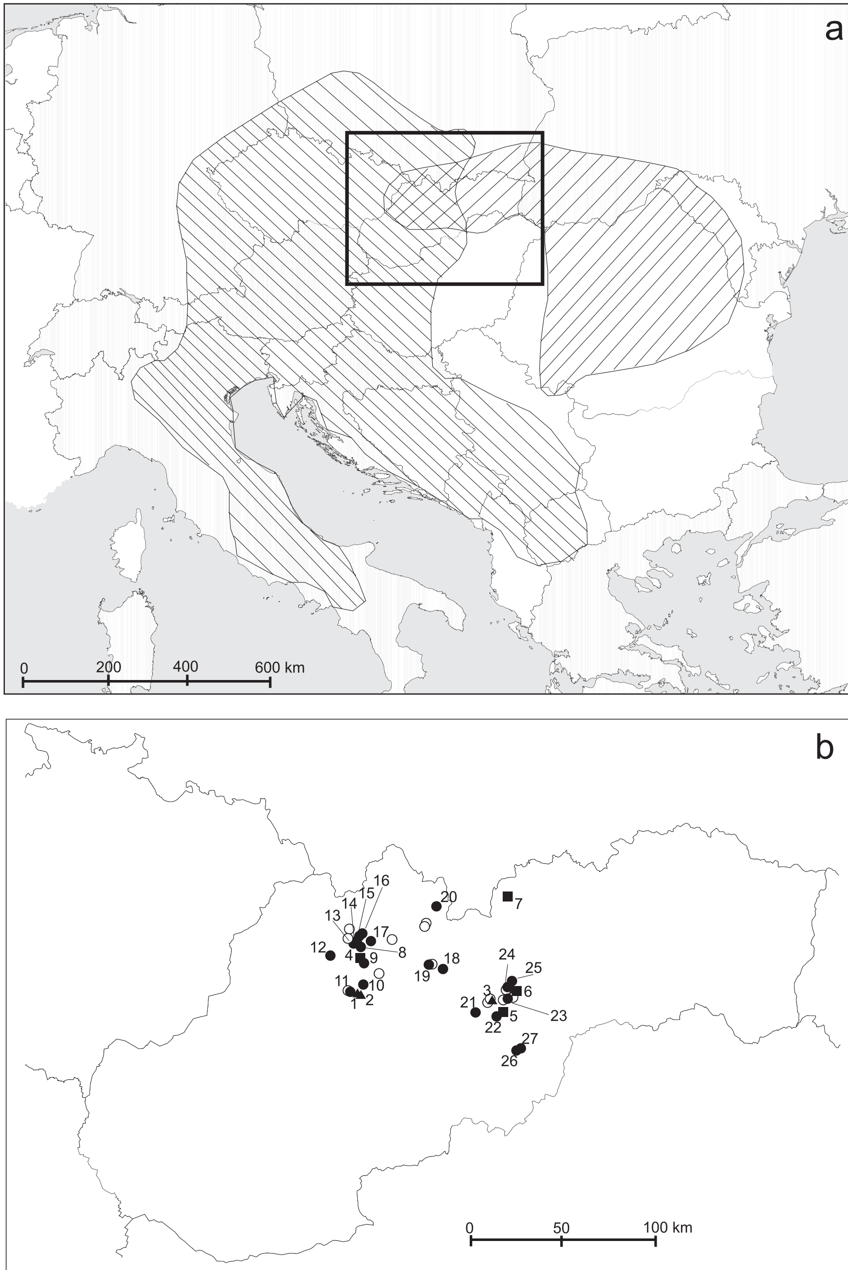

Fig. 1. – (a) Distribution areas of *Cardamine enneaphyllos* (left-hatched) and *C. glanduligera* (right-hatched) overlapping in Slovakia, S Poland and E Moravia (modified according to Jalas & Suominen 1994). (b) Distribution of *C.* ×*paxiana* in Slovakia and sample sites; empty circles – localities of *C.* ×*paxiana* documented by revision of herbarium material (for details on localities and specimens see Marhold & Kochjarová 2002); full circles – sample sites of *C.* ×*paxiana* together with one or both parental species; full triangles – sample sites of "pure" populations of *C. enneaphyllos*; full squares – sample sites of "pure" populations of *C. glanduligera*. For locality numbers see Appendix 1.

Hybridization is not a rare phenomenon in *Cardamine*, as numerous interspecific hybrids between *Cardamine* representatives are described (Lihová & Marhold 2006). A few hybridization events, both at the diploid and polyploid levels, have been recently subjected to detailed studies, employing both morphological and molecular techniques (Urbanska et al. 1997, Marhold et al. 2002, Lihová et al. 2006, 2007). Contrasting patterns of morphological, molecular and karyological variation were revealed in the cases studied and attributed to differences in relatedness and the evolutionary history of parental species, genome (in)compatibilities, chromosome numbers, reproduction modes, etc. In the present study we examined the putative hybrid *C.* ×*paxiana* from numerous populations in the West Carpathians in terms of its morphological variation, pollen fertility and molecular evidence of its hybrid origin. Intermediate morphology and high pollen sterility are strong indicators for hybrid origin, as they are features of many hybrids. Nevertheless, this may not always be the case, and hybrids can be morphologically much closer to one of the parents and/or exhibit transgressive phenotypic features (Rieseberg & Ellstrand 1993, Rieseberg & Carney 1998). Several molecular techniques are used to detect interspecific hybridization among plants and to prove the hybrid origin of suspect individuals, as e.g. fingerprinting methods (RAPD, AFLP, ISSR) and isozyme polymorphisms where intermediate genotypes and the additivity of fragments/alleles are expected in hybrids, or nuclear-encoded markers such as the multicopy nrDNA ITS region where divergent parental sequence variants are identified in a hybrid genome (Vriesendorp & Bakker 2005). Here we use the analyses of fragment-length polymorphisms of both the nuclear ITS region and the chloroplast *trnL* intron (PCR-RFLP). The former was used to document additive patterns in putative hybrid individuals and the latter to detect the direction of hybridization (maternal donor of the hybrid individuals). This approach was successfully applied e.g. in the study of hybrids in *Phlox* (Ferguson et al. 1999), *Potamogeton* (Kaplan & Fehrer 2004, 2006), and several other genera.

Material and methods

Plant material

The study area was located in the West Carpathians in Slovakia, where the distribution ranges of *Cardamine enneaphyllos* and *C. glanduligera* overlap, and the species occur sympatrically (Fig. 1a). For the data on their sympatric occurrence and the distribution of the putative hybrid we examined specimens from the following herbaria: BBZ, BP, BRA, BRNM, BRNU, KRA, KRAM, NI, POP, PR, PRC, SAV, SLO, SMBB, TNP, ZAM (acronyms follow Holmgren et al. 1990, Vozárová & Sutorý 2001). Plant material was sampled from 27 localities; three where only populations of *C. enneaphyllos* were present and four with only *C. glanduligera* ("pure" populations) were used to characterize phenotypes and genotypes not affected by interspecific gene flow, otherwise the sampling focused on localities where the parental species and putative hybrid individuals co-occurred (Appendix 1, Fig. 1b). For morphometric analyses usually 15–25 specimens per population were examined, only in some cases (depending on the population size) fewer plants were sampled. Altogether 1070 flowering stage individuals and 67 individuals at the fruiting stage were sampled and studied in respect of morphology. Two subsets of flowering specimens were further chosen for (i) determining pollen fertility (85 individuals drawn from 14 populations; dried

voucher specimens were used) and (ii) PCR-RFLP analyses (53 individuals from 12 populations; fresh leaf material was collected and dried using silica gel). The selection of populations and specimens for the above was from the whole study area and spanned the observed morphological variation as far as possible. The two subsets overlapped largely both at the population (Appendix 1) and individual levels: out of 48 individuals analysed for PCR-RFLP, 41 were also used for determining pollen fertility. This sampling allowed the direct (individual-level) comparison of the morphological, pollen fertility and molecular patterns. Voucher specimens are deposited in BBZ (Vozárová & Sutorý 2001).

PCR-RFLP analyses

The PCR-RFLP approach was employed to document hybridization between *Cardamine enneaphyllos* and *C. glanduligera* at multiple localities where both species co-occurred along with individuals morphologically identified as putative hybrids. The aim of the RFLP analysis of the ITS region of nrDNA (ITS1-5.8S-ITS2) was to obtain different restriction patterns for the parental species and to detect both parental variants within hybrid individuals (i.e. to detect additive patterns). The procedure successfully applied by Kaplan & Fehrer (2004) was followed. Similarly, the aim of the RFLP analysis of *trnL* intron of cpDNA was to obtain different restriction patterns for the parental species, and subsequently, to determine which species acted as the maternal parent for the individuals proved as hybrids by ITS additivity.

Total genomic DNA was extracted from silica gel dried leaf samples using the CTAB-procedure (Doyle & Doyle 1987) with minor modifications. PCR amplification of the ITS region of nrDNA and the *trnL* intron, was performed using the universal primers P1A, P4 developed by Francisco-Ortega et al. (1999) and the primers c, d by Taberlet et al. (1991), respectively. The PCR conditions were as follows: 10× reaction buffer including $MgSO_4$ at 2 mM, 0.2 mM of each dNTPs, 0.2 µM of each primer, and 0.75 or 1.25 unit of *Pfu* polymerase (MBI Fermentas) in a total reaction volume of either 25 or 40 µl. The PCR cycle profiles were the same as specified in Lihová et al. (2004). Amplification products were checked on 1.5% agarose gels. A few PCR products were selected for sequencing to assess differentiation between the studied species and their intraspecific variation: ITS products from 10 individuals (four per species, plus two from hybrids) and *trnL* products from four individuals (two per species), all sampled from different populations including those where only one of the parental species was growing (Appendix 1). PCR products subjected to sequencing were purified using the Spinprep PCR clean-up kit (Calbiochem) following the manufacturer's protocol. Sequencing, using the original PCR primers, was done at the Department of Molecular Biology, Comenius University, Bratislava (BITCET Consortium). The sequences were aligned manually using BioEdit (version 7.0.4.1; Hall 1999). The ITS sequences obtained from the putative hybrid individuals, however, were not readable; apparently due to the overlay of divergent ITS variants involving short length differences.

The aligned sequences were visually inspected for possible RFLP patterns differentiating between *C. enneaphyllos* and *C. glanduligera* by checking for palindromic motifs constantly present in one of the species but absent in the other, as well as by using the respective feature (restriction map) of BioEdit. Due to the pronounced sequence divergence observed between the species and low intraspecific variation (see Results), several restriction sites could be inferred. Among them, *Taq*I (T^CGA) and *Hae*III (= *Bsu*RI, GG^CC)

were chosen for the restriction of the ITS region, and *Xap*I (= *Apo*I, R^AATTY) for the restriction of the *trnL* intron (all enzymes from MBI, Fermentas). Restriction of PCR products was performed using 8 μl of PCR product, 10 units of the restriction enzyme, 1/10 of the recommended and supplied 10× restriction buffer, and incubated overnight at the recommended temperature (37 °C for *Hae*III and *Xap*I, and 65 °C for *Taq*I). The restriction products were separated and visualized on 2% agarose gels together with a DNA ladder (Low Range MassRuler DNA Ladder, MBI, Fermentas).

Pollen fertility

As a measure of male fertility, pollen viability of selected individuals was assessed using acetocarmine staining (Marks 1954, slightly modified). Anthers were removed from a single flower bud per individual and chopped in a drop of acetocarmine on a microscope slide to release pollen grains. 100–130 grains per flower were observed, and viable (well-stained) and nonviable (shrunken and unstained) grains were recorded. Pollen quality was expressed as the percentage of viable grains.

Morphometric analyses

Eight vegetative, seven floral, and eight fruit characters were measured or scored on the sampled specimens, plus five ratios were derived. One character was semiquantitative (glands on leaves), otherwise the characters were all quantitative (Appendix 2). For measurements on flowers, floral parts of one optimally developed flower randomly selected per plant were attached by adhesive tape to paper, dried, and then measured. Flowering and fruiting individuals were treated separately; within the former 18 characters were recorded (those listed as vegetative and floral), within the latter 19 characters (those listed as vegetative and fruit; Appendix 2). Spearman and Pearson correlation coefficients were calculated to reveal highly correlated pairs of characters, since very high correlations may distort results of multivariate morphometric analyses (Legendre & Legendre 1998). As a result (see below), three morphological characters were excluded from further analyses, except for the exploratory data analysis. Subsequently, five data matrices were assembled that were employed in the morphometric analyses: (1) matrix of flowering individuals of the parental species and the hybrid identified according to PCR-RFLP and/or pollen analyses (91 individuals × 16 characters; matrix hence forth called "matrix of plants analysed for PCR-RFLP and pollen fertility"); (2) matrix of flowering parental individuals (755 individuals × 16 characters; called "flowering parental matrix"); (3) matrix of flowering individuals of both parents and putative hybrids (1070 individuals × 16 characters; called "flowering complete matrix"); (4) matrix of fruiting parental individuals (56 individuals × 18 characters; called "fruiting parental matrix"); and (5) matrix of fruiting parental individuals plus a few hybrid ones with developed siliquae (61 individuals × 18 characters; called "fruiting complete matrix"). For flowering individuals (before they were pressed) the colour of petals was recorded. The following colour scale was used: 1 – white or almost white, 2 – yellow, 3 – yellowish pink or irregularly coloured, 4 – pale pink, 5 – purple. Because of the multistate nature of this character, it was not included directly in any of the morphometric analyses, but used only for the interpretation of ordination diagrams.

Hybrid individuals were tentatively determined prior to analyses based on the characters reported as diagnostic for the hybrid: flower colour, presence of glands on leaves,

presence and length of rhizome internodes (Kochjarová et al. 1999). Yellowish pink, irregularly coloured and white-coloured petals, never found in the parental species across their distribution ranges, were taken as the first criterion of hybrid origin; if the plants had pale pink or purple petals that have been recorded both in *C. glanduligera* and putative hybrids, presence of glands and morphology of rhizomes were examined and taxonomic assignment was made accordingly. While the rhizome of *C. glanduligera* is thickened with thin internodes usually a few cm long, that of *C. enneaphyllos* is equally thick along its length (these differences are depicted quite well in Slavík 1992: 113, Fig. 25); for the putative hybrid individuals very short thin internodes are reported. Glands on leaves are reported as sparse in putative hybrids, in contrast to their common presence both at the leaflet base and on the leaf margin in *C. glanduligera*, and their complete absence in *C. enneaphyllos* (Kochjarová et al. 1999). Classification based on these three criteria seemed to be unequivocal, but its reliability was tested in the following way. Canonical discriminant analysis (CDA) was performed on the "matrix of plants analysed for PCR-RFLP and pollen fertility" with three groups delimited as follows: hybrids were defined as individuals with additive PCR-RFLP pattern of ITS and/or with pollen fertility lower than 50%, whereas the assignment to the parental species was based on species-specific ITS and *trnL* RFLP patterns and/or pollen fertility above 85%. In this CDA we illustrated morphological differentiation among these three groups (91 individuals) and derived two discriminant functions classifying plants into the three groups. These functions were subsequently applied to the "flowering complete matrix", i.e. including also plants that were not employed in PCR-RFLP or pollen fertility analyses (altogether 1070 individuals). The position of plants tentatively identified using the above-mentioned three characters was depicted on the two-dimensional ordination diagram according to their canonical scores. Obviously, a direct analysis of all plants for PCR-RFLP and pollen fertility would be more precise, but it was rather unrealistic because of the cost and workload involved.

Next, principal component analyses (PCA) based on four data matrices (matrices 2–4, see above) were performed to show the overall morphological variation (as a contrast to CDA stressing only the differences among the taxa studied) and differentiation between the parental species, as well as to display the positions of hybrid relative to the parental individuals. An R type of PCA based on a correlation matrix of the characters was computed (Sneath & Sokal 1973, Krzanowski 1990). In addition, information on petal colour, data from pollen fertility and PCR-RFLP results were mapped onto the resulting PCA ordination of the "flowering complete matrix". The aim was to correlate this data with the morphological patterns as displayed by PCA. All analyses were performed using the SAS 9.1 software (SAS Institute 2000).

As the last step, an exploratory data analysis was performed on both complete matrices of flowering and fruiting individuals to obtain basic statistical parameters for individual characters separately for the two parental species and the hybrid.

Results

PCR-RFLP analyses

The DNA sequences obtained were submitted to the GenBank database (accession numbers EF136402–EF136409 for the ITS region of nrDNA, and EF136410–EF136413 for the *trnL*

intron). The alignment of nrDNA ITS sequences retrieved from eight individuals of *Cardamine enneaphyllos* and *C. glanduligera* showed rather high sequence divergence between the two species, in respect of both nucleotide substitutions (around 7.6% nucleotide divergence) and a few short (1 and 2 bp) indels. Except for a few intra-individual single-nucleotide polymorphisms detected in some individuals (inferred from the double peaks seen in electropherograms), no variation was found within the species. This facilitated the search for restriction enzymes that produce appropriate species-specific RFLP patterns.

As expected from the restriction maps of the two selected restriction enzymes (Figs 2b, 3b), RFLP patterns were resolved that allowed the differentiation between the ITS sequences of the parental species. No variation within species was found for the several individuals analysed (12 individuals of *C. glanduligera*, 10 of *C. enneaphyllos*). The restriction enzyme *Taq*I, with two restriction sites present in *C. glanduligera* and three in *C. enneaphyllos*, generated three fragments in the former species and four in the latter (Fig. 2). In putative hybrids, a clearly additive pattern was observed, indicating the simultaneous presence of both parental ITS variants. The *Hae*III enzyme had one restriction site in the ITS sequence of *C. glanduligera* and three sites in *C. enneaphyllos*, resulting in two and four restriction fragments, respectively (Fig. 3). The hybrid individuals (as suspected from morphology and shown by the additive RFLP patterns using *Taq*I enzyme) exhibited additive patterns with all the fragments found in the parental species, however, in all cases (altogether 31 hybrid individuals from eight populations were analysed) there was an additional band of about 450 bp (Fig. 3a). Its origin might be due to either one lost restriction site (from the *C. enneaphyllos* ITS variant) or one gained restriction site (in the *C. glanduligera* ITS variant), since in both scenarios an additional fragment of 455 or 446 bp, respectively, is generated (Fig. 3b). Intriguing, however, is that the additional band is present in all the individuals examined, and thus, the pattern seems to be fixed in hybrids.

The alignment of *trnL* intron sequences of cpDNA showed much lower divergence between the parental species (1.3% nucleotide divergence plus two indels of 2 and 4 bp), but it was possible to distinguish their haplotypes and to propose restrictions with different patterns. The restriction enzyme *Xap*I cut the *trnL* sequence of *C. enneaphyllos* at two sites, producing two short (40 bp, 89 bp) and one long (451 bp) restriction fragment, while an additional third restriction site is present in *C. glanduligera*, resulting in the restriction of the long fragment into two fragments of 273 and 176 bp lengths (Fig. 4). Most hybrid individuals examined (26 out of 31) possessed the RFLP pattern of *C. enneaphyllos* implying that this species was the maternal donor, and in only five individuals (collected from two localities no. 19, 25, Appendix 1) was *C. glanduligera* the maternal plant.

Pollen fertility

Male fertility, indicated by pollen stainability, was generally high in both *C. enneaphyllos* (25 individuals) and *C. glanduligera* (22 individuals), exceeding 85%. Only a single individual identified as *C. glanduligera* with 58% pollen stainability was found. On the other hand, most of the putative hybrid individuals investigated showed pollen stainability below 20% (26 individuals from eight populations), while 10 individuals (sampled from four populations) had pollen stainability in the range 20–50%. Only two specimens showed higher stainability of 78% and 57%, respectively.

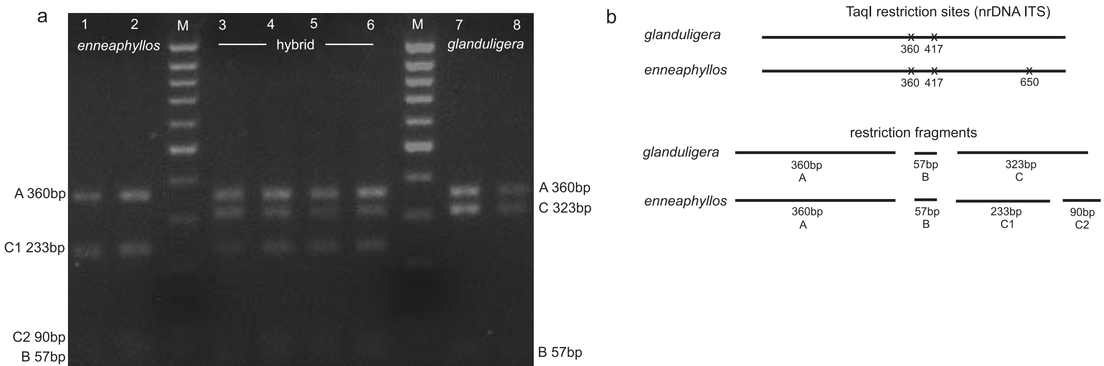

Fig. 2. – RFLP analysis of nrDNA ITS region (ITS1-5.8S-ITS2) using the *Taq*I restriction endonuclease. Picture (a) shows electrophoretic separation of the restriction fragments generated in parental taxa, *Cardamine glanduligera* and *C. enneaphyllos*, and the hybrid species *C.* ×*paxiana*; picture (b) indicates positions of restriction sites in the ITS sequences of parental species and the corresponding restriction fragments. The short fragments (90 bp and 57 bp) are faint, but still present. The hybrid individuals (3–6) show a clearly additive pattern, i.e. the simultaneous presence of the ITS variants of both parental species. Identity of samples: 1 – loc. Bobačka (loc. 23, ind. D75), 2 – loc. Šútovská dolina (loc. 14, ind. D25), 3 – loc. Bystrá (loc. 18, ind. D85), 4 – loc. Vyvieranie (loc. 19, ind. D42), 5 – loc. Bobačka (loc. 23, ind. D62), 6 – loc. Šútovská dolina (loc. 14, ind. D38), 7 – loc. Bystrá (loc. 23, ind. D109), 8 – loc. Vyvieranie (loc. 19, ind. D12). See Appendix 1 for the locality details.

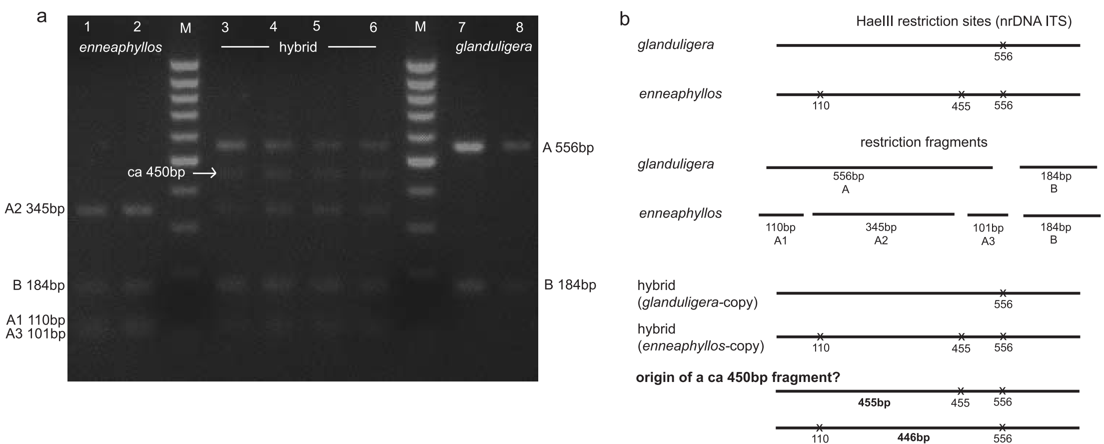

Fig. 3. – RFLP analysis of nrDNA ITS region (ITS1-5.8S-ITS2) using the *Hae*III restriction endonuclease. Picture (a) shows electrophoretic separation of the restriction fragments generated in parental taxa, *Cardamine glanduligera* and *C. enneaphyllos*, and the hybrid species *C.* ×*paxiana*; picture (b) indicates positions of restriction sites in the ITS sequences of parental species and the corresponding restriction fragments. The short fragments (110 bp and 101 bp) are faint, but still present. All the hybrid individuals (3–6) show an additional ca 450 bp fragment not present in the parents. Its origin might be explained as indicated in the text. Identity of samples: 1 – loc. Bobačka (loc. 23, ind. D75), 2 – loc. Šútovská dolina (loc. 14, ind. D25), 3 – loc. Bystrá (loc. 18, ind. D85), 4 – loc. Vyvieranie (loc. 19, ind. D42), 5 – loc. Bobačka (loc. 23, ind. D62), 6 – loc. Šútovská dolina (loc. 14, ind. D38), 7 – loc. Bystrá (loc. 18, ind. D109), 8 – loc. Vyvieranie (loc. 19, ind. D12). See Appendix 1 for the locality details.

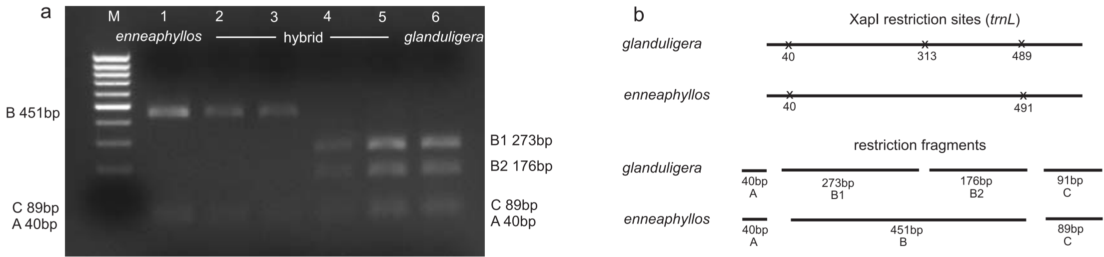

Fig. 4. – RFLP analysis of the *trnL* intron of chloroplast DNA using the *Xap*I restriction endonuclease. Picture (a) shows electrophoretic separation of the restriction fragments generated in parental taxa, *Cardamine glanduligera* and *C. enneaphyllos*, and the hybrid species *C.* ×*paxiana*; picture (b) indicates positions of restriction sites in the *trnL* intron sequences of parental species and the corresponding restriction fragments. The short fragments (40 bp and 89 bp) are faint, but still present. The individuals marked as hybrids were confirmed by ITS-RFLP analysis. All the samples shown originated from the locality Vyvieranie (loc. 19, see Appendix 1). First two hybrid individuals (2, 3) show the haplotype of *C. enneaphyllos*, while another two (4, 5) possess the haplotype of *C. glanduligera*. Identity of samples: 1 – ind. D17, 2 – ind. D92, 3 – ind. D91, 4 – ind. D43, 5 – ind. D42, 6 – ind. D12.

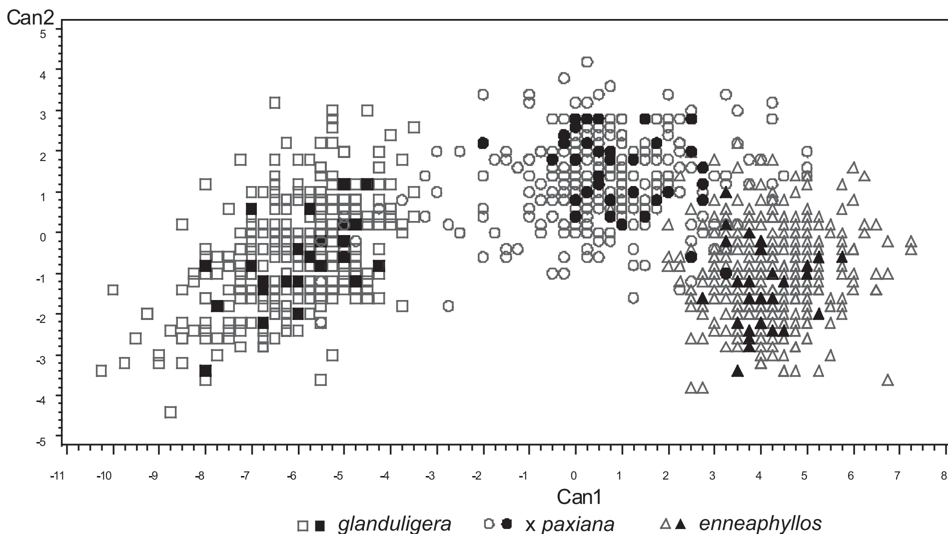

Fig. 5. – Canonical discriminant analysis of plants of *Cardamine enneaphyllos* (black triangles, n = 28), *C. glanduligera* (black squares, n = 25) and *C.* ×*paxiana* (black circles, n = 38) analysed for PCR-RFLP and/or pollen fertility. First two canonical axes extract 90.6% and 9.4% of variation among groups. The resulting canonical discriminant functions were used to compute positions of the remaining flowering plant individuals of *C. enneaphyllos* (open triangles, n = 383), *C. glanduligera* (open squares, n = 319), and *C.* ×*paxiana* (open circles, n = 277), identified by petal colour, rhizome morphology and presence of glands. Positions of individual plants are schematized to decrease overlap of symbols of the same type (“round” command in the SAS statistical package).

Morphometric analyses

Computation of Spearman and Pearson correlation coefficients yielded similar values. Three pairs of highly correlated characters were identified (r > 0.95): length of stem leaf lamina – length of middle leaflet of stem leaf, length of shorter stamen – length of longer

stamen, width of mature seeds – length of mature seeds. The latter characters from each pair were therefore excluded from further analyses (Appendix 2), except the exploratory data analysis.

The CDA performed on the "matrix of plants analysed for PCR-RFLP and pollen fertility" showed three distinct groups corresponding to the parental species and hybrid (Fig. 5, black symbols). The groups were clearly separated along the first and partly also along the second axis, with only two hybrid plants apparently misplaced in the *C. enneaphyllos* group (both these plants expressed a clearly additive PCR-RFLP profile). The first axis, contributing most to group separation, was highly correlated with the presence of glands (correlation –0.95) and with the presence and length of rhizome internodia (correlation –0.85); i.e. two of the three characters recognized as diagnostic (note that the colour of petals, because of its multistate character, was not included in the data matrix). The second canonical axis (expressing a slight shift of the hybrid) was influenced simultaneously by several characters, none of them being particularly strongly correlated with this axis. When the two canonical discriminant functions derived from this analysis were applied to the "flowering complete matrix" and our tentative identification was indicated by different symbols, three groupings were still apparent (Fig. 5, open symbols representing non-analysed plants and black ones plants analysed for PCR-RFLP and pollen fertility). This approach was used to test the reliability of our plant identification (mainly in respect of the hybrid individuals). In spite of the slight overlap of these three groups it is apparent that our identification on morphological grounds represents a fairly good estimate of the parental vs. hybrid status of the plants. In addition, the petal colour, not used in CDA, significantly contributes to the differentiation of the groups.

PCA based on "flowering parental matrix" showed two groups of specimens corresponding to *C. enneaphyllos* and *C. glanduligera* with only a few specimens in the zone of overlap (Fig. 6). Only a two-dimensional ordination graph is shown, since the third axis did not contribute anything further to differentiation. The parental species were separated mainly along the first axis. The vegetative characters showing highest correlations with the first axis and most contributing to species differentiation are height of stem, length of rhizome internodes, width of middle leaflet of stem leaf, and presence of glands; three additional floral characters are significant as well: number of flowers, length of stamens and length of petals. The former two exhibit higher values in *C. enneaphyllos*, while petals are longer in *C. glanduligera* (see also Table 1).

Figure 7 shows the results of PCA based on "flowering complete matrix". In contrast to CDA, it depicts the position of the parental taxa and hybrids based on their overall morphology, not just a few characters, which best separate the groups. The resulting picture is therefore different from that of CDA. Hybrid individuals were placed in intermediate positions but also largely overlap with parental phenotypes, especially with *C. enneaphyllos*. This picture reflects morphology of hybrid individuals that, indeed, display diverse phenotypes; it corresponds also to variation in the patterns of individual characters, where hybrids are more similar to *C. enneaphyllos* in most characters (Table 1). This ordination diagram illustrates the positions of hybrid specimens according to the colour of their petals. Four different classes of colour of petals were recognized in hybrids; most common are yellowish pink or irregularly coloured petals, less common are white or almost white (recorded in four populations) and pale pink petals (found in two populations, but mainly in population no. 14), and occasionally also purple petals can be found (scattered individuals

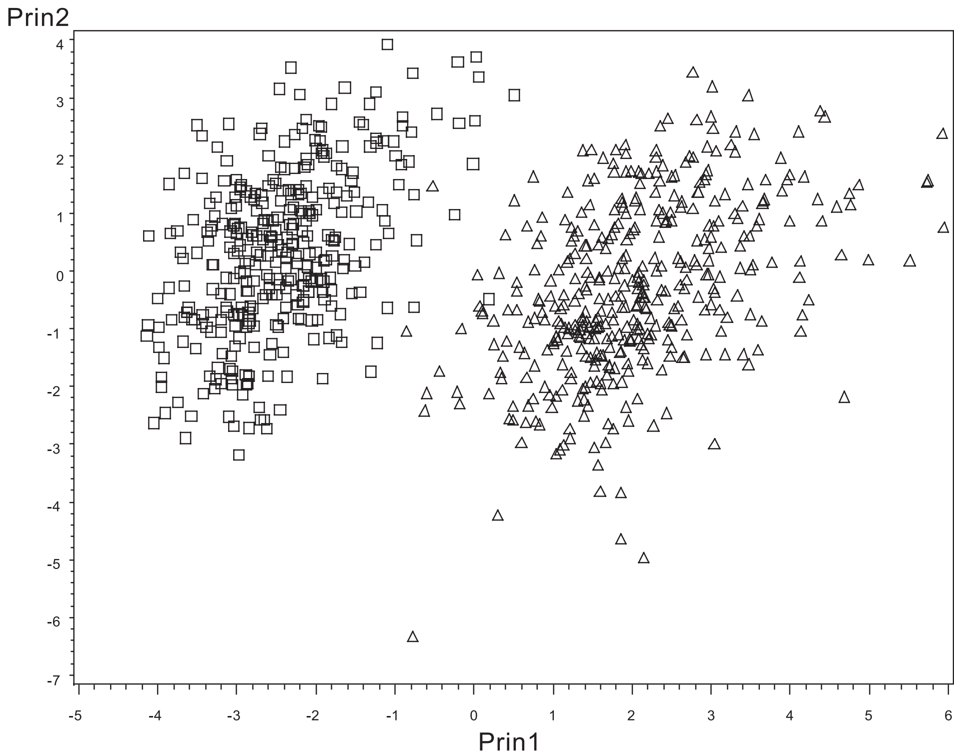

Fig. 6. – Principal component analysis of flowering specimens of *Cardamine enneaphyllos* (triangles, n = 411) and *C. glanduligera* (squares, n = 344). The analysis is based on nine vegetative and seven floral characters. First two components extract 37.5% and 13.9% of total variation.

in three populations). Hybrid individuals, as shown in the graph, do not completely intermingle, but there is some tendency for specimens to group according to petal colour, i.e. a correlation between colour of petals and other morphological characters should exist. Analyses (both PCA and CDA) run with hybrid individuals only (figures not shown) indicated that pale-pink-flowered hybrid specimens possess larger petals and sepals than other hybrid individuals. White-flowered and yellowish-pink-flowered specimens, in turn, tend to be mutually shifted in respect of the number of flowers, stem leaves and petal width.

Data from PCR-RFLP superimposed onto the PCA ordination graph of morphological data (based on "flowering complete matrix") show that genetically confirmed hybrids exhibit large morphological variation (Fig. 8). Hybrids with *C. glanduligera* as the maternal parent were found scattered across phenetic space, thus there is no apparent correlation between morphology and the direction of interspecific crosses. Pollen fertility data were superimposed onto the ordination graph of PCA (based on "flowering complete matrix") to show the position of the corresponding specimens in phenetic space (Fig. 9). Hybrid specimens with high fertility were found mainly in two populations (population no. 14, 19; Appendix 1), and tend to be morphologically closer to *C. glanduligera*.

Table 1. – Results of the exploratory data analysis of *Cardamine enneaphyllos* (411 individuals in flower/28 individuals in fruit), *C. glanduligera* (344/28 individuals) and their hybrid *C.* ×*paxiana* (315 individuals in flower). Of the 28 characters examined only those best differentiating between the parental species are presented, plus the colour of petals not included in the morphometric analyses. Mean ± standard deviation, and a range between 5th and 95th percentiles (with 1st and 99th in brackets) are shown.

Character/taxon	*C. enneaphyllos*	*C.* ×*paxiana*	*C. glanduligera*
Height of stem (cm)	33.78 ± 5.37 (21) 25–42 (47)	32.51 ± 7.05 (16) 22–45 (47)	23.77 ± 4.52 15 (17)–32 (37)
Length of the first rhizome internode (cm)	0	0.80 ± 0.84 (0) 0–2 (3)	4.58 ± 2.21 (1) 2–9 (11)
Length of stem leaf petiole (mm)	22.48 ± 7.91 (10) 12–38 (50)	24.25 ± 8.01 (10) 14–38 (50)	15.46 ± 4.61 (6) 9–23 (28)
Length of stem leaf lamina (mm)	83.54 ± 21.65 (45) 54–123 (145)	84.26 ± 23.87 (37) 48–122 (154)	65.49 ± 14.51 (38) 42–90 (105)
Length of middle leaflet of stem leaf (mm)	77.94 ± 20.36 (41) 50–114 (135)	79.78 ± 22.76 (34) 45–117 (145)	62.21 ± 14.00 (35) 40–87 (99)
Width of middle leaflet of stem leaf	26.76 ± 8.32 12 (15)–42 (50)	28.04 ± 9.57 (12) 15–47 (61)	19.32 ± 5.10 (9) 12–29 (32)
Number of flowers	6.94 ± 1.85 (4) 4–10 (11)	6.21 ± 2.13 (2) 3–10 (11)	3.08 ± 1.12 (1) 2–5 (6)
Length of petals (mm)	15.24 ± 1.80 (12) 13–18 (20)	16.69 ± 2.51 (12) 13–22 (23)	20.04 ± 2.17 (15) 17–24 (25)
Width of petals (mm)	5.95 ± 0.95 (4) 5–8 (8)	7.24 ± 1.24 (5) 5–10 (10)	7.53 ± 0.96 (6) 6–9 (10)
Length of shorter stamen (mm)	14.16 ± 1.94 (10) 11–17 (19)	11.03 ± 2.01 (6) 8–14 (15)	9.22 ± 1.21 (6) 8–11 (13)
Length of longer stamen (mm)	15.71 ± 1.94 (11) 12–19 (20)	12.59 ± 2.04 (8) 10–16 (16)	11.17 ± 1.34 (8) 9–14 (14)
Number of developed siliquae	5.46 ± 1.55 (3) 3–8 (9)	—	2.07 ± 1.09 (1) 1–4 (6)
Number of seeds per siliqua	3.43 ± 0.5 (3) 3–4 (4)	—	6.71 ± 1.82 (4) 4–10 (10)
Length of siliqua beak (mm)	14.21 ± 2.11 (12) 12–18 (19)	—	6.96 ± 2.01 (5) 5–10 (15)
Glands on leaves	absent	scarce intermediate presence	present at leaflet base, leaf margin
Colour of petals	yellow	yellowish pink or irregularly coloured, white, pale pink, rarely purple	purple, rarely pale pink

PCA based on "fruiting parental matrix", which aimed to show variation patterns and differentiation of plants also at the fruiting stage, revealed two distinct groups corresponding to *C. enneaphyllos* and *C. glanduligera*, clearly separated along the first axis (figure not shown, but see Fig. 10). The vegetative characters with the highest values of eigenvectors and thus contributing most to this separation were the same as in PCA based on flowering specimens, and additionally the number of siliquae and length of the siliqua beak were also significant. Of the many hybrid individuals surveyed only five had almost

fully developed fruits. PCA based on this matrix ("fruiting complete matrix") is shown in Fig. 10. The hybrid individuals are located in between the parents along the first axis (correlated with the same characters as given above), but shifted along the second axis in respect of both parents. The characters with the highest eigenvectors for the second axis are the length of siliquae, number of seeds per siliquae and length of seeds. Even if a few siliquae are formed in hybrids, they are much shorter, with fewer and smaller seeds. It remains to be determined, if any of these seeds are viable and able to germinate.

Finally, the results of the exploratory data analysis are summarized in Table 1 showing the descriptive statistics of selected characters that were found to differentiate between *C. enneaphyllos* and *C. glanduligera*. In most quantitative characters, hybrid individuals are apparently closer to *C. enneaphyllos*, only in the width of petals do they show a closer resemblance to *C. glanduligera* and in the length of stamens they are clearly intermediate.

Discussion

Evidence for interspecific hybridization and morphological variation of hybrids

To determine whether a particular plant individual is a hybrid or just variation of a non-hybrid (assumed parental) species is not always easy and straightforward. The main and most commonly used criterion for inferring hybridity is morphological intermediacy, although morphology alone can be potentially misleading due to complex and often unpredictable character expression in hybrids, or is insufficient in cases where parental species are morphologically very similar (Rieseberg & Ellstrand 1993, Rieseberg 1995, Rieseberg & Carney 1998). There is need also for convincing evidence from data, such as karyology, ecology, geographic distribution and especially DNA markers (see e.g. review by Hegarty & Hiscock 2005). Here we conducted a study on plants commonly determined as hybrids between *Cardamine enneaphyllos* and *C. glanduligera* (*C.* ×*paxiana*) based on their morphology, mainly the colour of petals and the absence of siliquae after flowering (Kochjarová et al. 1999 and references therein). We performed detailed morphometric evaluation, taking into account several other floral and vegetative characters, of a large collection of specimens, and used pollen fertility, nrDNA and cpDNA markers to test the assumed hybridity. Low pollen fertility and additive nrDNA profiles observed in a subset of specimens determined to be hybrids from their morphology, together with the canonical discriminant analysis results showed that our preliminary plant identification was correct. It was not realistic to analyse all 714 specimens of putative hybrids for pollen fertility and/or PCR-RFLP analyses, but the selection of 38 and 31 specimens, respectively (drawn from several populations spanning the geographical area and covering the range of morphological variation), yielded consistent results, confirming the hybrid status of each specimen within that subset. Detailed morphometric analysis revealed that except for the three characters used for hybrid identification – colour of petals, character of rhizome, intermediate presence of glands on leaves (see Methods) – intermediacy is reflected also in the length of stamens (Table 1). In most of the other characters examined, however, the hybrids seem to resemble more *C. enneaphyllos* as can be seen from the descriptive statistics of individual characters (Table 1). This is evident also from the ordination graph of PCA (Fig. 7) where the hybrid individuals occupied not only intermediate positions, but were found scattered across the morphological space of the parental species, but mainly that of *C. enneaphyllos*.

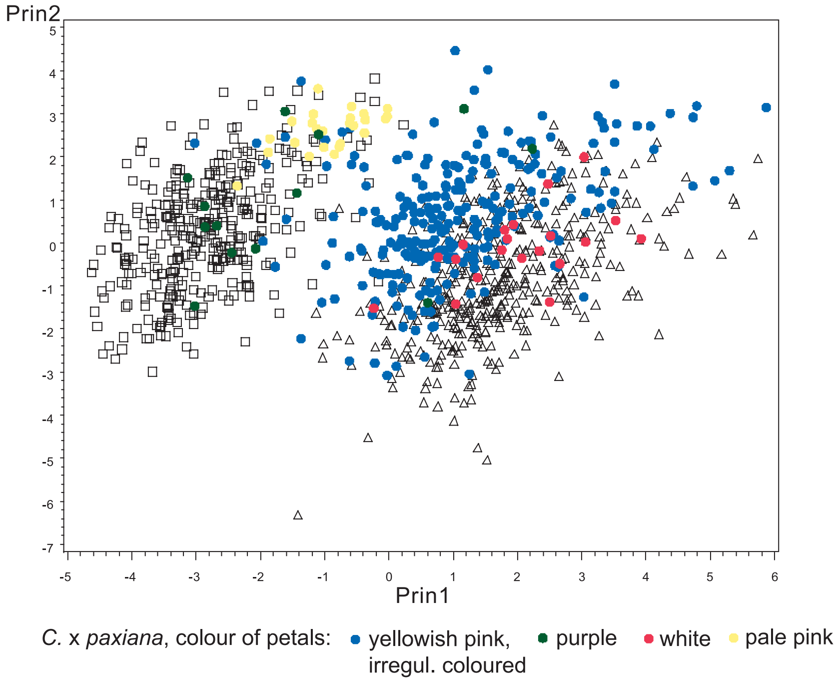

Fig. 7. – Principal component analysis of flowering specimens of *Cardamine enneaphyllos* (triangles, n = 411), *C. glanduligera* (squares, n = 344) and *C. ×paxiana* (circles, n = 315). Colour of the circles indicates colour of petals of individual plants of *C. ×paxiana.* The analysis is based on nine vegetative and seven floral characters. First two components extract 33.3% and 14.8% of total variation.

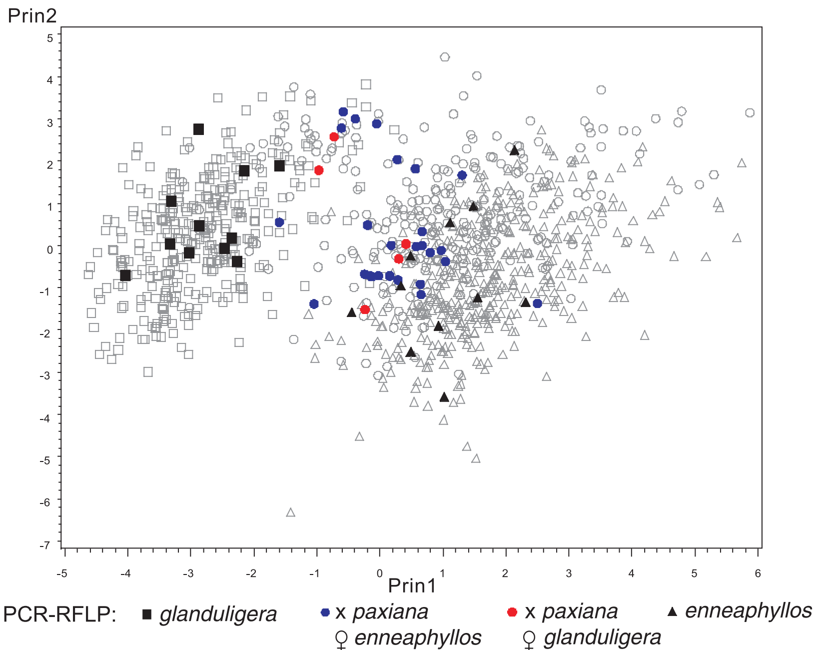

Fig. 8. – PCR-RFLP results mapped onto the ordination of the principal component analysis of flowering specimens of *Cardamine enneaphyllos* (triangles, n = 411), *C. glanduligera* (squares, n = 344), and *C. ×paxiana* (circles, n = 315). Open grey symbols refer to specimens not analysed by PCR-RFLP; the black squares and triangles refer to the analysed samples of *C. glanduligera* and *C. enneaphyllos*, respectively; the blue circles indicate samples of hybrids (based on ITS additive patterns) and the cpDNA haplotype of *C. enneaphyllos*; the red circles indicate samples of hybrids (based on ITS additive patterns) and the cpDNA haplotype of *C. glanduligera.*

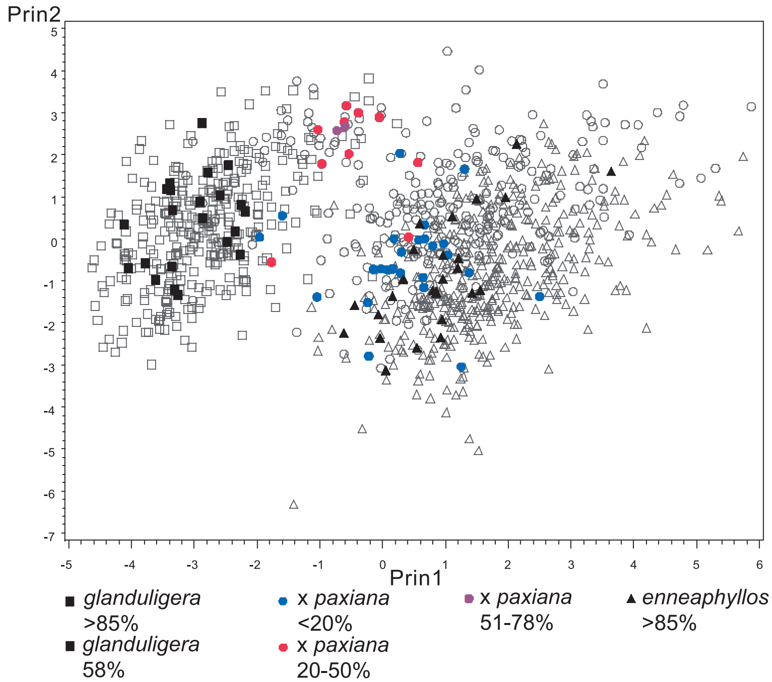

Fig. 9. – Pollen fertility data mapped onto the ordination of the principal component analysis of flowering specimens of *Cardamine enneaphyllos* (triangles, n = 411), *C. glanduligera* (squares, n = 344) and *C.* ×*paxiana* (circles, n = 315). Open grey symbols refer to specimens not analysed for pollen fertility; the coloured symbols indicate the percentage pollen fertility range/value.

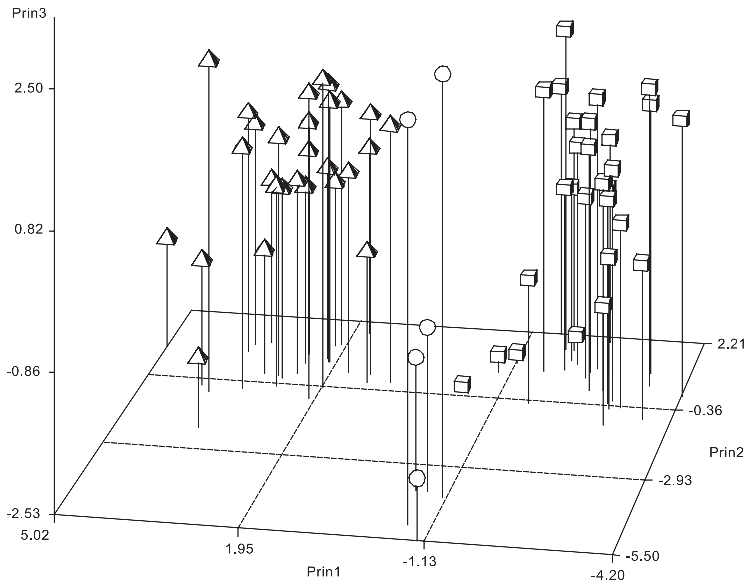

Fig. 10. – Principal component analysis of fruiting specimens of *Cardamine enneaphyllos* (pyramids, n = 28), *C. glanduligera* (cubes, n = 28) and a few plants of *C.* ×*paxiana* with developed fruits (circles, n = 5). The analysis is based on eight vegetative and six fruit characters. First three components extract 48.0%, 17.8% and 8.0% of total variation.

Detailed morphological studies of populations in hybrid zones are rather rare, but three recent studies performed on *Cardamine* (Marhold et al. 2002, Lihová et al. 2006, 2007) allow us to compare and discuss the patterns observed. In contrast to the present study, the morphology of the Pyrenean diploid *C.* ×*enriquei* (*C. amara* subsp. *pyrenaea* × *C. crassifolia*; Marhold et al. 2002), and the SW Alpine pentaploid *C.* ×*ferrarii* (hexaploid *C. asarifolia* × diploid *C. amara* subsp. *amara*; Lihová et al. 2006) is much more homogeneous, uniform and distinct from that of the parents. These hybrids clearly display intermediate phenotypes, showing intermediacy mainly in the characters best discriminating parental taxa, plus a mixture of parental ones in a balanced manner. Considering also the high pollen sterility and restricted genetic variation, it is suggested that mating of hybrid individuals or backcrossing, if it occurs, is rather limited. Recurrent hybridizations and persistence of first-generation hybrids through vegetative propagation probably best explains the observed patterns at these localities (Marhold et al. 2002, Lihová et al. 2006). A contrasting case of interspecific hybridization is that between the polyploids *C. pratensis* and *C. raphanifolia* in NW Spain, where extensive gene flow resulted in a dynamic hybrid swarm characterized by a wide range of morphological, karyological and genetic variation. Phenotypes observed in those populations span the whole morphological space within and between the parental species, yielding vast and continuous morphological variation (Lihová et al. 2007).

Several scenarios can be proposed to explain the patterns of morphological variation found in the hybrid *C.* ×*paxiana*. First, it might be speculated that reciprocal crosses can produce different phenotypes, i.e. depending on which species acted as the maternal species, the resulting hybrids may differ morphologically. Significant morphological differences between the two progeny groups resulting from reciprocal crosses are reported e.g. in *Rosa dumalis* × *R. rubiginosa* hybrids (Werlemark et al. 1999). This was assumed for a long time to be the case for two morphologically distinct hybrids of *Spartina alternifolia* and *S. maritima* in UK and France, respectively, but later detailed investigations rejected this hypothesis. Genetic and epigenetic changes following hybridization appear to be responsible for phenotypic differences in *Spartina* hybrids (Salmon et al. 2005). In the present study bidirectional hybridization is indeed taking place, although it is mainly in one direction; *C. glanduligera* acted as the maternal parent in only five cases in two populations. Still, these five individuals differed morphologically and were found scattered in ordination space (Fig. 8). The second explanation for the morphological patterns observed might be that the morphology can be attributed to different classes of hybrids, i.e. not only F_1 but also later generation hybrids and backcross progeny were present in the population. Given the reduced pollen fertility of hybrids and the almost complete sterility manifested by the absence of siliquae, mating and backcrossing are strongly restricted, and thus this scenario appears unlikely. Nevertheless, several hybrid plants with higher pollen fertility (20–78%) were found in two populations, and they may have played the role at least of pollen donors. Artificial hybridization, experimental backcrosses and comparative morphological evaluation of the obtained progeny may be a useful way of testing this assumption. The third possible explanation may refer to the ploidy levels and chromosomal constitutions of hybrid individuals. The two parental species *C. enneaphyllos* and *C. glanduligera* are high polyploids, differing in ploidy levels (deca- and hexaploid, respectively), thus, it may be interesting to ascertain the ploidy level(s) of the hybrid. There are no published records of the exact chromosome numbers of *C.* ×*paxiana*. Only an approximate record 2n = ca

60–66 for one population was published (Kochjarová & Bernátová 1995). Assuming that segregation of meiotic chromosomes occurs in the parental species in a regular and equal way, octoploids may be expected among the hybrids. Although there are no studies on meiosis in the parental species, considering their high ploidy levels some irregularities resulting in an unequal segregation of meiotic chromosomes and consequently different gametic and zygotic chromosomal constitutions may be expected. It is unknown whether all hybrid individuals are of the same ploidy level. Variation in chromosome numbers and unbalanced chromosomal constitutions are reported in several highly polyploid North American species of *Dentaria* (Harriman 1965, Spooner 1984), as well as in the recently examined hybrid populations of *C. raphanifolia* and *C. pratensis* in NW Spain (Lihová et al. 2007). Chromosomal variation together with complex gene expression in highly polyploid genomes may account for the large morphological variation in hybrids. These issues remain to be resolved.

The utility of the nrDNA ITS region for hybrid detection

Important part of the present study is the evidence for hybridization provided by PCR-RFLP analysis of the nrDNA ITS region. Several molecular tools are available and are successful in detecting hybrids (Vriesendorp & Bakker 2005). Generally, biparentally inherited unequivocal genetic markers are needed to differentiate each of the proposed progenitor species and search for additive profiles in putative hybrids. The ITS region of nrDNA has proved useful in many previous studies, as it is easily amplifiable using universal primers and exhibits relatively high levels of phylogenetically informative sequence variation differentiating between closely related taxa. On the other hand, it is known that a number of molecular genetic processes impact ITS sequence evolution. Concerted evolution of nrDNA, a process by which intra- and intergenomic differences among nrDNA copies are typically homogenized, may lead to the recombination between the parental repeat types and/or preferential deletion of one of them. In addition, some repeats can degenerate into pseudogenes, which are released from functional constraints and evolve independently (Álvarez & Wendel 2003). Even if these processes can proceed in an unpredictable and fast fashion (Fuertes Aguilar et al. 1999, Franzke & Mummenhoff 1999), F_1 and first generation hybrids are expected to retain both repeat types and display additivity. Several methods are used to detect both parental ITS repeat types within a genome of a putative hybrid, as reviewed and discussed by Rauscher et al. (2002). These include direct sequencing of PCR products and inspection of nucleotide additivity (i.e. double peaks in electropherograms) that suggests the presence of more than one repeat type (Sang et al. 1995, Hodkinson et al. 2002, Saito et al. 2006); cloning of PCR products and screening for parental repeat types among the clones (Nieto Feliner et al. 2004, Zhou et al. 2005, Lihová et al. 2006); and the use of specific primers that only amplify the ITS repeat of a particular parental species (repeat-specific primers; Rauscher et al. 2002, 2004). Finally, digestion of PCR products using restriction enzymes that produce different restriction patterns for the parental repeats may also be useful to prove their simultaneous presence in the hybrid (Hardig et al. 2000, Kaplan & Fehrer 2004, 2006). The first and second approaches have limitations for detecting rare ITS repeats, since they are not sensitive enough to reveal signal from a repeat type present at a low frequency (Rauscher et al. 2002). This may be crucial for later generation hybrids or backcrossed plants, where one of the parental repeat

types may be retained in the genome only at a very low frequency. Another drawback of all methods except repeat-specific primers is the occurrence of artificial recombinants generated during PCR of heterogeneous templates (Bradley & Hillis 1997, Cronn et al. 2002). When the sequences of parental species are known, however, recombinant sequences can be distinguished and interpreted for most cases.

In the present study we took advantage of the considerable divergence between the ITS sequences of the parental species, including differentiation at several potential restriction sites. The PCR-RFLP method applied here allowed a convenient and rapid screening of numerous putative hybrid individuals without the need to generate sequence information and/or cloning. This method is especially suitable for first generation hybrids, where perfect additivity is expected and the resulting restriction patterns can be interpreted unequivocally. After partial sequence homogenization through concerted evolution, however, complex restriction patterns would be generated. Using the restriction enzyme *Taq*I we obtained a clearly interpretable additive restriction pattern in all 31 hybrid individuals analysed, which accords with the assumed hybrid status of these plants (Fig. 2). Surprisingly an additional ca 450 bp long fragment was obtained when the ITS amplification product was digested using the enzyme *Hae*III (Fig. 3). There are two possible explanations, a partial sequence homogenization leading to the loss or gain of one restriction site, or just an artificial recombination between heterogeneous templates in PCR. Nevertheless, intriguing is that the pattern with the additional band was present consistently in each of the hybrid individual analysed, and therefore, cannot be explained just by artefacts generated by PCR. Cloning these PCR products and sequencing could help resolve the origin of the additional band in the hybrid.

Concluding remarks on hybrids and hybrid zones

The present paper documents another case of interspecific hybridization in the genus *Cardamine*. In the context of previous hybridization studies on this genus (Urbanska et al. 1997, Marhold et al. 2002, Lihová et al. 2006, 2007) and other plant genera, our study contributes to the complexity of the observed morphological, genetic and ecological patterns in hybrids. The hybrid *C.* ×*paxiana* studied here displayed morphological intermediacy only to a certain extent, but using the three main characters, colour of petals, morphology of rhizomes and presence of glands on leaves (plus the sterility of plants seen in populations at the fruiting stage) it can be quite easily recognized. Geographically it is restricted to the areas where the parental species' distributions overlap, and almost exclusively to the localities where both parental species co-occur. Hybridization in such areas is observed in several other cases where prezygotic isolation barriers are weak or can be occasionally broken. In such hybrid zones different outcomes can be found, ranging from the occurrence of incidental mostly sterile hybrids, through the formation of long-lasting dynamic hybrid swarms to hybrid speciation. Examples of the occurrence of hybrid swarms with extensive gene flow, where morphological and genetic differentiation between the parental species is largely blurred, are e.g. sympatric European oak species (Petit et al. 2003), *Rorippa amphibia* × *R. sylvestris* populations (Bleeker & Hurka 2001, Bleeker 2004, 2007) or *Cardamine* populations in NW Spain (Lihová et al. 2007). On the other hand, there are cases where hybrids are formed repeatedly, but are largely sterile so that backcrosses or later generation hybrids are exceptionally rare. The integrity of parental species

is little affected, and such hybrids with strong postzygotic barriers usually have little evolutionary potential unless their fertility is restored. This latter scenario applies to the hybrid studied here, and has been reported also for other hybridization events e.g. between *Quercus crassifolia* and *Q. crassipes* (Tovar-Sánchez & Oyama 2004), *Rorippa amphibia* and *R. palustris* (Bleeker 2007) and some other *Cardamine* species (Marhold et al. 2002, Lihová et al. 2006). The final outcome of interspecific hybridization is still hardly predictable, since we know little about the mechanisms or conditions that promote or counteract successful hybrid establishment, long-term existence of hybrid swarms or hybrid speciation. Considering the contrasting patterns in *Cardamine* hybrids (localized largely sterile hybrids vs. hybrid swarms) we might hypothesize that isolation barriers due to genetic incompatibilities occurring between either two diploids or a diploid and a polyploid parent (as is the case in the diploid *Cardamine* ×*enriquei*, Marhold et al. 2002; and pentaploid *C.* ×*ferrarii*, Lihová et al. 2006) are much stronger than can be expected when the two hybridizing species are both high polyploids with assumed close evolutionary history (polyploid hybrid swarms between *C. pratensis* and *C. raphanifolia*; Lihová et al. 2007). As for the present study *Dentaria* species, irrespective of their polyphyletic origin, are high polyploids of unknown polyploid origin and evolutionary history; it has just been assumed that they are rather old polyploids where the original diploid genomes are difficult to recognize or identify among the extant species (see review by Lihová & Marhold 2006). Therefore, it is not clear to what extent *C. enneaphyllos* and *C. glanduligera* are closely related, and how distinct their genomes are. Despite the polyphyletic origin of *Dentaria*, what all these high polyploid taxa have in common is complicated reticulate evolution. Its reconstruction is admittedly a challenge for future studies and is relevant to various aspects of interspecific hybridization.

Acknowledgements

This study was supported by the Slovak Research and Development Agency, Bratislava, Slovakia (grant no. APVT-51-026404). We are grateful to Judith Fehrer (Institute of Botany, Academy of Sciences of the Czech Republic, Průhonice) for advice and discussion on PCR-RFLP analyses and Jaromír Kučera (Institute of Botany, Slovak Academy of Sciences, Bratislava) for technical help with Fig. 1. Tony Dixon (Norwich) kindly improved our English.

Súhrn

V predloženej štúdii sa autori zaoberajú hybridizáciou medzi dvomi polyploidnými druhmi rodu *Cardamine*: dekaploidom *C. enneaphyllos* a hexaploidom *C. glanduligera* (oba boli predtým zaraďované do rodu *Dentaria*). Študované územie bolo situované do slovenskej časti Západných Karpát, kde sa prekrývajú areály výskytu oboch rodičovských taxónov a kde sa oba druhy vyskytujú na spoločných lokalitách. U predpokladaného hybrida *C.* ×*paxiana* bola študovaná na viacerých lokalitách morfologická variabilita, fertilita peľu, a PCR-RFLP profil. Hybridné jedince boli determinované na základe troch morfologických znakov, ktoré sa považujú za diferenciálne: farba kvetov, prítomnosť žliazok na listoch a dĺžka internódií podzemku. Táto predbežná identifikácia hybridov bola potvrdená výrazne zníženou fertilitou peľu a aditívnym PCR-RFLP profilom ITS regiónu jadrovej DNA. Morfometrickými analýzami sa zistila značná morfologická variabilita hybridov, pričom sa v článku diskutujú možné zdroje variability. Na základe výsledkov PCR-RFLP analýz cpDNA, bola dokázaná obojsmerná hybridizácia, pričom však druh *C. enneaphyllos* vystupoval oveľa častejšie ako materská rastlina. Geografické rozšírenie a sterilita hybridných jedincov naznačujú, že tieto vznikajú opakovane prostredníctvom kríženia rodičovských druhov a reprezentujú F_1 alebo včasné generácie hybridov, udržujúce a šíriace sa prostredníctvom vegetatívneho rozmnožovania.

References

Álvarez I. & Wendel J. F. (2003): Ribosomal ITS sequences and plant phylogenetic inference. – Molec. Phylogenet. Evol. 29: 417–434.
Al-Shehbaz I. A. (1988): The genera of *Arabidae* (*Cruciferae*; *Brassicaceae*) in the southeastern United States. – J. Arnold Arbor. 69: 85–166.
Bleeker W. (2004): Genetic variation and self-incompatibility within and outside a *Rorippa* hybrid zone (*Brassicaceae*). – Plant Syst. Evol. 246: 35–44.
Bleeker W. (2007): Interspecific hybridization in *Rorippa* (*Brassicaceae*): patterns and processes. – Syst. & Biodiversity (in press).
Bleeker W., Franzke A., Pollmann K., Brown A. H. D. & Hurka H. (2002): Phylogeny and biogeography of Southern Hemisphere high-mountain *Cardamine* species (*Brassicaceae*). – Austral. Syst. Bot. 15: 575–581.
Bleeker W. & Hurka H. (2001): Introgressive hybridization in *Rorippa* (*Brassicaceae*): gene flow and its consequences in natural and anthropogenic habitats. – Molec. Ecol. 10: 2013–2022.
Bradley R. D. & Hillis D. M. (1997): Recombinant DNA sequences generated by PCR amplification. – Molec. Biol. Evol. 14: 592–593.
Cesca G. & Peruzzi L. (2002): A new species of *Cardamine* subgen. *Dentaria* (*Cruciferae*), apoendemic in Calabria (Southern Italy). – Plant Biosystems 136: 313–320.
Cronn R., Cedroni M., Haselkorn T., Grover C. & Wendel J. F. (2002): PCR-mediated recombination in amplification products derived from polyploid cotton. – Theor. Appl. Genet. 104: 482–489.
Detling L. E. (1936): The genus *Dentaria* in the Pacific States. – Amer. J. Bot. 23: 570–576.
Doyle J. J. & Doyle J. L. (1987): A rapid DNA isolation procedure for small quantities of fresh leaf tissue. – Phytochem. Bull. Bot. Soc. Amer. 19: 11–15.
Ferguson C. J., Levin D. A. & Jansen R. K. (1999): Natural hybridization between an outcrossing and a selfing *Phlox* (*Polemoniaceae*): the maternal species of F1 hybrids. – Plant Syst. Evol. 218: 153–158.
Francisco-Ortega J., Fuertes-Aguilar J., Gómez-Campo C., Santos-Guerra A. & Jansen R. K. (1999): Internal transcribed spacer sequence phylogeny of *Crambe* L. (*Brassicaceae*): molecular data reveal two Old World disjunctions. – Molec. Phylogenet. Evol. 11: 361–380.
Franzke A. & Mummenhoff K. (1999): Recent hybrid speciation in *Cardamine* (*Brassicaceae*) – conversion of nuclear ribosomal ITS sequences in statu nascendi. – Theor. Appl. Genet. 98: 831–834.
Franzke A., Pollmann K., Bleeker W., Kohrt R. & Hurka H. (1998): Molecular systematics of *Cardamine* and allied genera (*Brassicaceae*): ITS and non-coding chloroplast DNA. – Folia Geobot. Phytotax. 33: 225–240.
Fuertes Aguilar J., Roselló, J. A. & Nieto Feliner G. (1999): Nuclear ribosomal DNA (nrDNA) concerted evolution in natural and artificial hybrids of *Armeria* (*Plumbaginaceae*). – Molec. Ecol. 8: 1341–1346.
Futák J. (1984): Fytogeografické členenie [Phytogeographical division]. – In: Bertová, L. (ed.), Flóra Slovenska [Flora of Slovakia] 4(1) (Appendix), Veda, VSAV, Bratislava.
Hall T. A. (1999): BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. – Nucl. Acids Symposium Series 41: 95–98.
Hardig T. M., Brunsfeld S. J., Fritz R. S., Morgan M. & Orians C. M. (2000): Morphological and molecular evidence for hybridization and introgression in a willow (*Salix*) hybrid zone. – Molec. Ecol. 9: 9–24.
Harriman N. A. (1965): The genus *Dentaria* L. (*Cruciferae*) in eastern North America. – Ph.D. thesis [Depon. in: Vanderbilt University, Nashville, Tennessee].
Hegarty M. J. & Hiscock S. J. (2005): Hybrid speciation in plants: new insights from molecular studies. – New Phytol. 165: 411–423.
Hodkinson T. R., Chase M. W., Takahashi C., Leitch I. J., Bennett M. D. & Renvoize S. A. (2002): The use of DNA sequencing (ITS and *trnL-F*), AFLP, and fluorescent in situ hybridization to study allopolyploid *Miscanthus* (*Poaceae*). – Amer. J. Bot. 89: 279–286.
Holmgren P. K., Holmgren N. H. & Barnett L. C. (1990): Index Herbariorum. Part I: The Herbaria of the World. Ed. 8. – Regnum Veg. 120: 1–693.
Jalas J. & Suominen J. (1994): Atlas florae europaeae, vol. 10. – The Committee for Mapping the Flora of Europe and Societas Biologica Fennica Vanamo, Helsinki.
Jones B. M. G. & Akeroyd J. (1993): *Cardamine* L. – In: Tutin T. G. Heyword V. H., Burges N. A., Moore D. M., Valentine D. H., Walters S. M. & Webb D. A. (eds), Flora Europaea,ed. 2, 1: 346–351, Cambridge University Press, Cambridge.
Kaplan Z. & Fehrer J. (2004): Evidence for the hybrid origin of *Potamogeton ×cooperi* (*Potamogetonaceae*): traditional morphology-based taxonomy and molecular techniques in concert. – Folia Geobot. Phytotax. 39: 431–453.

Kaplan Z. & Fehrer J. (2006): Comparison of natural and artificial hybridization in *Potamogeton*. – Preslia 78: 303–316.

Kochjarová J. & Bernátová D. (1995): Chromosome numbers of several rare, endangered and endemic species of the flora of Slovakia. – Biologia (Bratislava) 50: 27–31.

Kochjarová J., Blanár D., Janovicová K. & Kliment J. (1999): Nové lokality výskytu, morfologická charakteristika a fytocenologická väzba zaujímavého kríženca zubačky Paxovej – *Dentaria* ×*paxiana* (O. E. Schulz) Jáv. na Muránskej planine a v Slovenskom rudohorí [The new localities, morphological characteristic and phytosociological relationship of the interesting hybrid *Dentaria* ×*paxiana* (O. E. Schulz) Jáv. in the Muránska planina and Slovenské rudohorie Mts]. – In: Uhrin M. (ed.), Výskum a ochrana prírody Muránskej planiny [Research and nature protection in the Muránska planina], 2: 55–69, Ministerstvo životného prostredia SR & Správa národného parku Muránska planina, Revúca.

Kotov M. I. (1979): Zubyanka – *Dentaria*. – In: Fedorov A. A. (ed.), Flora evropeiskoi chasti SSSR [Flora of the European part of the USSR], 4: 91–93, Nauka, Leningrad.

Krzanowski W. J. (1990): Principles of multivariate analysis. – Clarendon Press, Oxford.

Legendre P. & Legendre L. (1998): Numerical ecology. Ed. 2. – Elsevier, Amsterdam.

Lihová J., Fuertes Aguilar J., Marhold K. & Nieto Feliner G. (2004): Origin of the disjunct tetraploid *Cardamine amporitana* (*Brassicaceae*) assessed with nuclear and chloroplast DNA sequence data. – Amer. J. Bot. 91: 1231–1242.

Lihová J., Kučera J., Perný M. & Marhold K. (2007): Hybridization between two polyploid *Cardamine* (*Brassicaceae*) species in northwestern Spain: discordance between morphological and genetic variation patterns. – Ann. Bot. (Oxford) (in press, doi 10.1093/mcm056).

Lihová J. & Marhold K. (2006): Phylogenetic and diversity patterns in *Cardamine* (*Brassicaceae*) – a genus with conspicuous polyploid and reticulate evolution. – In: Sharma A. K. & Sharma A. (eds), Plant genome: biodiversity and evolution. Vol. 1C: Phanerogams (Angiosperms – Dicotyledons), pp. 149–186, Science Publishers, Inc., Enfield, New Hampshire.

Lihová J., Shimizu K. K. & Marhold K. (2006): Allopolyploid origin of *Cardamine asarifolia* (*Brassicaceae*): incongruence between plastid and nuclear ribosomal DNA sequences solved by a single-copy nuclear gene. – Molec. Phylogenet. Evol. 39: 759–786.

Marhold K., Lihová J., Perný M., Grupe R. & Neuffer B. (2002): Natural hybridization in *Cardamine* (*Brassicaceae*) in the Pyrenees: evidence from morphological and molecular data. – Bot. J. Linn. Soc. 139: 275–294.

Marhold K. & Kochjarová J. (2002): 19. *Cardamine* L. – In: Goliašová K. & Šípošová H. (eds), Flóra Slovenska [Flora of Slovakia] V/4: 316–382, Veda, Bratislava.

Marks G. E. (1954): An aceto-carmine glycerol jelly for use in pollen fertility counts. – Stain Technol. 29: 277.

Nieto Feliner G., Gutiérrez Larena B. & Fuertes Aguilar J. (2004): Fine-scale geographical structure, intra-individual polymorphism and recombination in nuclear ribosomal internal transcribed spacers in *Armeria* (*Plumbaginaceae*). – Ann. Bot. (Oxford) 93: 189–200.

Pawłowski B. (1956): Flora Tatr, rośliny naczyniowe [Flora of the Tatry Mts., vascular plants]. Vol. 1. – PWN, Warszawa.

Petit R. J., Bodénès C., Ducousse A., Roussel G. & Kremer A. (2003): Hybridization as a mechanism of invasions in oaks. – New Phytol. 161: 151–164.

Rauscher J. T., Doyle J. J. & Brown A. H. D. (2002): Internal transcribed spacer repeat-specific primers and the analysis of hybridization in the *Glycine tomentella* (*Leguminosae*) polyploid complex. – Molec. Ecol. 11: 2691–2702.

Rauscher J. T., Doyle J. J. & Brown A. H. D. (2004): Multiple origins and nrDNA internal transcribed spacer homeologue evolution in the *Glycine tomentella* (*Leguminosae*) allopolyploid complex. – Genetics 166: 987–998.

Rieseberg L. H. (1995): The role of hybridization in evolution: old wine in new skins. – Amer. J. Bot. 82: 944–953.

Rieseberg L. H. & Carney S. E. (1998): Tansley Review No. 102. Plant hybridization. – New Phytol. 140: 599–624.

Rieseberg L. H. & Ellstrand N. C. (1993): What can molecular and morphological markers tell us about plant hybridization? – Crit. Rev. Plant Sci. 12: 213–241.

Rollins R. C. (ed.) (1993): The *Cruciferae* of Continental North America. – Stanford University Press, Stanford.

Saito Y., Möller M., Kokubugata G., Katsuyama T., Marubashi W. & Iwashina T. (2006): Molecular evidence for repeated hybridization events involved in the origin of the genus ×*Crepidiastrixeris* (*Asteraceae*) using RAPDs and ITS data. – Bot. J. Linn. Soc. 151: 333–343.

Salmon A., Ainouche M. L. & Wendel J. F. (2005): Genetic and epigenetic consequences of recent hybridization and polyploidy in *Spartina* (*Poaceae*). – Molec. Ecol. 14: 1163–1175.
Sang T., Crawford D. J. & Stuessy T. F. (1995): Documentation of reticulate evolution in peonies (*Peonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: implications for biogeography and concerted evolution. – Proc. Natl. Acad. Sci. USA 92: 6813–6817.
SAS Institute (2000): SAS OnlineDoc®, version 8 (available online). – SAS Institute, Cary, NC, USA.
Schulz O. E. (1903): Monographie der Gattung *Cardamine*. – Bot. Jahrb. Syst. 32: 280–623.
Slavík B. (1992): *Dentaria.* – In: Hejný S. & Slavík B. (eds), Květena České republiky [Flora of the Czech Republic], 3: 110–115, Academia, Praha.
Sneath P. H. & Sokal R. R. (1973): Numerical taxonomy. Priciples and practice of numerical classification. – W. H. Freeman and Company, San Francisco.
Spooner D. M. (1984): Reproductive features of *Dentaria laciniata* and *D. diphylla* (*Cruciferae*), and the implications in the taxonomy of the eastern North American *Dentaria* complex. – Amer. J. Bot. 71: 999–1005.
Sweeney P. W. & Price R. A. (2000): Polyphyly of the genus *Dentaria* (*Brassicaceae*): evidence from *trnL* intron and *ndhF* sequence data. – Syst. Bot. 25: 468–478.
Taberlet P., Gielly L., Pautou G. & Bouvet J. (1991): Universal primers for amplification of three non-coding regions of chloroplast DNA. – Plant Molec. Biol. 17: 1105–1109.
Tovar-Sánchez E. & Oyama K. (2004): Natural hybridization and hybrid zones between *Quercus crassifolia* and *Q. crassipes* (*Fagaceae)* in Mexico: morphological and molecular evidence. – Amer. J. Bot. 91: 1352–1363.
Urbanska K. M., Hurka H., Landolt E., Neuffer B. & Mummenhoff K. (1997): Hybridization and evolution in *Cardamine* (*Brassicaceae*) at Urnerboden, Central Switzerland: biosystematic and molecular evidence. – Plant Syst. Evol. 204: 233–256.
Vozárová M. & Sutorý K. (2001): Index herbariorum Reipublicae bohemicae et Reipublicae slovacae. – Zpr. Čes. Bot. Společ., Příl. 2001/1: 1–95; Bull. Slov. Bot. Spoloč. 2001, Suppl. 7: 1–95.
Vriesendorp B. & Bakker F. T. (2005): Reconstructing patterns of reticulate evolution in angiosperms: what can we do? – Taxon 54: 593–604.
Werlemark G., Uggla M. & Nybom H. (1999): Morphological and RAPD markers show a highly skewed distribution in a pair of reciprocal crosses between hemisexual dogrose species, *Rosa* sect. *Caninae*. – Theor. Appl. Genet. 98: 557–563.
Zhou R., Shi S. & Wu Ch-I. (2005): Molecular criteria for determining new hybrid species – an application to the *Sonneratia* hybrids. – Molec. Phylogenet. Evol. 35: 595–601.

Received 17 December 2006
Revision received 7 March 2007
Accepted 11 March 2007

Appendix 1. – Origin of the plant material analysed in this study. First seven localities represent pure populations of *Cardamine enneaphyllos* or *C. glanduligera*, otherwise the sampling focused on localities where the parental species co-occurred along with putative hybrid individuals (arranged geographically from the west to the east). The localities are given as follows: population no., phytogeographical region according to Futák (1984) (all localities are in Slovakia), the name of the closest village, specification of the locality, altitude, geographic coordinates (WGS-84), collection date, the name of the collector (JK = J. Kochjarová), additional notes in brackets. For morphometric analyses material from all listed populations was used. Superscripts indicate the populations that were used for pollen fertility analyses ([p]) and molecular PCR-RFLP analyses ([m]).

1 Veľká Fatra Mts, Blatnica, Gaderská dolina valley, Dedošova, ca 730 m a.s.l., 48°55'50" N, 19°01'50" E, 8 V 1996, coll. JK ("pure" *C. enneaphyllos*)

[mp]**2** Veľká Fatra Mts, Blatnica, Gaderská dolina valley, Škap, 750 m, 48°55'25" N, 19°02'12" E, 8 V 1996, 6 V 2005, coll. JK ("pure" *C. enneaphyllos*)

[mp]**3** Muránska planina plain, Veľká Stožka, 1237 m, 48°47'01" N, 19°59'38" E, 10 VI 1999, 13 V 2005, coll. JK ("pure" *C. enneaphyllos*)

4 Veľká Fatra Mts, Nolčovo, Veľká dolina valley, foot of Ostrý hill, ca 500 m, 49°05'30" N, 19°05'20" E, 20 IV 1999, coll. JK ("pure" *C. glanduligera*)

5 Slovenské rudohorie Mts, Muránska Lehota, alluvium bed of Lehotský potok stream, 395 m, 48°43'30" N, 20°01'30" E, 1 V 1999, coll. JK & D. Blanár, ("pure" *C. glanduligera*)

6 Muránska planina plain, Červená Skala, dolina Župkov valley, 840 m, 48°48'30" N, 20°08'30" E, 14 VI 1996, coll. JK ("pure" *C. glanduligera*)

[mp]**7** Belianske Tatry Mts, Ždiar, Monkova dolina valley, ca 950 m, 49°15'52" N, 20°14'16" E, 31 V 2005, coll. JK ("pure" *C. glanduligera*)

8 Veľká Fatra Mts, Krpeľany, Kopa – elevation point Sokol, N foot above Krpelianska priehrada dam, ca 440 m, 49°08'40" N, 19°06'20" E, 28 IV 1999, coll. JK (occurrence of all three taxa, but only *C. enneaphyllos* was analysed)

[mp]**9** Veľká Fatra Mts, Nolčovo, Veľká dolina valley, alluvium bed near "Pod Brdcom", 773 m, 49°03'59" N, 19°06'53" E, 20 IV 1999, 27 IV 1999, 6. 5. 2005, coll. JK

[p]**10** Veľká Fatra Mts, Belá, Belianska dolina valley, side gorge Horný Borišov, ca 900 m, 48°57'54" N, 19°04'57" E, 14 V 1999, 6 V 2005, coll. JK (only *C. enneaphyllos* and *C.* ×*paxiana*)

11 Veľká Fatra Mts, Blatnica, Gaderská dolina valley, upper part of side valley Lubená, ca 1260 m, 48°56'08" N, 18°58'40" E, 10 V 1999, coll. JK (only *C. enneaphyllos* and *C.* ×*paxiana*)

12 Lúčanská Malá Fatra Mts, Vrútky, alluvium bed of Chrapový potok stream, ca 680 m, 49°07'40" N, 18°52'30" E, 27 IV 1999, coll. JK

13 Krivánska Malá Fatra Mts, Šútovo, Šútovská dolina valley, bottom of the valley, ca 700 m, 49°10'40" N, 19°05'00" E, 16 VI 1999, coll. JK (occurrence of all three taxa, but only *C. enneaphyllos* and *C. glanduligera* were analysed)

[mp]**14** Krivánska Malá Fatra Mts, Šútovo, Šútovská dolina valley, alluvium bed of the stream**,** 733 m, 49°11'05" N, 19°05'05" E, 21 IV 1999, 6 V 2005, coll. JK

[p]**15** Krivánska Malá Fatra Mts, Šútovo, Šútovská dolina valley, 738 m, 49°10'55" 19°05'08", 21 IV 1999, 6. 5. 2005, coll. JK (occurrence of all three taxa, but only *C.* ×*paxiana* was analysed)

16 Krivánska Malá Fatra Mts, Šútovo, Šútovská dolina valley, side valley Tesnô, ca 860 m, 49°11'25" N, 19°05'00" E, 5 V 1999, coll. JK

[mp]**17** Chočské vrchy Mts, Stankovany, Šíp (ellevation point 1170 m), N slopes below the peak, 1033 m, 49°09'58" N, 19°11'08" E, 19 V 1999, 20 V 2005, coll. JK

[mp]**18** Nízke Tatry Mts, Liptovský Ján, Bystrá valley, above the gamekeeper house Pred Bystrou, 918 m, 48° 58' 17" N, 19° 40' 40" E,11 V 1999, 10 V 2005, coll. JK

[mp]**19** Nízke Tatry Mts, Demänová, Demänovská dolina valley, dolina Vyvieranie valley above the cave jaskyňa Mieru, 765 m, 49°00'14" N, 19°34'56" E, 11 V 1999, 10 V 2005, coll. JK

[mp]**20** Západné Tatry Mts, Osobitá, Kremenná – above the saddle Bôrik, below the rocks Radové skaly, 1023 m, 49°16'34" N, 19°42'32" E, 6 V 1999, 10 V 2005, coll. JK

[mp]**21** Muránska planina plain, Tisovec, Čertova dolina valley, 721 m, 48°44'20" N, 19°51'18" E, 1 V 1999, coll. JK & D. Blanár, 13 V 2005, coll. JK

22 Slovenské rudohorie Mts, Tisovec, saddle Dielik, upwards to Čierťaž hill, ca 680 m, 48°42' N, 20°00' E, 1 V 1999, coll. JK & D. Blanár (occurrence of all three taxa, but only *C. enneaphyllos* was analysed)

[mp]**23** Muránska planina plain, Muránska Huta, Bobačka, 771 m, 48°46'48" N, 20°06'20" E, 30 IV 1999, 4 VI 1999, coll. JK & D. Blanár, 12 V 2005, coll. JK

24 Muránska planina plain, Muránska Huta, saddle Javorinka, at the crossroad towards Veľká lúka, ca 920 m, 48°47'30" N, 20°07'40" E, 30 IV 1999, coll. JK & D. Blanár

mp**25** Muránska planina, Červená Skala, above the railway station, 790 m, 48°49'12" N, 20°07'54" E, 4 VI 1999, coll. JK & D. Blanár , 12 V 2005, coll. JK

26 Slovenské rudohorie Mts, Rybník, údolie Drienku valley**,** ca 290 m, 48°31'30" N, 20°06'00" E, 14 IV 1999, coll. JK & J. Kliment

27 Slovenské rudohorie Mts, Španie Pole, Pod skalou, nearby the cave Špaňopolská jaskyňa, ca 300 m, 48°31'40" N, 20°07'10" E, 14 IV 1999, coll. JK & J. Kliment

Appendix 2. – List of morphological characters examined. Characters marked with asterisks were excluded from multivariate morphometric computations due to very high correlations (Spearman rank coefficient > 0.95) with other characters.

Vegetative characters

height of stem (cm)
number of stem leaves
length of the first rhizome internode (closest to the stem base) (cm)
length of stem leaf petiole (mm)
*length of stem leaf lamina (mm)
length of middle leaflet of stem leaf (mm)
width of middle leaflet of stem leaf, measured at maximum width including teeth (mm)
glands on leaves (0 – glands absent, 1 – scarce occurrence of glands at either base of leaflets or on leaf margin, 2 – glands present both at base of leaflets and between teeth on leaf margin)
ratio length/width of middle leaflet of stem leaf

Floral characters

number of flowers
length of sepals (mm)
width of sepals (mm)
length of petals (mm)
width of petals (mm)
*length of shorter stamen (mm)
length of longer stamen (mm)
ratio length/width of sepals
ratio length/width of petals

Fruit characters

number of potential siliquae (equal to the number of flowers above)
number of developed siliquae
length of siliqua (mm)
width of siliqua measured at the maximum width (mm)
number of seeds per siliqua
length of siliqua beak (mm)
*width of mature seed (mm)
length of mature seed (mm)
ratio length/width of siliqua
ratio length of siliqua/length of siliqua beak