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Phylogenetic and Diversity Patterns in *Cardamine* (Brassicaceae) – A Genus with Conspicuous Polyploid and Reticulate Evolution

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ABSTRACT

The genus *Cardamine* is one of the largest genera of the family Brassicaceae, comprising at least 200 species distributed worldwide. In the past years, numerous studies focusing on various aspects of its genetic and genomic diversity have been published. The objective of the present review is to summarize the most relevant investigations addressing: (1) cytogenetic diversity within the genus, (2) origin and evolution of particular polyploid species and complexes, (3) hybridization and introgression among species, (4) phylogeny reconstruction and biogeography, and (5) population genetics and phenotypic plasticity. Polyploidization and reticulation are among the most significant speciation processes in the genus. Several examples of detailed studies on the origin and biogeographic history of polyploids are presented and discussed. Molecular evidences on interspecific hybridization and hybrid speciation are summarized, pointing to the role of habitat disturbance and human activities for the hybrid establishment. Hypotheses on interspecific relationships are reviewed in the light of recent molecular phylogenetic studies.

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Finally, a few population genetic studies published provide insights into diversity patterns within and among populations. Research questions and topics neglected in previous studies have been examined, and some new techniques and approaches have been referred to, which may in future significantly enhance our knowledge on *Cardamine*.

Key Words: Cruciferae, molecular markers, polyploidy, phylogeny reconstruction, reticulate evolution

INTRODUCTION

The genus *Cardamine* L., along with *Draba* L., *Lepidium* L. and *Alyssum* L. is one of the largest genera of the family Brassicaceae, distributed worldwide on all continents except Antarctica [3]. Several classification systems proposed for the family Brassicaceae have consistently placed it within the tribe Arabideae DC. [39, 45, 119]. Recent molecular studies, however, showed that the tribe is not monophyletic, as seen in the case of many tribes of Brassicaceae, delimited solely on morphology [55]. Monophyly of the genus *Cardamine* (including *Dentaria* L., mentioned below) has been proved in several studies, also showing its close position to the genera *Nasturtium* W.T. Aiton, *Barbarea* R.Br., *Armoracia* P. Gaertn., B. Mey. & Scherb., and *Rorippa* Scop. [55, 59, 93, 126]. According to two most recent estimates, the number of species within *Cardamine* are reported around 160 [51] to at least 200 [3]. The number of species recognized depends on the taxonomic treatment of several complicated groups, which can vary among the authors. In turn, the taxonomic treatment is considerably affected by the current knowledge of morphological and genetic variation patterns in the genus in various parts of its distribution area. This is illustrated by recent taxonomic revisions in Europe, particularly in the European Mediterranean. In 1993, the account of the genus in Flora Europaea [48] listed 31 species for Europe. Since that time, detailed morphological, molecular and karyological investigation led to the recognition of a few new species [23, 74, 75, 91, 92] or their re-discovery [87]. Furthermore, the subspecific rank of some taxa was shown to contradict their evolutionary history and their species-level treatment has been favoured [74, 73, 104, J. Kučéra, unpubl.]. As a result, 54 well-defined species can be recognized today in Europe (authors' compilation). Another example indicating much higher species diversity than assumed in the past is from New Zealand. Only five native and three naturalized species are listed in the *Cardamine* account of the most

recent flora of New Zealand [140]. However, the actual number of recognizable, but so far undescribed entities, is about 20-25 [93, 111, 140].

The cosmopolitan distribution of the genus, presence of several endemics on each continent, as well as only scarce data on genetic diversity and phylogenetic relationships within the genus on worldwide scale do not allow to identify the centre(s) of origin with confidence. Nevertheless, major centres of diversity, inferred from the total number of species and abundance of local endemics, appear to be in the Far East and the Himalayas with about 70 representatives [3]. Besides this, important is the area including the European Mediterranean and Caucasus with around 49 species (authors' compilation), as well as North and Central America with about 50 species [115]. Although there are several widespread taxa across Europe and/or Asia (e.g. *C. flexuosa* With., *C. hirsuta* L, *C. impatiens* L, *C. amara* L, *C. macrophylla* Willd. [43, 44]) and North America (e.g. *C. bulbosa* (Schreb. ex Muhl.) Britton, Sterns & Poggenb., *C. concatenata* (Michx.) O. Schwarz, *C. diphylla* (Michx.) Alph. Wood, *C. pensylvanica* Muhl. ex Willd. [115]), only a few are actually cosmopolitan, growing indigenously in both Eurasia and America (e.g. *C. pratensis* sensu lato, *C. bellidifolia* L., *C. parviflora* L.). Some species have been reported to spread beyond their indigenous distribution area, becoming naturalized or often occurring as noxious garden and greenhouse weeds (e.g. *C. hirsuta* in North America, Eastern Asia, Australia and New Zealand, *C. flexuosa* subsp. *debilis* O.E. Schulz in North America, *C. corymbosa* Hook.f. in Great Britain and Belgium [18, 42, 63, 115, 140, 142], J. Lihová, unpubl.).

Annuals, winter annuals, biennials as well as perennials, are represented in *Cardamine*, but the majority of taxa are perennials with thickened or tuberous rhizomes [3]. The species grow in diverse, mostly mesic habitats, from lowlands up to the alpine belt, those from the southern hemisphere and equatorial regions are mostly shifted to high montane and alpine habitats. Perennial *Cardamine* species show several modes of vegetative propagation. Examples are as follows: via branching and fragmentation of rhizomes (*C. concatenata* group [94, 120], *C. crassifolia* Pourr. [78], *C. resedifolia* L., pers. observ.), via stolons (*C. amara* [79]), adventitious shoots on the surface of leaf blades and in the leaf axils (*C. pratensis* and *C. dentata* Schult. [78, 116], *C. corymbosa* [18]), bulbils (*C. bulbifera* (L.) Crantz [120]) or

long leafy shoots in the axils of cauline leaves (*C. yezoensis* Maxim., *C. schinziana* O.E. Schulz; Marhold and Lihová, pers. observ.). Few detailed studies on mating systems are available presently [9, 54, 78, 131, 136, 139], but they already demonstrate the whole range from self-incompatibility with high outcrossing rate (e.g. *C. amara* [131], *C. pratensis* [9, 78], *C. lyrata* Bunge [54], *C. californica* (Nutt.) Greene [109]) to autogamy (e.g. *C. hirsuta* [54], *C. oligosperma* Nutt. [109]), including some cases of cleistogamy (*C. corymbosa* [18], *C. chenopodifolia* Pers. [24, 130]). Population genetic studies addressing life history strategies and impact of different mating systems on the patterns of genetic diversity have been published in several Brassicaceae species [117], but are very scarce in *Cardamine* so far (refer to the section on Population Genetic Studies and Phenotypic Plasticity in this chapter).

Morphologically, the genus is well-characterized by flat siliques with a marginate or narrowly winged replum and spirally coiling valves. Otherwise, large morphological diversity is present in the genus, particularly in leaf morphology. The only monograph covering the whole distribution area of *Cardamine* was published by O.E. Schulz [118], who also proposed a taxonomic concept of the genus with 13 sections, six of them monotypic. This sectional classification, based on the rhizome morphology, funiculus, placenta, and septum characters, cotyledon arrangements, and number of ovules per ovary, apparently does not reflect phylogenetic relationships. Monophyly of several sections has already been rejected in recent molecular studies (sections *Cardamine* L., *Papyrophylum* O.E. Schulz, *Dentaria* (L.) O.E. Schulz, *Cardaminella* Prantl [14, 36, 126]), but only a small fraction of the genus has been analyzed up to now to be able to propose a natural subdivision.

In the past, numerous studies focusing on various aspects of genetic and genomic diversity of the genus *Cardamine* have been published. The objective of the present review is to summarize the most relevant investigations addressing: (1) cytogenetic diversity within the genus, its sources and observed patterns, (2) origin and evolution of particular polyploid species and complexes, (3) hybridization and introgression as revealed by molecular markers, (4) phylogeny reconstruction and biogeography, and (5) population genetic structures and phenotypic plasticity. In this context we also examine research questions and topics that were neglected in previous studies, as well as available

techniques, tools and approaches so far not applied in the *Cardamine* research. The genus *Cardamine* in its worldwide diversity in any biological aspect undoubtedly offers an excellent study object for genetic and genomic studies.

Chromosome Number Diversity and Cytogenetic Studies

Chromosome number variation in the genus *Cardamine* is enormous due to occurrence of several cytogenetic phenomena as polyploidy, aneuploidy and dysploidy[1] up to the highest ploidy level known within the family Brassicaceae. Several authors have studied the chromosome numbers of *Cardamine*, since the very first counts in the 1920s-1930s [68, 83, 114, 120]. The mitotic chromosomes are very small (often less than 1 μm long) making any cytogenetic study extremely difficult. Detailed karyotype investigations have been performed only in the *C. pratensis* group [138, 139], which revealed meta- to submetacentric chromosomes of 0.7 to 2.1 μm. Five different base chromosome numbers (x = 6, 7, 8, 10, 12) have been reported [3]. However, the majority of chromosome numbers recorded are based on x = 8, and only two records for diploids with less than 16 chromosomes were published. The number 2n = 12 was published for the Caucasian species *C. seidlitziana* Albov [27] from the region of Svanetia in the Greater Caucasus. Our chromosome survey of Caucasian plants, although not from the same locality, revealed only 2n = 16 for this species so far [70]. Two records exist for north Italian *C. asarifolia*, 2n = 16 [83] and 2n = 14 [68], but apparently based on plants of unknown origin obtained from botanical gardens. Our recent studies (J. Lihová et al. unpubl.) definitely rejected the diploid status of the species. We counted exclusively 48 chromosomes from six populations across its distribution range, and the single ploidy level was also confirmed by flow cytometric ploidy screening of a large number of individuals. Thus, the numbers x = 6 and 7 can be most likely discarded as the base chromosome numbers at the diploid level in *Cardamine*. There is probably a different situation at higher ploidy

[1] Aneuploidy: loss or addition of one or more chromosomes of a euploid complement, usually caused by mitotic or meiotic irregularities; dysploidy: occurrence of a secondary base chromosome number, as a result of structural chromosomal rearrangements (for details see [112]).

levels. While only 2n = 16 is found at the diploid level in the *C. pratensis* group (apart from aneuploidy resulting in 2n = 17-21), two tetraploid chromosome numbers 2n = 30 and 2n = 32 have been repeatedly reported for this species group from different parts of Europe [71, 78, 84, 86]. Already in 1931, on the basis of chromosome morphology, chromosome fusion giving rise to the chromosome set of 7 chromosomes was suggested by Lawrence [68]. Two significantly longer chromosomes, most likely resulting from chromosome fusion, were observed in plants with 30 chromosomes, indicating the genomic composition of two 7- and two 8-chromosome sets. The same karyotype characteristics supporting the hypothesis of dysploidy were later shown also in cytogenetic studies of Urbanska-Worytkiewicz and Landolt [138]. This finding also seems to be supported by the chromosome number variation at higher ploidy levels within the *C. pratensis* group, where the numbers like the hypohexaploid 2n = 44 (along with usual hexaploid 2n = 48) can be explained by the combination of sets of 8 and 7 chromosomes [78]. Nevertheless, Lawrence's hypothesis has not yet been proved by more recent approaches to the genome study that would unequivocally corroborate the proposed chromosome fusion and explain the chromosome variation at higher ploidy levels. Especially the application of the state-of-the-art techniques as comparative genetic mapping [38], comparative chromosome painting and genomic in situ hybridization (GISH) would certainly be very helpful not only in addressing this issue, but also e.g. to explain the origin of the assumed higher base chromosome numbers x = 10 and 12 [3], and to trace the karyotype evolution in the genus. These techniques have been successfully applied in *Arabidopsis thaliana* (L.) Heynh. and its close or more distant relatives (e.g. *Cardaminopsis* (C.A. Mey.) Hayek, *Capsella* Medik., *Arabis* L. [16, 61, 67, 81, 82]), but not in *Cardamine*.

Aneuploidy has been observed in diploid representatives of the *Cardamine pratensis* group [84, 138]. Most likely it is common also in other members of the genus, and especially at higher ploidy levels. Recent flow cytometric measurements of nuclear DNA content in Eastern Asian polyploids *C. yezoensis* and *C. torrentis* Nakai revealed not only two different ploidy levels within both species, but also nuclear DNA content differences within a single ploidy level up to several percents (Fig. 1) that are most likely attributable to aneuploidy (K. Marhold et al. unpubl.).

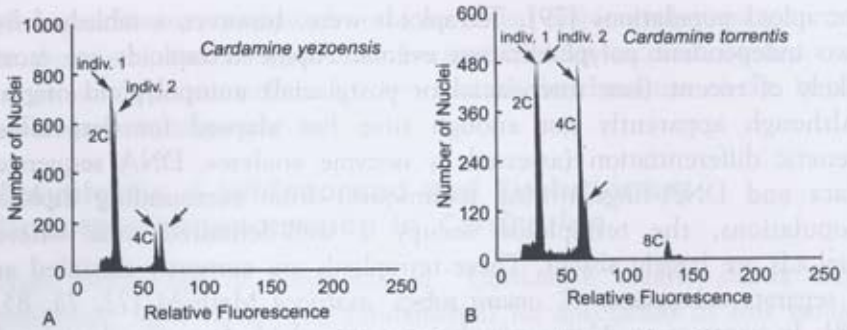

Fig. 1 Histograms of relative nuclear DNA content obtained from flow cytometric analyses of DAPI-stained nuclei. Two individuals originating from the same population were analyzed simultaneously; double peaks indicate differences in their nuclear DNA contents, attributable to aneuploidy. A. *Cardamine yezoensis*, loc. Japan, Hokkaido, Ishikari-gun, Tohbetsu-cho, Aoyamaokunibangawa; 6.8% difference. B. *Cardamine torrentis*, loc. Japan, Hokkaido, Kato-gun, Shikaoi-cho, Yamada-onsen; 6.2% difference between the two individuals. K. Marhold et al., unpubl.

About 58% of *Cardamine* species are exclusively polyploid (authors' compilation). Very high polyploid chromosome numbers (>2n = 100) have been reported in several species, mostly those previously assigned to the separate genus or subgenus *Dentaria* (e.g. ca. 208 in *Cardamine maxima* (Nutt.) Alph. Wood [94] and 2n = ca. 160 in *C. battagliae* Cesca & Peruzzi [23]). The highest chromosome numbers known in the family were reported in North American *C. diphylla* and *C. concatenata* (= *D. laciniata* Willd.), both up to 2n = 256 or n = 128, corresponding to 32-ploidy level [3, 30]. However, in the former species there is considerable intrapopulational and even intraindividual variation in the chromosome numbers reported, and thus, it is difficult to assess whether the high numbers really represent regular and stabilized chromosome sets of the species.

About 8% of *Cardamine* species include both diploid and polyploid populations (authors' compilation). In some cases, multiple cytotypes reported within a single species might point to unresolved taxonomy, i.e. detailed morphological and molecular investigation might bring recognizable patterns, reveal geographic or ecological correlations, and suggest that the different cytotypes represent in fact different taxonomic and evolutionary entities. This was recently shown in the Eurasian species *C. amara*, known to include both diploid and

tetraploid populations [79]. Tetraploids were, however, established by two independent polyploidization events. Alpine tetraploids are most likely of recent (late interglacial or postglacial) autopolyploid origin. Although apparently not enough time has elapsed for detectable genetic differentiation (assessed by isozyme analyses, DNA sequence data and DNA-fingeprinting techniques) from surrounding diploid populations, the tetraploids occupy a well-delimited area where diploids are largely absent. These tetraploids are currently classified as a separate subspecies, *C. amara* subsp. *austriaca* Marhold [72, 73, 85, 88]. In contrast to Alpine populations, tetraploids from Catalonia and central Italy surely originated much earlier, possibly in preglacial time. They show well-pronounced genetic differentiation from diploid *C. amara*, and are nowadays treated as a separate species *C. amporitana* [73]. Detailed studies in other species including several cytotypes may bring similar results. This can be expected in polymorphic species like *C. macrophylla* Willd. from Asia [52, 143] or South American-African *C. obliqua* Hochst. ex A. Rich. [40, 49]. On the other hand, many polyploid species harbouring two or more cytotypes probably represent dynamic systems, with gene flow across ploidy levels, finally resulting in fully viable and stable hybrid swarms (reported in *C. dentata* and *C. nymanii* Gand. [78]). Analyses of molecular variance based on AFLP (amplified fragment length polymorphism) data of *C. pratensis* s.str. in Europe (including eight different cytotypes) revealed patterns indicating that: (1) at least some of the cytotypes occurring in different parts of Europe arose independently, and (2) there is considerable gene flow across ploidy levels [74]. The latter finding is in accordance with early crossing experiments between plants of different higher ploidy levels, resulting in largely fertile progeny [78].

Along with chromosome number determination and karyotype studies, techniques allowing to measure DNA amount per nucleus (flow cytometry or microdensitometry) provide useful tools to study cytogenetic diversity [11, 29]. Among the diploids of *Cardamine*, only three species, *C. amara*, *C. hirsuta* and *C. impatiens*, were analyzed for genome size. Very small values were revealed, being among the smallest ones within angiosperms (1C = 0.21-0.24 pg [10, 46]). Examples from other genera show that considerable variation usually occurs within a single genus [20, 22, 110], and variation in the genome size can be thus expected also in *Cardamine*. Variation reflecting differences in the monoploid genome size mapped onto a robust

phylogenetic tree of diploid taxa can reveal major evolutionary trends in the genus and its particular lineages towards increase or decrease of genome size, as well as to demonstrate the dynamics of the large-scale genome organization.

Polyploidy: A Widespread and Evolutionary Important Phenomenon in *Cardamine*

High percentage of polyploids in *Cardamine* clearly shows that polyploidy must have been fundamental for speciation in this genus. First attempts to reveal the polyploid origin of a particular species and to identify its progenitors mostly relied on morphological and cytogenetic data (karyotype, chromosome behaviour at meiosis, degree of fertility, etc.), crossing experiments, artificially induced polyploidy, and ecogeography [69]. Despite the wide occurrence of polyploidy in *Cardamine*, only a few such early studies were performed on its representatives. Phenotype differences among diploids and artificially produced autotetraploids and autooctoploids were studied in *C. amara* by Lövkvist [79], but the observations were not related to the wild tetraploid populations. *Cardamine flexuosa*, a Eurasian widespread tetraploid, was first proposed to be an autopolyploid derivate of diploid *C. hirsuta* [8], based solely on morphological resemblance. Later, however, Ellis and Jones [31] produced autotetraploids of *C. hirsuta* as well as hybrids between *C. hirsuta* and *C. flexuosa*. Their study considered morphological features and data on geographical distribution from several potential diploids and suggested an allotetraploid origin of *C. flexuosa*, with diploids *C. hirsuta* and *C. impatiens* as progenitors. Furthermore, a comprehensive study of the *C. pratensis* polyploid complex was undertaken by Lövkvist [78]. Although several diploid populations were identified, no unequivocal diploid-polyploid affinities were found, so the origin and evolution of polyploids remained unresolved. It was suggested that polyploidization events are still going on within the complex, and chances of establishing a new polyploid population might have differed in time and space.

In many cases, identification of the polyploid origin can be hampered by the fact that the original diploid progenitors either have become substantially differentiated since the polyploid formation, or are now extinct. The latter is most probably the case of the North American *C. concatenata* group in which only high polyploids are currently known and diploid ancestors died out most probably a long

time ago. Several studies examining members of this complex have shown morphological intermediacy for some populations and taxa, meiotic irregularities, partial sterility due to the abortion of embryo-sacs in some populations, and efficient vegetative reproduction [94, 124, 127]. On the other hand, recent allopolyploidization events can be easy to reconstruct as the affinities to diploids and overall additivity are still retained. This was shown in Swiss autoallohexaploid *C. schulzii* Urbanska (2n = 6x = 48) that arose by autopolyploidization of a natural triploid hybrid (2n = 3x = 24) between two diploid species. The origin of *C. schulzii* was manifested first through its karyotype structure: the size differentiation between chromosomes of diploid species allowed to identify genomes derived from either parental species (referred to below). In addition, the hexaploid species grows in close vicinity of both diploids and their triploid hybrid [134].

With the advent of new cytogenetic and molecular techniques (isozymes, DNA-based molecular markers, genomic in situ hybridization), significant progress towards the study of polyploids, their origin and evolution has been made [69, 123]. It has been shown, and this holds true for *Cardamine* as well that many reticulation and polyploidization events have been associated with Pleistocene climatic oscillations, or that glaciation and deglaciation periods significantly shaped genetic variation patterns and current geographic distribution of several polyploids of pre-glacial origin.

Allopolyploid speciation has been documented for Calabrian (southern Italy) endemic *C. silana* Marhold & Perný [105]. Nuclear DNA sequences, AFLP markers and morphological data provided convincing evidence that two diploid species were involved in the origin of this hexaploid species (2n = 48): central Italian *C. apennina* Lihová & Marhold and Balkan *C. acris* Griseb. (Fig. 2). Sequences of internal transcribed spacer (ITS) of nuclear ribosomal DNA (nrDNA) obtained from *C. silana* indicate that this multi-copy DNA region, typically subjected to sequence homogenization, still retained (at least) two different sequence variants. Intraindividual polymorphisms detected in *C. silana* displayed additive patterns, strongly suggesting that one ITS sequence variant comes from *C. apennina* [70, 105], while the other is shared by several species. AFLP (amplified fragment length polymorphism) fingerprinting profile, representing variation distributed throughout the whole genome, on the other hand, revealed strong

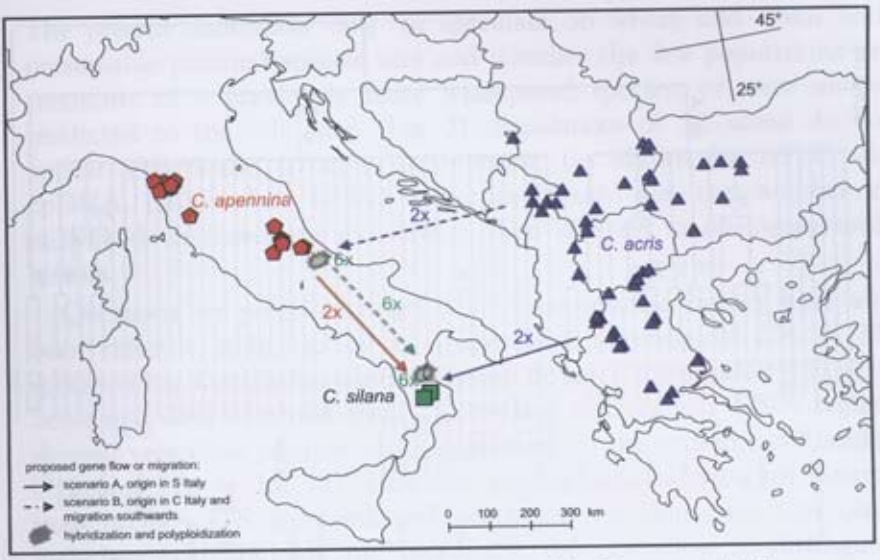

Fig. 2 Scenario of allopolyploid speciation in the European Mediterranean. *Cardamine silana*, a hexaploid currently restricted to the Sila Mts. in Calabria (southern Italy), is expected to have originated through hybridization of diploids from central Italy (*C. apennina*) and from the Balkans (*C. acris*) and subsequent polyploidization. Area contraction-expansion cycles during Quaternary climatic changes and lower Adriatic sea level probably allowed hybridization between the diploids (indicated by arrows), but neither the past distribution areas of the diploids nor the place of origin of *C. silana* are known. Distribution data taken from [75, 103].

affinity to the Balkan *C. acris* (Fig. 3). Phenotypically, *C. silana* shares similarities with both diploids. Cytogenetic analyses of the species triangle by genomic in situ hybridization (GISH) could eventually corroborate the origin of *C. silana*. Geographic separation of both assumed parental species from *C. silana*, especially that of Balkan *C. acris* being isolated not only by terrestrial disjunction, but also by the Channel of Otranto (Adriatic Sea), might be at first sight surprising. During the last glacial maximum, the sea level, however, was considerably lower [1, 37]. There is clear evidence for past migration and gene flow between the peninsulas seen in phylogeographic studies [33] as well as Italian-Balkan distribution patterns of numerous taxa [105]. In addition, Calabria is well-known as an important refugium during Pleistocene climatic changes, where populations of several species distributed in higher latitudes (including *C. apennina*) could have found favourable conditions during colder periods [33, 102, 128].

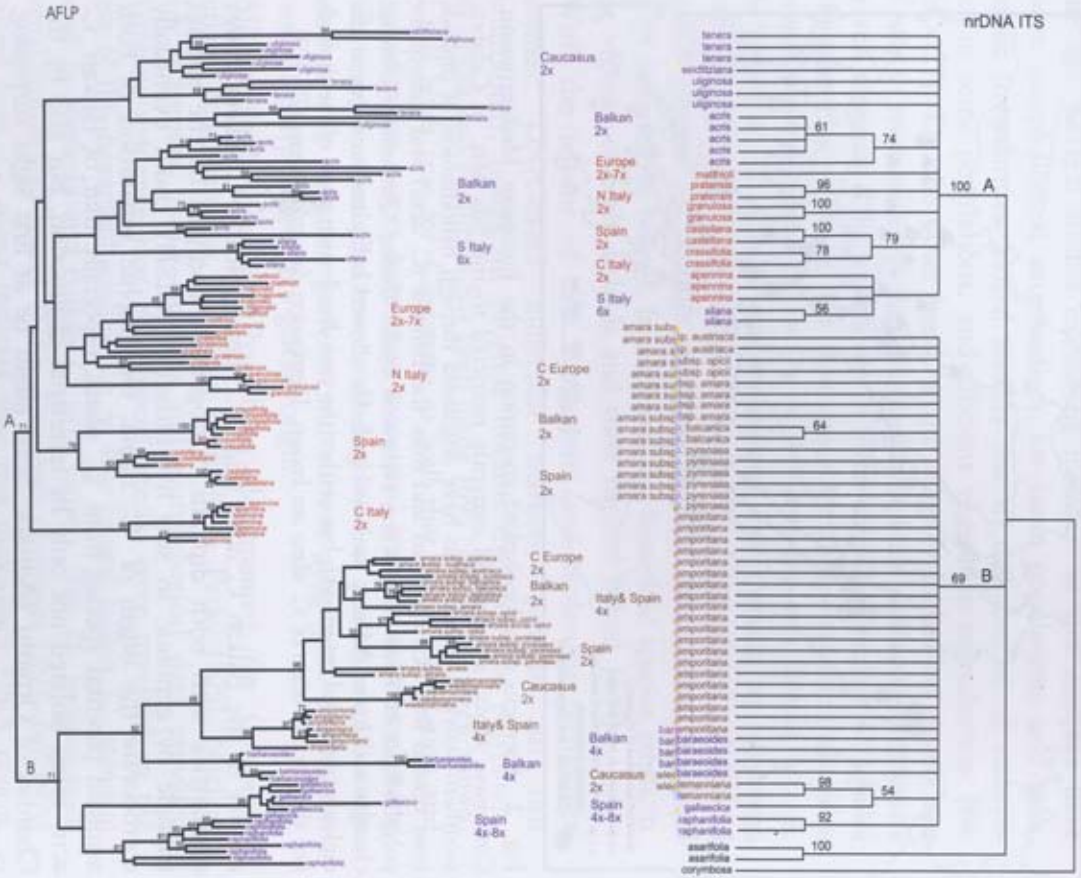

Fig. 3 Neighbour-joining tree of AFLP data (left) and strict-consensus tree of parsimony analysis of nrDNA ITS sequence data (right) including diploid and polyploid taxa from three traditionally recognized polyploid complexes of mainly European distribution. Assignment to the complexes is indicated with colours: blue - *Cardamine raphanifolia*, red - *C. pratensis*, brown - *C. amara*. Bootstrap values are shown above the branches; ploidy level and distribution area are indicated. The nrDNA ITS tree is based on data from [70]; the AFLP tree on re-analyses of separate data sets published in [73, 74, 104].

The present data allow only to speculate on where and when both presumable parental species met and whether the few populations are remnants of a previously more widespread species, or were always restricted to the Sila Mts. (Fig. 2). Populations of *C. silana* do not appear genetically strongly depauperate (as shown by AFLP and cpDNA [chloroplast DNA] sequence data), but the number of individuals analyzed was too low to shed light on its phylogeographic history.

Questions on polyploid origin and biogeographic history have also been recently addressed in *C. amporitana*, a tetraploid (2n = 32) species with Catalonian-central Italian disjunct distribution [70, 73]. Sequence data obtained from chloroplast and nuclear DNA regions showed very close position of *C. amporitana* to the widespread Eurasian *C. amara* (2n = 16, 32). However, phylogenetic information inferred from nrDNA ITS and *trnL-trnF* sequences for that particular clade including both diploids and polyploids, did not allow to distinguish between several morphologically well-distinct species. Much more resolution was provided by AFLP data (Fig. 3): *C. amporitana* was resolved as a well-supported distinct lineage characterized by numerous diagnostic AFLP fragments and clustered together with *C. amara* and the Caucasian diploid *C. wiedemanniana* Boiss. All available data suggest that the origin of *C. amporitana* is within the *C. amara* group (i.e. *C. amara* and *C. wiedemanniana*), with a possible involvement of tetraploid Balkan *C. barbaraeoides* or its putative diploid progenitor. Furthermore, both cpDNA sequence data and AFLP markers revealed low genetic variation in Catalonian populations, in comparison to the Italian ones. The genetic depauperation was prominent especially in AFLP data, as the Catalonian populations possessed a small subset of total variation present in this species. Two biogeographic hypotheses that may have developed the present pattern were discussed in the context of molecular data [70, 73]: (1) origin in Italy and later colonization of Catalonia either by long-distance dispersal or migration through the regions connecting both peninsulas, associated with the reduction of genetic diversity through founder effects; (2) fragmentation of original larger distribution area, disappearance of connecting populations and genetic impoverishment in the western colonization route, most probably due to Quaternary climatic oscillations.

Incongruent phylogenetic topologies based on nuclear and chloroplast DNA data offer an effective means to indicate past

allopolyploidization and to identify species involved in the hybridization. Similar patterns of discordance, however, may arise from population genetic processes (e.g. lineage sorting). Thus, additional data are needed to prove the hybrid speciation unequivocally [76]. Two individuals of north Italian hexaploid *C. asarifolia* (2n = 48) analyzed in our recent study showed strongly conflicting positions in the nrDNA ITS and cpDNA *trnL-trnF* phylogenies [70]. In the cpDNA haplotype network they were placed within the *C. amara* group, whereas their ITS sequences were strongly differentiated from any taxon included in the study and formed their own well-supported clade. As this species is known to co-occur with *C. amara*, either the scenario of a recent chloroplast capture or allopolyploid origin was suggested. The latter would imply ITS sequence homogenization towards a strongly divergent variant of another parent [70]. To explain the pattern detected, we increased the sampling to cover the whole species distribution range and employed a single-copy nuclear gene (chalcone synthase) not subjected to sequence homogenization (J. Lihová et al. unpubl.). Our preliminary results strongly support the scenario of allopolyploid origin of *C. asarifolia* with *C. amara* as one progenitor. The second parent could not be identified so far and may even represent an extinct taxon.

Recent studies from various angiosperm lineages indicate that autopolyploidy is much more common than traditionally considered and apparently has played an important role in plant evolution [69, 123]. A few cases with evidence or indication for autopolyploidy have been documented in *Cardamine*. Tetraploid populations of *C. amara* occupying the area of the Eastern Alps and adjacent regions (*C. amara* subsp. *austriaca*) have been studied using several molecular markers, including isozymes, nuclear and chloroplast DNA sequences, as well as RAPD (random amplified polymorphic DNA) and AFLP fingerprinting [72, 73, 85, 88]. The results show very low genetic differentiation between diploid *C. amara* subsp. *amara* and tetraploid subsp. *austriaca*. This, together with only slight morphological differentiation and clear geographical patterns, strongly favours autopolyploid origin. Several populations of *C. amara* across its distribution range (currently classified as five subspecies) were investigated for variation at eight isozyme loci, where a total of 23 alleles were observed and analyzed [88]. Tetraploid *C. amara* subsp. *austriaca* showed a very similar isozyme profile to that of diploid *C. amara* subsp. *amara* (widespread in

Europe). Out of 17 alleles found in each subsp. *amara* and subsp. *austriaca*, 15 were shared by both subspecies. The analysis of cpDNA sequence variation (based on *trn*L-*trn*F intergenic spacer) revealed several haplotypes present in subspecies of *C. amara* ([70] and J. Lihová, unpubl.). Two individuals of subsp. *austriaca* analyzed so far showed haplotypes (differing by a single mutational step) identical to those present in subsp. *amara*, and differed from haplotypes detected in Balkan and Pyrenean diploid taxa (*C. amara* subsp. *balcanica* Marhold, Ančev & Kit Tan, subsp. *pyrenaea* Sennen). RAPD and AFLP-derived banding patterns of subsp. *amara* and subsp. *austriaca* were very similar, with only a few private bands found in both subspecies. No fixed bands of the diploid, which would be absent from the tetraploid, and vice versa, were observed. Slightly more bands per individual and in total were resolved in tetraploid subsp. *austriaca*, a result that is expected for an autotetraploid derivate from an outcrossing diploid. Altogether molecular data are fully consistent with a hypothesis of recent autotetraploid origin of *C. amara* subsp. *austriaca*. As the distribution of subsp. *austriaca* coincides with the area heavily affected by Pleistocene glaciation, we assume that the autopolyploidization and establishment of this new tetraploid taxon took place in the last interglacial or postglacial period when new habitats became available after glacier retreat.

Another tetraploid taxon for which autopolyploid origin appears as most probable is *C. majovskii* Marhold & Záborský, a recently described species occurring in Central and southeastern Europe [92]. *C. majovskii* and its assumed progenitor, diploid *C. matthioli* Moretti, were included in the complex molecular systematic and biogeographic study of the *C. pratensis* group by Franzke and Hurka [34], and later by us using AFLP data [74]. All analyses involving isozymes, nuclear and cpDNA sequences, RAPD and AFLP markers showed very close relationship of both species. Although for some parts of the distribution area of *C. majovskii* details on its occurrence are not completely known, large sympatry with *C. matthioli* is already apparent. This fact, together with slight morphological differences observed among populations of *C. majovskii* from different parts of its distribution area ([71] and K. Marhold, unpubl.) might indicate its recurrent and independent origins. Thorough sampling and high-resolution molecular markers will be needed to corroborate this assumption in future studies.

Franzke and Hurka [34] in their study on the *C. pratensis* group addressed the question invoked early by Lövkvist [78]: the origin of widely distributed and karyologically highly variable polyploids of this group. From the results of several molecular markers they concluded that the polyploids evolved repeatedly from Central European diploids, and could be considered as autopolyploids. Indeed, all diploids from the so-called Derived Group [34] are treated as a single species *C. pratensis* s.str., so at least taxonomically the process can be described as autopolyploidization. Brochmann and Elven [19] had already pointed out the discrepancy between the taxonomic and genetic autopolyploidy on an example of cryptic species in the genus *Draba*. A similar situation may exist in diploid *Cardamine pratensis* s.str. Diploid populations from the Alps and adjacent areas have been reported to be ecologically and even cytogenetically (based on karyotype characteristics) mutually differentiated [139]. Molecular markers applied [34], however, did not yield enough resolution to find genetic divergence among those populations.

The above recent studies focusing on the origin and evolution of polyploids illustrates that the genus *Cardamine* harbours polyploids with very different evolutionary and biogeographic histories with an immense resource for future studies. As new molecular markers and techniques for genomic studies become available (e.g. chromosome painting based on fluorescent in situ hybridization [26, 82]), the spectrum of powerful tools to study the genomic composition of polyploids increases. One of the most efficient approaches to elucidate the composition of a polyploid genome (along with DNA sequences and other molecular markers) has been undoubtedly genomic in situ hybridization (GISH). In plants with small genome size, however, the utilization of this technique remained hitherto rather limited. Successful application of the modified GISH procedure to *Arabidopsis thaliana* and related species [2] appears to be promising also for studies in other plant species with small genomes.

Natural Interspecific Hybridizations: Molecular Evidences

Many examples of hybridization, introgression and hybrid speciation reported from the Brassicaceae (e.g. *Rorippa* [15], *Lepidium* [99], *Boechera* A. Löve & D. Löve [58]) indicate that this is a significant evolutionary phenomenon in the family. The occurrence of

interspecific hybrids between *Cardamine* representatives has been described since early studies [50, 80, 118]. Traditionally, the hybrids were determined by their morphology, and rather frequently a single morphological deviation was considered to be a sign of hybridization. Closer examination of such putative hybrids often led to the rejection of the hybrid status [78, 90]. Morphological intermediacy is indeed expected in natural hybrids, but extreme and novel characters can appear as well [113]. Morphological characters alone are of limited values and additional data are needed to prove the hybrid origin. Cytogenetic data, such as intermediate chromosome numbers, meiotic disturbances or male sterility, have often been used as further supporting evidences. Pollen sterility was reported already by Kerner [50] for *Cardamine* ×*keckii* A. Kern. (*C. amara* L. × *C. flexuosa* With.), or by Bongini [17] when discussing the putative hybrid origin of *C. ferrarii* Burnat (*C. amara* L. × *C. asarifolia* L.). Triploid chromosome numbers were presented as evidence for hybridization between the diploids *C. matthioli* Moretti and *C. pratensis* L. (*C.* ×*smejkalii* Tomšovic [129]). In addition, artificial synthesis of hybrids between *C. flexuosa* With. and *C. hirsuta* L. supported the occurrence of natural hybridization between these two species [12, 47]. More recently, several molecular tools have been applied to document recent hybridization events as well as diploid and polyploid hybrid speciation.

Interspecific hybridization reported between two *Cardamine* diploids, *C. pratensis* s. str. (reported mostly under the name *C. rivularis*) and *C. amara* (subsp. *amara*), in central Switzerland and successful establishment of triploid hybrids has become a model example of hybrid speciation associated with habitat disturbance caused by human activities [137]. Autopolyploidization of these hybrids (later described as *C.* × *insueta* Urbanska) led to the formation of a highly fertile and viable hexaploid population, named *C. schulzii* Urbanska [135]. Since the discovery of this hybrid complex, extensive biosystematic and molecular investigations have been performed to gain insights into its origin and ongoing microevolutionary processes. Initially, studies were focused on cytogenetic and reproduction aspects (chromosome numbers, karyotype, meiosis and chromosome segregation, pollen quality), as well as population biology (size and spatial structure of populations, population dynamics, flowering intensity), and the role of human interference [134, 135, 136, 137]. It was concluded that the triploid hybrid arose by fertilization of an unreduced *C. pratensis* gamete with

a reduced *C. amara* gamete, and has been only recently established and expanded into suitable man-made habitats. Although its reproduction is mainly vegetative and the plants are largely male-sterile, a limited sexual reproduction apparently plays a significant role. More recent molecular investigations included several markers, RFLP of cpDNA, isozymes, RAPD fingerprinting, and nrDNA ITS sequences [35, 100, 133]. CpDNA restriction site mutations allowed the differentiation between the chloroplast genomes of the progenitor species *C. pratensis* and *C. amara*, and showed that all individuals of *C.* × *insueta* and *C. schulzii* displayed patterns identical with *C. pratensis*, herewith providing the evidence for the maternal parent [133]. Both RAPD markers and isozymes revealed additivity in the triploid and hexaploid, supporting their hybrid origin. Distance analysis based on RAPD markers clearly showed the intermediate position of *C.* × *insueta*. As might be expected from the assumed genomic composition of *C.* × *insueta* and *C. schulzii*, the predominance of *C. pratensis* RAPD bands was observed in both. Predominantly vegetative reproduction, however, somewhat contradicts the high level of genetic diversity found in *C.* × *insueta*, being comparable to that in the outcrossing parental species. High genetic variation in the triploid can be most likely attributed to backcrossing and recurrent hybridizations between the parental species. The possibility of backcrossing has been indicated already in early studies, resulting from crossing experiments. In accordance with its assumed recent origin, only a few non-parental RAPD bands were detected in *C.* × *insueta*, indicating that the genome of *C.* × *insueta* has not evolved far from the parental ones [100]. Sequencing of the internal transcribed spacer region (ITS) of nrDNA revealed a rather pronounced sequence divergence between the diploids *C. pratensis* and *C. amara* (23 differing positions, representing ca. 5% sequence divergence). In recent hybrids, both parental nrDNA sequence types are expected to be found, although concerted evolution and population genetic processes can quickly eliminate one of the parental type or produce a recombinant one [4]. In *C.* × *insueta* and *C. schulzii* as many as 20 out of these 23 positions showed conversion to the nucleotide present in *C. pratensis*, and the remaining three sites displayed either the paternal (*C. amara*) nucleotide or presence of both parental nucleotides. Given the recent origin of hybrids (dated not earlier than 1900), this shows very rapid sequence homogenization with a strong bias towards the maternal sequence type [35].

As a counterpoint to the scarcity of polymorphic sites retained in ITS sequences of *C.* × *insueta*, 24 polymorphic nucleotide positions were found in an individual from a central Italian population of the tetraploid species *C. amporitana* [70]. Almost all of the positions (23) displayed an additive pattern in respect to the sequences of *C. amporitana* and the co-occurring diploid *C. apennina* (from the *C. pratensis* group), implying the presence of ITS copies from both species. The pattern detected can be unambiguously interpreted as a result of a very recent hybridization event (presumably F1 generation). The hybrid individual shared the chloroplast type with *C. amporitana* [70].

It seems that despite genetic differentiation between *Cardamine* species, incompatibility barriers can occasionally breakdown, and plants with similar ecological requirements and sympatric occurrence hybridize. Nevertheless, hybrid establishment and persistence implicate that either the hybrid is able to outcompete the parental species in the ancestral habitats, or that it spreads into new habitats [5, 6, 132]. The role of environmental disturbance for creating new niches available for hybrids is widely recognized, and apparently was also crucial for the origin of *C.* × *insueta* and *C. schulzii*. This is, however, most likely not a rare case in *Cardamine*. We can report on at least two other similar events from Spain, both on the polyploid and diploid levels. Inferring from our preliminary morphological and molecular (AFLP markers) investigation, extensive hybridization, backcrossing and introgression occur on several sites between high polyploids of *C. pratensis* s.str. and *C. raphanifolia* Pourr. s.str. in the Cordillera Cantabrica Mts. (NW Spain; J. Lihová et al. unpubl.). Parental species together with the scarce occurrence of intermingled hybrids or introgressants have been observed to grow along brooks on pastures and wet meadows providing relatively stable and less disturbed habitats, while dense populations consisting exclusively of hybrids are spreading in ditches along the road (Fig. 4). Natural hybridization at the diploid level was documented between two eastern Pyrenean endemics, *C. amara* subsp. *pyrenaea* and *C. crassifolia* [90], with very similar ecological preferences and roughly the same distribution area. Two hybrid populations (named as *C.* × *enriquei* Marhold, Lihová & Perný) were found, which formed small but rather dense populations, to a certain extent spatially separated from the parents, and growing on somewhat man-influenced sites. Morphology, pollen sterility, and AFLP markers proved the hybrid status. Similarly to *C.* × *insueta*, the hybrid populations of *C.* × *enriquei*

exhibited considerable morphological and genetic variation, suggesting a recurrent origin and/or backcrossing with parents. Again, vegetative propagation has most likely made the major contribution to the establishment of *C.* × *enriquei*.

All these examples illustrate recent and still ongoing interspecific hybridizations, where the hybrids have remained localized in the vicinity or sympatry with parental species and have not been observed to spread beyond their distribution range so far. Interspecific hybridization and subsequent recombination in hybrids can, however, generate novel genotypes prone to invasive behaviour and quickly colonizing new areas [32], as reported e.g. in the closely related genus *Rorippa* [13, 15]. We assume that interspecific hybridization in *Cardamine* took place frequently also in the past, and represented a major evolutionary force in the genus.

Reconstruction of the Genus Phylogeny, its Evolutionary and Biogeographic History

The worldwide distribution of the genus *Cardamine*, together with the high number of species recognized, invokes the question of the evolutionary history and phylogenetic relationships within the genus. Early phylogenetic hypotheses and infrageneric classifications were based on selected morphological traits, chromosome numbers and the analysis of distribution patterns [51, 52, 53, 118]. Molecular-based phylogenetic inferences, however, often contradict traditional phylogenetic and taxonomic concepts, as shown in many other Brassicaceae genera (e.g. *Thlaspi* L., *Lepidium*, *Cochlearia* L., *Draba*, [55]). Several molecular phylogenetic studies have been recently published addressing the family-level phylogeny, the traditional tribal classifications or focusing on particular lineages within Brassicaceae, which also included a few *Cardamine* representatives [41, 57, 59, 93]. Although more insights into the relationships within the genus *Cardamine* could not be obtained through those studies, they documented its monophyletic origin, in contrast to several other large Brassicaeae genera (e.g. *Arabidopsis* Heynh. *Arabis*, *Sisymbrium* L., [56]). *Nasturtium* was resolved as the most closely related genus, and an early radiation within *Cardamine* shortly after its separation from *Nasturtium* was hypothesized [126].

In the study of New Zealand endemic Brassicaceae representatives based on nrDNA ITS sequences, the monotypic genus *Iti* Garn.-Jones &

P.N. Johnson was placed within the clade of the five *Cardamine* species analyzed [93]. The phylogenetic position of *Iti lacustris* Garn.-Jones & P.N. Johnson (2n = 48) was previously a matter of dispute, as it could not be related morphologically to any genus. It was hypothesized that characters, which could indicate closer relationship to *Cardamine* or any other genus, have been lost due to the ecological adaptation. The lack of recoiling valves and explosive fruit dehiscence in *Iti lacustris*, which have been accepted as a morphological synapomorphy for all *Cardamine* species, might indeed question such placement, suggested from the analysis of a single nuclear region.

Molecular phylogenetic studies within the genus *Cardamine* are presently very scarce. The studies published in recent years addressed different questions and were based on different taxon sampling [14, 36, 70, 89, 126]. A few sections out of 13 recognized by Schulz [118] were covered by those studies, indicating that the infrageneric classification proposed by him is largely artificial. The need for the whole-genus phylogenetic hypothesis based on molecular markers is apparent. Reconstruction of evolutionary history in a genus like *Cardamine*, strongly affected by reticulate evolution and polyploidization, definitely will not be an easy task. Forcing reticulate evolutionary events to be displayed in the branching topology of a phylogenetic tree might lead to biased patterns, lack of support for the resolved clades, and collapse of hierarchical structure. To decrease the risk of producing false phylogenies in such cases, one could suggest to remove all known polyploids from the phylogenetic analyses, e.g. strongly advocated by Bachmann [7]. We followed this approach in the study on perennial species from the European Mediterranean and Caucasus, traditionally treated within three different polyploid complexes by including only diploids in the analyses [89]. The lack of supported hierarchical structure, together with the evidence for ancient hybridization and introgression among Caucasian diploids, strongly suggested extensive past reticulate evolution even at the diploid level (referred to below).

When inferring phylogenetic relationships, detailed knowledge on the species analyzed (morphology, ploidy level, distribution area, etc.) is also inevitable, as unresolved taxonomy or misinterpretation of some taxa might lead to wrong conclusions. Populations assigned to a single species might, under detailed analysis, turn out to represent distinct lineages. This seems to be the case of Eastern Asian populations assigned to *C. flexuosa*, Chinese populations treated as *C. hirsuta* (J.

Lihová et al. unpubl.), or the southern hemisphere species *C. africana* L. (referred to below). The genus includes several taxonomically critical aggregates, which might include populations and taxa only distantly related, as in *C. raphanifolia* in the European Mediterranean. This widely conceived species or species group consisting of several diploids and polyploids is apparently of polyphyletic origin and comprises lineages with different evolutionary history ([70], K. Marhold et al. unpubl. data, Fig. 3). And finally, adequate sampling is also important, as stressed by Sweeney and Price [126] when discussing the sister position of morphologically dissimilar *C. clematitis* Shuttlew. and *C. bellidifolia* L. It is apparent that their close position, as resolved in the tree [126], was due to the absence of close relatives of both species in the phylogenetic analysis, rather than reflecting their immediate sister relationship.

The first molecular phylogenetic study of the genus was published by Franzke et al. [36], who included 19 *Cardamine* species originating from both northern and southern hemispheres, apparently representing different evolutionary lineages of the genus. The study, based on sequence data from the nuclear rDNA transcribed spacer region (ITS) and non-coding cpDNA, provided the first insights into the phylogenetic relationships within the genus, affinities between northern and southern hemisphere species, and identified several clades of related taxa. Relationships among the obtained groups, however, were only partially resolved, as the inferred phylogenetic trees displayed large basal polytomies among the clades.

About 20 North American and Eurasian species have been recognized within *Dentaria*, a group of high polyploid woodland perennials with pronounced vegetative reproduction. Its systematic position has long been controversial. Several authors, both in Europe and North America, treated *Cardamine* and *Dentaria* as two distinct genera [21, 28, 122, 124], while in other treatments, e.g. in Flora Europaea [48], *Dentaria* was recognized as a subgenus within *Cardamine*. Originally, separate taxonomic treatment of *Dentaria* at the genus level was claimed by Linnaeus [77]. In the taxonomic concept of Schulz [118, 119], a separate section *Dentaria* (L.) O.E. Schulz was recognized, but some species originally described or combined under the genus *Dentaria* were placed into three other sections (sect. *Eutreptophyllum* O.E. Schulz, sect. *Sphaerotorrhiza* O.E. Schulz, and sect. *Macrophyllum* O.E. Schulz), implying that the delimitation of

Dentaria species was not fully consistent in earlier literature. More recently some authors of local floras in Europe [62, 122, 141] favoured the separate status of the genus *Dentaria* on the basis of its different rhizome morphology, leaf type, lower number of cauline leaves, and larger flowers. On the other hand, Al-Shehbaz [3] and Rollins [115] did not recognize *Dentaria* at any taxonomic level and included all species within *Cardamine*. In the study of Sweeney and Price [126] chloroplast sequence data (*trn*L intron and *ndh*F gene) were used to the examine phylogenetic position of *Dentaria* and *Cardamine*, and to elucidate relationships among *Dentaria* species from three main disjunct biogeographic groups (eastern North American, western North American, and Eurasian). Although only nine *Dentaria* species were included, the results clearly showed that the group is polyphyletic with at least three separate origins corresponding to the three main biogeographic groups. It was suggested that morphological characters, often used to delimit *Dentaria* as a separate taxon, are probably the subject of convergent evolution, as also found in several other Brassicaceae genera (e.g. *Thlaspi* [98], *Lepidium* [97]). Six eastern North American dentarias were resolved as a strongly supported monophyletic group, but clearly distinct from the western North American species *C. californica* (Nutt.) Greene (= *Dentaria californica* Nutt.). Only two out of eight recognized European species of *Dentaria* were analyzed (*C. pentaphyllos* (L.) Crantz and *C. waldsteinii* Dyer), which formed a well-supported clade. Further studies are needed to address the question whether all European representatives of *Dentaria* have a single origin and could be recognized as a single lineage. The results of the above-mentioned study by Franzke et al. [36], however, do not support their monophyly, as two European *Dentaria* species included, *C. bulbifera* (L.) Crantz (= *Dentaria bulbifera* L.) and *C. pentaphyllos* (L.) Crantz (= *D. pentaphyllos* L.), were placed within two different *Cardamine* clades. Still, the sister position of *C. bulbifera* to the morphologically extremely different biennial diploid *C. impatiens* found in that study is intriguing. Although the authors suggested the scenario of allopolyploid origin of dodecaploid *C. bulbifera* to explain the puzzling pattern, occasional hybridization between these two species occupying the same habitat cannot be ruled out. Broader sampling from larger areas and additional markers will be useful to prove either of the hypotheses.

Bleeker et al. [14] focused on mountain and alpine *Cardamine* species from the southern hemisphere to infer their biogeographic history and phylogenetic relationships, as well as affinities to northern hemisphere species. One of the central questions was the origin of Australian high-mountain *Cardamine* species, as the Australian alpine flora is known to be of recent origin. The investigations were based on sequence data from the previously studied DNA regions, ITS nrDNA and non-coding *trn*L and *trn*L-*trn*F regions. Close position and low sequence divergence were found between the South American *C. glacialis* (Forster) DC. and Australian and New Zealand species, supporting transoceanic dispersal from South America to Australia and New Zealand, or vice versa. Assuming from chloroplast sequence data, *C. obliqua* from East Africa showed a close position to European species, being in favour of early assumptions of their relatedness based on morphological data. Three accessions identified as *C. africana* originating from South America, New Guinea and Africa were surprisingly resolved in different positions in the phylogenetic trees and a polyphyletic origin of this diploid species was suggested. While in the nuclear ITS phylogenetic tree, South American and African accessions of this species grouped together, being distinct from the New Guinean accession, in the cpDNA tree each of the three accessions had a different position. However, *C. africana* appears to be a taxonomically critical taxon with uncertain circumscription. Therefore, an alternative explanation to the polyphyletic origin might be that the accession from New Guinea represents another distinct species from the accessions from South America and Africa. Furthermore, when studying herbarium material of African *Cardamine* species at the herbarium in Kew, U.K. (K), we found specimens morphologically intermediate between *C. africana* and another African species *C. trichocarpa* Hochst., tempting us to assume hybridization between these two species (K. Marhold, unpubl.). Such contrasting nuclear and cpDNA placement of African accession of *C. africana* could be explained by introgression from another *Cardamine* species.

Most recently, we aimed to elucidate phylogenetic relationships among representatives of three polyploid complexes of mainly European distribution (*C. amara*, *C. raphanifolia* and *C. pratensis* groups), previously proved to be closely related in the study of Franzke et al. [36]. Each of the groups has always been, at least implicitly, considered a coherent, monophyletic group. Two independent molecular data sets

were used, ITS sequences of nrDNA and AFLP markers, but as mentioned above, only diploid taxa were included [89]. Phylogenetic inferences from both data sets were largely congruent, and revealed two main lineages. While the *C. amara* group (consisting of four subspecies of *C. amara* and one separate species) was resolved as a well-defined monophyletic group, neither of the markers supported current taxonomic separation of remaining diploids into two groups (*C. pratensis* and *C. raphanifolia*). Instead, all these taxa formed a single clade with poorly resolved relationships. It was hypothesized that either hybridization and introgression between the taxa obscured genetic differentiation amid the two groups, or that traditional taxonomic treatment is incorrect and the taxa form a single monophyletic group with a common ancestor. When adding polyploid representatives of these groups (Fig. 3) to both data sets, the resolved pattern was largely retained. The distinction between the two main lineages remained, but interestingly, polyploids from the *C. raphanifolia* group were split between the two clades. Iberian and Balkan polyploids showed high affinity to the *C. amara* representatives, a pattern that would indicate their progenitor(s) in the *C. amara* group, whereas south Italian polyploid *C. silana* (as discussed in more detail above) apparently originated within the sister group (Fig. 3). The traditionally recognized polyploid complexes evidently do not represent isolated lineages with their own evolutionary history, as putative parents of at least some polyploid taxa are likely to be found in more than one of these groups. Although two very different markers were used (ITS sequences known to undergo concerted evolution, and AFLP markers representing polymorphisms across the major part of the genome), results of cladistic and distance-based analyses of respective data sets were mostly congruent and revealed a similar pattern.

Further approach to the study of the evolutionary and biogeographic history of the genus would be to focus on (supposedly) related lineages distributed worldwide, to address speciation events in the genus, and to identify main colonization routes. Our preliminary results from the investigation of a group of taxa taxonomically often associated with tetraploid *C. flexuosa* and diploid *C. hirsuta* and distributed on all continents, show interesting patterns. The data suggest very different genetic diversity distribution among the analyzed species, showing e.g. much higher diversity in local Eastern Asian endemics than in several much more widespread species, implying rapid colonization across large

areas and/or bottleneck effect in the latter. Expectedly, Eastern Asian representatives seem to show more genetic affinity to North American species than to the European ones. Examples on human-mediated recent introduction to new areas and even continents can be demonstrated (J. Lihová et al. unpubl.).

This survey of molecular phylogenetic studies published in the genus *Cardamine* so far shows the spectrum of employed markers to be very narrow. Only a few cpDNA regions have been utilized (*trn*L, *trn*L-*trn*F, *ndh*F) until now, and as yet published nuclear DNA-based sequence analyses relied entirely on nrDNA ITS region. On one hand, it allows direct comparison and enlargement of data sets with previously published sequences available in GenBank; on the other hand, inferred phylogenies might reflect evolution of a few loci only, rather than that of the organisms themselves. Several other cpDNA regions with available (semi-)universal primers can be employed nowadays [121], thus enabling a choice of uniparentally-inherited markers with the most adequate resolution for the addressed question. Considering drawbacks and limitations of the nrDNA ITS region [4], it is highly desirable that future phylogenetic inferences will include data also from other nuclear regions. Low- and single-copy nuclear genes, overcoming several limitations reported for nrDNA, appear to be the markers of choice for future studies [4, 95]. Initial results of our analyses of a single copy gene in the study of origin of *C. asarifolia* (J. Lihová et al. unpubl., referred to above) confirm this fact.

Population Genetic Studies and Phenotypic Plasticity

Isozyme analyses and DNA-fingerprinting techniques are among the most frequently used methods to assess the level of genetic variation in populations, to investigate spatial genetic structure, extent of intra- and interpopulational gene flow, impact of mating systems on diversity patterns, and other population genetic questions. Chloroplast or nuclear microsatellites have become popular and preferred mainly because of their high resolution (providing more polymorphisms than isozymes), reproducibility and codominance (in contrast to dominant RAPD and AFLP markers [25, 96, 125]).

Distribution of genetic variation was recently studied in populations of the outcrossing perennial *C. amara* in northwestern Germany, using isozyme analyses [60]. Allelic variation of six isozyme systems has been

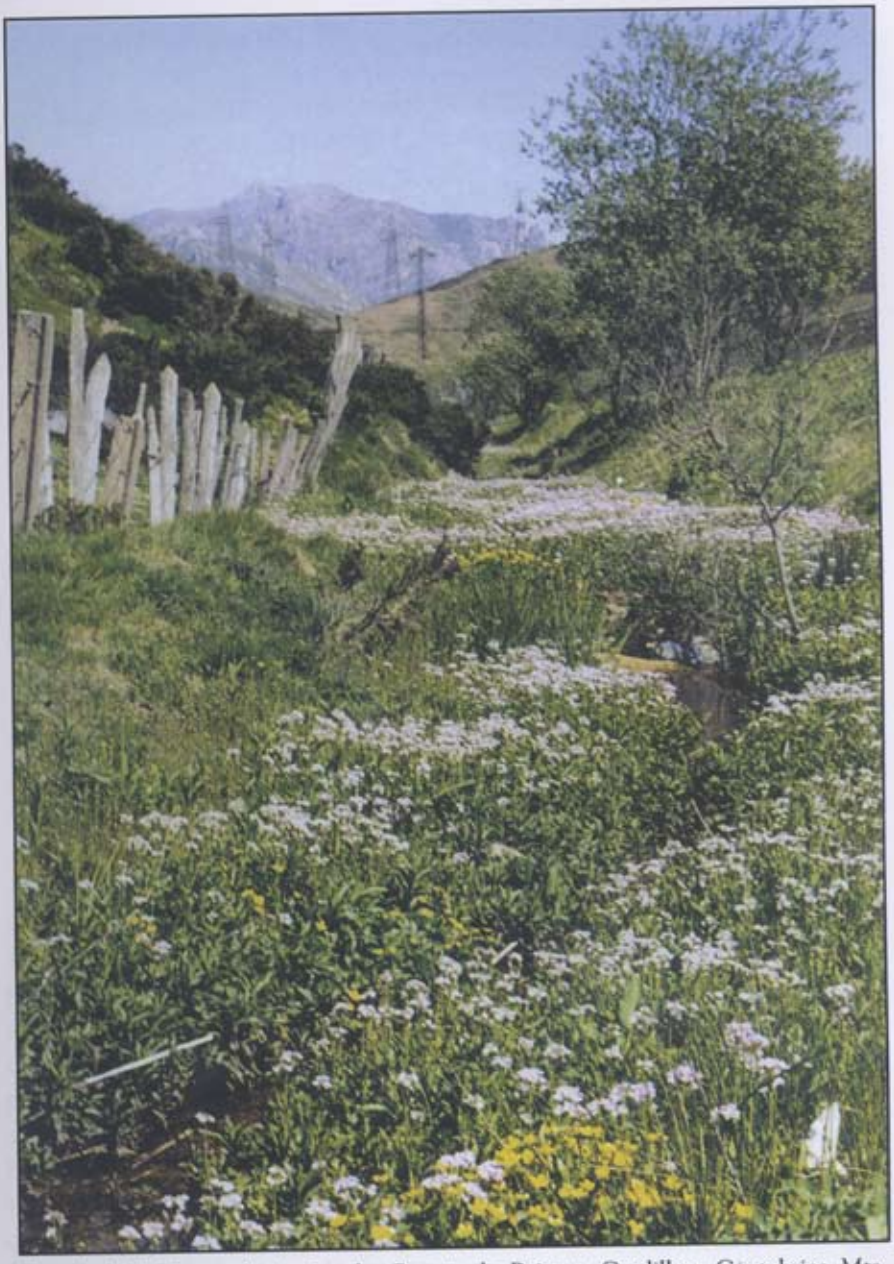

Fig. 4 Hybrid population in the Puerto de Pajares, Cordillera Cantabrica Mts. (NW Spain). The plants apparently originated through hybridization between polyploids *Cardamine pratensis* and *C. raphanifolia* growing in vicinity, and invaded disturbed habitats. Photo by J. Lihová.

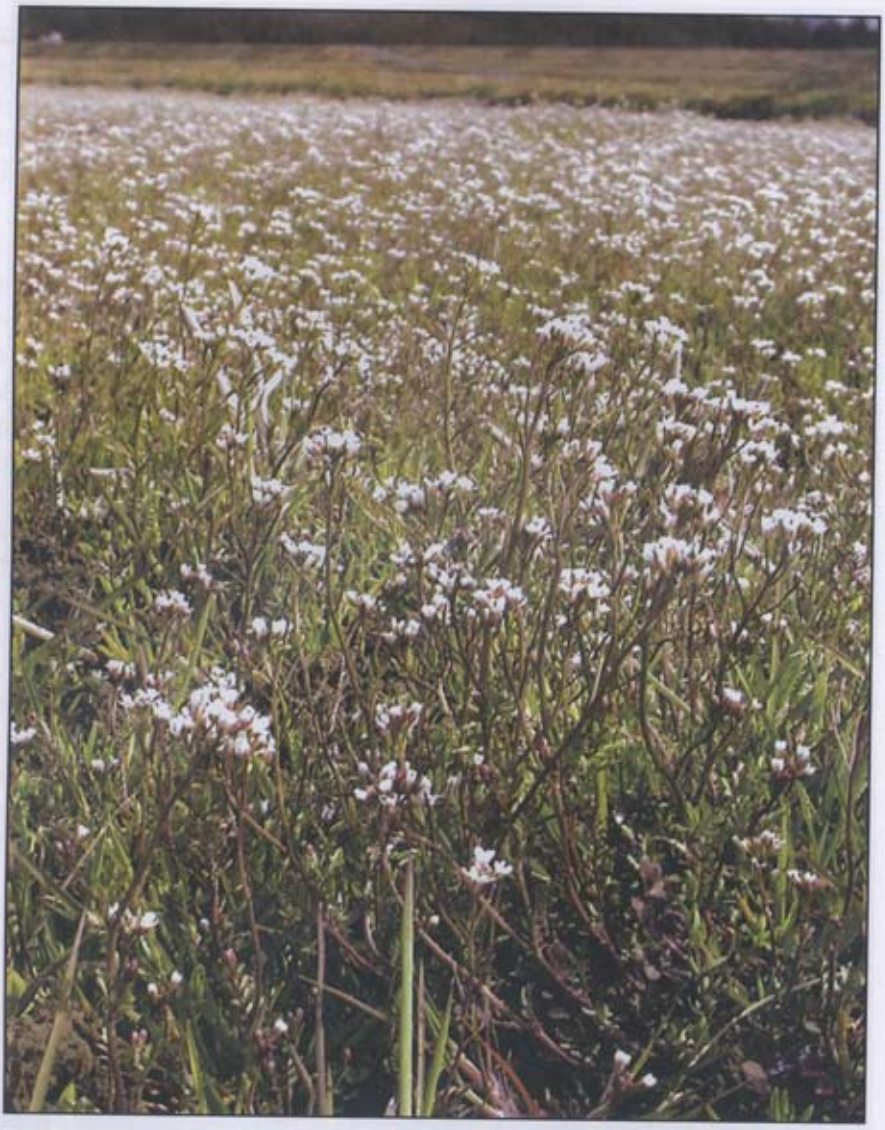

Fig. 5 Japanese populations considered conspecific with the European tetraploid *Cardamine flexuosa* typically grow in rice paddy fields. The plants are well-adapted to strong seasonal disturbances associated with rice cultivation. Photo by H. Kudoh.

explored to address the potential influence of habitat dynamics on genetic differentiation, as well as to compare genetic diversity present in the soil seed bank and in the established populations. Low population differentiation and no geographical structuring of genetic

variation were observed, consistent with the mating system of this plant. The study indicated an impact of environmental disturbances on the level of heterozygosity in the established populations and on the genetic homogeneity of the seed bank.

Genetic variation based on RAPD markers was investigated in an Australian endemic, hexaploid *C. lilacina* Hook. [101]. Four morphologically distinct ecotypes have been recognized within this species [42], apparently being also genetically differentiated. Populations of one of them, the alpine snow-patch variant, are found in three geographically close (2-6 km apart), but separated alpine regions in southeastern Australia. The extant populations are assumed to be relics of a previously more widespread population(s). RAPD data were generated to determine the level of genetic variation and differentiation among the regions. The proportion of polymorphic RAPD bands within the analyzed populations, together with the RAPDs scoring in the seed progeny indicated outbreeding. Interestingly, most of the variation (84%) was attributable to the intrapopulational variation, and only negligible proportion (0.93%) was assigned to the differences among the regions. To explain the absence of spatial genetic structure among the regions, three hypotheses were proposed: (1) ongoing gene flow among the sites and regions, (2) maintenance of original genetic variation since area fragmentation, and (3) independent acquirement of similar types of variation.

Phenotypic plasticity, i.e. the ability of a genotype to express different phenotypes in response to different environmental conditions, has undoubtedly strong ecological and evolutionary implications. Detailed studies on phenotypic variability and plasticity have been performed in *Cardamine* populations in Japan, focused on populations assigned to *C. flexuosa*, which typically grow there as a weed in cultivated fields (rice paddy fields, crop fields and orchards, Fig. 5) providing different ecological conditions [64, 65, 66]. Seasonal disturbance associated with rice cultivation (plowing, flooding and herbicide application) strongly affects phenology, reproduction and survival of plants growing in paddy fields. Seed germination characteristics appeared to be crucial for the establishment of *C. flexuosa* in such habitats [142]. Considerable variability in life history traits and phenology was observed when comparing populations from different habitats. Populations from paddy fields showed the life cycle of a winter-annual, in contrast to crop and orchard populations

behaving as a yearlong annual. Subsequently, differences in population structure were recorded among populations from different habitats. Furthermore, seasonal differences observed in the growth form and phenology irrespective of the habitat type, suggested the impact of seasonally oscillating temperatures and photoperiod regimes [64]. This aspect was further examined, and plastic responses to different chilling and photoperiod treatments were studied in four populations of *C. flexuosa* [65]. The results showed considerable variation in plasticity of all investigated traits among the populations. However, rather than differences in the pattern of response, differences in the magnitude of response were found. Similar results were obtained when studying phenotypic plasticity in response to vegetation shade in *Arabidopsis thaliana* and related species [107], showing different magnitude of plasticities. Much of the recent investigation of phenotypic plasticity focuses on *Arabidopsis thaliana* to elucidate the genetic basis of plastic responses, which indicates different genetic mechanisms involved [106, 107, 108].

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