*Annals of Botany* **93**: 507–520, 2004
doi:10.1093/aob/mch073, available online at www.aob.oupjournals.org

# Comparative ITS and AFLP Analysis of Diploid *Cardamine* (Brassicaceae) Taxa from Closely Related Polyploid Complexes

KAROL MARHOLD[1,2,*], JUDITA LIHOVÁ[1], MARIÁN PERNÝ[1] and WALTER BLEEKER[3]

[1]*Institute of Botany, Slovak Academy of Sciences, Dúbravská cesta 14, SK-845 23 Bratislava, Slovak Republic,* [2]*Department of Botany, Charles University, Benátská 2, CZ-128 01 Praha 2, Czech Republic and* [3]*Department of Systematic Botany, University of Osnabrück, Barbarastrasse 11, D-49076 Osnabrück, Germany*

Received: 21 July 2003 Returned for revision: 24 September 2003 Accepted: 13 January 2004 Published electronically: 22 March 2004

• *Background and Aims* Diploid representatives from the related polyploid complexes of *Cardamine amara*, *C. pratensis* and *C. raphanifolia* (Brassicaceae), were studied to elucidate phylogenetic relationships among the complexes and among the individual taxa included.
• *Methods* Two independent molecular data sets were used: nucleotide sequences from the internal transcribed spacers (ITS) of nrDNA, and amplified fragment length polymorphism (AFLP) markers. Seventeen diploid taxa from the studied groups were sampled.
• *Key Results* Both ITS and AFLP analyses provided congruent results in inferred relationships, and revealed two main lineages. While the *C. amara* group, consisting of *C. wiedemanniana* and four subspecies of *C. amara*, was resolved as a well-supported monophyletic group, taxa from the *C. pratensis* and *C. tenera* groups (the latter representing diploid taxa of the complex of *C. raphanifolia*) all appeared together in a single clade/cluster with no support for the recognition of either of the groups. Intra-individual polymorphisms and patterns of nucleotide variation in the ITS region in *C. uliginosa* and *C. tenera*, together with the distribution of AFLP bands, indicate ancient hybridization and introgression among these Caucasian diploids.
• *Conclusions* The lack of supported hierarchical structure suggests that extensive reticulate evolution between these groups, even at the diploid level, has occurred (although an alternative explanation, namely ancestral polymorphism in ITS data, cannot be completely excluded). Several implications for the investigation of the polyploid complexes of concern are drawn. When tracing origins of polyploid taxa, a much more complex scenario should be expected, taking into account all relatives as potential parents, irrespective of the group in which they are classified.


**Key words:** AFLP fingerprinting, *Cardamine pratensis*, *Cardamine raphanifolia*, *Cardamine amara*, internal transcribed spacers, molecular phylogeny, nrDNA, reticulation.

## INTRODUCTION

The genus *Cardamine* L. (Brassicaceae) comprises about 200 species with indigenous taxa on all continents except Antarctica (Al-Shehbaz, 1988). In several molecular phylogenetic studies involving selected *Cardamine* taxa, the genus was shown to be a well-supported monophyletic group [*ndhF* and *trnL* intron of plastid DNA (Sweeney and Price, 2000); *matK* of plastid DNA and *Chs* of nuclear DNA (Koch *et al*., 2001)]. In others, representatives of *Cardamine* were nested with taxa of the closely related genera *Nasturtium* W.T.Aiton, *Rorippa* Scop. and *Armoracia* P.Gaertn., B.Mey. & Scherb. [ITS of nuclear ribosomal (nr)DNA, *trnT-trnL* spacer and *trnL* intron of plastid DNA (Franzke *et al*., 1998); ITS of nrDNA (Yang *et al*., 1999)]. Infrageneric relationships in *Cardamine*, as proposed by Schulz (1903) in his sectional classification based on morphology, were not supported, as several sections recognized by him appeared to be polyphyletic (e.g. sections *Cardamine*, *Dentaria* (L.) O.E.Schulz, *Cardaminella* Prantl and *Papyrophyllum* O.E.Schulz; Sweeney and Price, 2000; Bleeker *et al*., 2002).

From molecular analyses, several groups of closely related taxa can be delimited within *Cardamine*, among them a clade composed of members of three traditionally recognized polyploid complexes, *C. amara*, *C. pratensis* and *C. raphanifolia*, together with the tetraploid *C. flexuosa* With. (Franzke *et al*., 1998), all being probably related to two species of mostly southern hemispheric distributions, *C. obliqua* Hochst. and *C. africana* L. (Bleeker *et al*., 2002). The close evolutionary position of these taxa can also be demonstrated by weak reproductive barriers among some of them, indicated by recent natural hybridization reported between the *C. amara* and *C. pratensis* groups (Urbanska *et al*., 1997; Marhold *et al*., 2002*b*), between *C. amara* and *C. flexuosa* (Kerner, 1870; Lövkvist, 1956), between *C. flexuosa* and the *C. pratensis* group (K. Mummenhoff and T. C. G. Rich, unpubl. res.), and probably also between *C. raphanifolia* and the *C. pratensis* group (M. Perný *et al.*, unpubl. res.).

The *C. amara* group comprises diploid and tetraploid taxa treated at subspecies and species levels. While the typical subspecies of *C. amara* L., subsp. *amara* ($2n = 16$) is widespread throughout most of Europe and extends far into Asia, other taxa are mostly restricted to certain European mountainous regions: *C. amara* subsp. *opicii* (J.Presl &

* For correspondence. E-mail karol.marhold@savba.sk

C.Presl) Čelak. (2*n* = 16), subsp. *pyrenaea* Sennen (2*n* = 16), subsp. *balcanica* Marhold, Ančev & Kit Tan (2*n* = 16), subsp. *austriaca* Marhold (2*n* = 32), *C. amporitana* Sennen & Pau (2*n* = 32). The diploid *C. wiedemanniana* Boiss. (2*n* = 16) is restricted to western Transcaucasia and northern Anatolia (Spasskaya, 1978; Marhold, 1995, 1999; Marhold *et al*., 1996; Lihová *et al*., 2000, 2004*a*). Support for such a taxonomic treatment, originally proposed on the basis of morphology, was recently provided by isozymes and AFLP analyses (Marhold *et al*., 2002*a*; Lihová *et al*., 2004*a*). The taxa included are morphologically characterized by a long horizontal rhizome bearing stolons, all stem leaves pinnate with short stalked to sessile leaflets, white petals, violet (all subspecies of *C. amara*) or yellow anthers (*C. amporitana* and *C. wiedemanniana*) and attenuate stigmas (Spasskaya, 1978; Marhold, 1992, 1998, 1999; Rico, 1993; Lihová *et al*., 2000).

The *C. pratensis* group is an assemblage of taxa with much more confusing patterns of karyological and morphological variation. It comprises diploids, polyploids up to the dodecaploid level, aneuploids and dysploids, mostly distributed in Europe, but extending to Asia, North Africa and northern North America. It has always been considered, at least implicitly, a coherent, monophyletic group of taxa at species or subspecies level (e.g. Lövkvist, 1956; Urbanska-Worytkiewicz and Landolt, 1974; Marhold, 1993, 1994; Rico, 1993; Franzke and Hurka, 2000). Recent morphometric and molecular studies provided a more detailed picture of this complex and identified diploid (2*n* = 16) Mediterranean species *C. crassifolia* Pourr. [= *C. pratensis* subsp. *nuriae* (Sennen) Sennen], *C. rivularis* Schur [≡ *C. pratensis* subsp. *rivularis* (Schur) Nyman], *C. penzesii* Ančev & Marhold, *C. castellana* Lihová & Marhold (Lihová *et al*., 2003) and *C. apennina* Lihová & Marhold (Lihová *et al*., 2004*b*), as early branching taxa, while the others, *C. pratensis* L. (≡ *C. pratensis* subsp. *pratensis*, 2*n* = 16–56), *C. granulosa* All. [≡ *C. pratensis* subsp. *granulosa* (All.) Arcang., 2*n* = 16], *C. matthioli* Moretti [≡ *C. pratensis* subsp. *matthioli* (Moretti) Nyman, 2*n* = 16], *C. majovskii* Marhold & Záborský (2*n* = 32), *C. dentata* Schult. [= *C. pratensis* subsp. *paludosa* (Knaf) Čelak., 2*n* = 56–96] and *C. nymanii* Gand. (≡ *C. pratensis* subsp. *polemonioides* Rouy, 2*n* = 64–80), as those which evolved more recently (Franzke and Hurka, 2000; Lihová *et al*., 2003, 2004*b*). The group is defined by several diagnostic morphological characters, although with exceptions in some taxa: a short or slightly elongated thickened rhizome without stolons (except *C. crassifolia* with thin, creeping and stoloniferous rhizome), leaf dimorphism, with basal leaves pinnate, forming a rosette (except *C. crassifolia* without a true rosette) and upper stem leaves pinnatisect (except *C. dentata* with all leaves pinnate), yellow anthers (violet anthers present only in *C. rivularis*), and a conspicuously enlarged stigma (Lövkvist, 1956; Marhold, 1993, 1994; Rico, 1993).

The third polyploid complex comprises several diploid to octoploid taxa distributed in the European Mediterranean, usually classified either as separate species or as subspecies of *C. raphanifolia* Pourr.: *C. raphanifolia* (≡ *C. raphanifolia* subsp. *raphanifolia*, 2*n* = 48, 64), *C. gallaecica* (M.Laínz) Rivas. Mart. & Izco (≡ *C. raphanifolia* subsp. *gallaecica* M.Laínz, 2*n* = 32, 48), *C. silana* Marhold & Perný (2*n* = 48), *C. barbaraeoides* Halácsy [≡ *C. raphanifolia* subsp. *barbaraeoides* (Halácsy) Strid, 2*n* = 32], and *C. acris* Griseb. [≡ *C. raphanifolia* subsp. *acris* (Griseb.) O.E. Schulz, 2*n* = 16] (Cullen, 1965; Strid, 1986; Jones and Akeroyd, 1993; Rico, 1993; Jalas and Suominen, 1994; Marhold *et al*., 2003). In addition to these taxa, three diploid species (2*n* = 16) from the Caucasus and neighbouring areas have also been, at least implicitly, included in this complex: *C. tenera* C.A.Mey., *C. uliginosa* M.Bieb., and *C. seidlitziana* Albov (Khatri, 1988; chromosome number data from M. Perný, unpubl. res.). Khatri (1988) did not recognize *C. seidlitziana*, merging it with the Balkan species *C. acris*. He included these three taxa, i.e. *C. tenera*, *C. uliginosa* and *C. acris* into a single subsection *Tenerae* Spasskaja. The taxa forming this polyploid complex are characterized by a combination of the following morphological characters: thick and creeping rhizome, cauline and basal leaves similar in shape, terminal leaflet much larger than lateral ones (slightly larger in *C. gallaecica* and *C. uliginosa*) and yellow anthers. Unlike the *C. amara* and *C. pratensis* groups, no detailed morphological or molecular systematic studies of this Mediterranean–Caucasian group have been published so far, and little if anything is known about true phylogenetic relationships and relatedness among the taxa included.

In previous studies on the two above-mentioned groups of *C. amara* and *C. pratensis*, attention was paid to relationships and evolution within each of them separately (Franzke and Hurka, 2000; Marhold *et al*., 2002*a*; Lihová *et al*., 2003, 2004*b*), but no broad-scale phylogenetic study including taxa of these groups together has been conducted. For convenience we refer to these polyploid complexes as 'species groups' (as they were traditionally treated) throughout this paper, even though results of our analyses do not fully support such treatment. For diploid taxa of the polyploid complex of *C. raphanifolia*, which were included by Khatri (1988) in *Cardamine* subsect. *Tenerae*, we provisionally use the name the *C. tenera* group.

The main objective of this study was to analyse these related taxa together, and to elucidate relationships within and among the groups. In polyploid complexes with high polyploids represented, evolutionary histories with complicated reticulate patterns can be expected. To decrease the disruptive effect of hybridization and allopolyploidization on phylogeny reconstruction, and biased phylogenetic signal because of reticulation, we restricted this study to diploid representatives only. We used two independent molecular data sets: sequences of the internal transcribed spacers (ITS1 and ITS2) of nrDNA (Baldwin *et al*., 1995), and amplified fragment length polymorphisms of total DNA (amplified fragment length polymorphism: AFLP™) (Vos *et al*., 1995). Removing all known polyploids from a sample before constructing a branching tree, even if the tree is simply a representation of similarity and distance, to avoid false phylogenies due to reticulate evolution is also advocated by Bachmann (2000). He argues that a cladistic treatment of data from diploid and allopolyploid taxa together will inevitably produce a wrong result. A detailed search for the origin of polyploid taxa in the above-

mentioned three polyploid complexes is the subject of our further studies (e.g. *C. silana*; M. Perný, K. Marhold, A. Tribsch, T. F. Stuessy, unpubl. res.). In addition, in this paper we compare the data from these two molecular markers and evaluate to what extent they are congruent and/or complementary.

## MATERIALS AND METHODS

### *Plant material*

Leaf material was collected either directly in the field or from plants transferred and cultivated in the experimental garden of the Institute of Botany, Bratislava. Fresh leaf samples were dried and preserved in silica gel. Voucher specimens are deposited in the herbarium SAV. For ITS analyses, all diploid taxa from the three polyploid complexes were sampled and sequenced (17 taxa in total). In the AFLP analyses two species from the *C. pratensis* group (*C. rivularis* and *C. penzesii*) group were not included, because of the lack of fresh material (Table 1). In the ITS analyses each taxon was represented by one to five individuals from different populations, whereas broader sampling was used for AFLP, due to higher inter- and intrapopulational variation revealed by this marker. In both ITS and AFLP analyses the number of accessions of Balkan *C. acris* exceeded those of other taxa, because of the need to include several geographic–morphological entities observed in this species. These probably deserve formal subspecific recognition (M. Perný, A. Tribsch, M. Anchev, unpubl. res.)

### *DNA extraction, ITS amplification and sequencing*

Total DNA was isolated from leaf material dried with silica gel (Chase and Hills, 1991) using the 2X CTAB method of Doyle and Doyle (1987). Internal transcribed spacers of nrDNA (ITS1, ITS2) and the 5.8S rDNA gene were amplified with primers ITS4 and ITS5 (White *et al*., 1990). Amplification followed the protocol described in Franzke *et al*. (1998), with the cycle profile comprising 40 cycles of 1 min at 94 °C (first cycle 3 min), 45 s at 50 °C, and 2 min at 72 °C (last cycle 6 min). Amplification products were then purified (Qiaquick PCR Purification Kit; Qiagen, Hilden, Germany). Sequencing reactions were performed on both strands resulting in a 100 % overlap using the ABI PRISM BigDye Ready Reaction Terminator Cycle Sequencing Kit (Applied Biosystems, Vienna, Austria). The cycle profile for sequencing reactions was: 30 cycles, 30 s at 96 °C, 15 s at 50 °C, and 4 min at 60 °C. The products were separated electrophoretically on an ABI 377 sequencer (Applied Biosystems). Alignment was done manually, and the occurrence of within-individual polymorphisms, when observed on both strands, was recorded using the IUPAC ambiguity symbols. GenBank accession numbers of the new and the previously published sequences are listed in Table 1.

### *AFLP analyses*

The AFLP procedure (Vos *et al*., 1995) followed the general protocol of Applied Biosystems (PE Applied Biosystems, 1996) with a few modifications, described in detail in Schönswetter *et al*. (2004). Total genomic DNA was restricted with the endonucleases *Eco*RI and *Mse*I (Promega, Madison, WI, USA) and double-stranded adaptors (Applied Biosystems) were ligated in one step at 37 °C. Amplification of the generated fragments was performed in two amplification cycles. First, preselective amplification was performed using two primers with 1-bp pair extensions with the following PCR conditions: 2 min at 72 °C, 20 cycles of 1 s at 94 °C, 30 s at 56 °C, 2 min at 72 °C, and final 30 min at 60 °C. On the basis of a primer test with 15 different primer combinations, three primer pairs were chosen and used for selective amplification: *Eco*RI–AAG, *Mse*I–CTG; *Eco*RI–ATC, *Mse*I–CAG; *Eco*RI–AGC, *Mse*I–CTG. The PCR conditions for selective amplification were: 2 min at 94 °C followed by 32 cycles of 1-s denaturing at 94 °C, 30 s annealing, and 2 min extension at 72 °C, ending with 2 min at 72 °C for complete extension. Annealing was initiated at a temperature of 65 °C, which was then reduced by 1 °C for the next eight cycles and maintained at 56 °C for the subsequent 23 cycles. The amplified AFLP fragments were separated electrophoretically on an ABI 377 sequencer, and then analysed with GeneScan® software (version 3.2; Applied Biosystems). Presence or absence of fragments ranging from 70 to 500 bp were scored for each sample (only well scorable, unambiguous fragments were analysed) and transferred into a binary matrix using GenoGrapher (version 1.6.0, ©Montana State University 1999; http://hordeum.msu.montana.edu/genographer/).

### *Analyses of ITS sequences*

For phylogenetic analyses, only the ITS1 and ITS2 sequences were used. The 5.8S rDNA gene sequence (which showed almost no variation and was lacking in the previously published sequences) was excluded. Combined ITS1 and ITS2 were analysed by Fitch parsimony using the heuristic search in PAUP* (version 4.0b10; Swofford, 2001) with the following options: gaps treated as missing data, single-nucleotide polymorphisms as uncertainties, tree construction with stepwise addition, 100 replicates with random taxon addition and unlimited number of trees saved per replicate, tree–bisection–reconnection (TBR) branch swapping, no MaxTrees limits, MulTrees option in effect, and DELTRAN for character optimization. In addition, a heuristic search with 1000 replicates with random taxon addition but with a limit of 1000 trees saved per replicate was applied to allow a more extensive search in tree space, and to minimize the risk that any island of most-parsimonious trees had been missed. The evolutionary direction of sequence changes was inferred by outgroup comparison. For this purpose, two Australasian *Cardamine* species (*C. corymbosa* Hook.f. and *C. debilis* Banks ex DC.) were used. In a previous analysis (Bleeker *et al*., 2002), *C. corymbosa* and *C. debilis* were placed in a distinct clade closely related to other clades containing species of the *C. pratensis* group and the *C. amara* group. The consistency index (CI) of Kluge and Farris (1969) estimating the level of homoplasy in the characters, and the retention index (RI) of

TABLE 1. *List of* Cardamine *taxa and origin of samples included in the present study*

| Population code, origin and collection data | ITS1, ITS2 GenBank accession numbers | AFLP no. of indiv. |
|---|---|---|
| *Cardamine tenera* group | | |
| *Cardamine acris* | | |
| AR – Bulgaria, Zapadni Rodopi Mts, Pamporovo, Ardashla, 1450 m, 25 June 2000, Perný | | 1 |
| OC – Bulgaria, Zapadni Rodopi Mts, Pamporovo, Orlitsa, 1600 m, 25 June 2000, Perný | | 1 |
| GE2 – Bulgaria, Osogovska planina Mts, from Gramadite (W of 'Trite Buky' chalet) to the 'Ruen' Hill, approx. 1600 m, 28 June 2000, Perný & Anchev | | 1 |
| OG – Bulgaria, Osogovska planina Mts, approx. 3 km W of 'Osogovo' chalet, 1740 m, 28 June 2000, Perný & Anchev | | 1 |
| RP – Bulgaria, Stara planina Mts, Tetevenska planina, the 'Ribarishki prokhod' pass, 1650 m, 22 June 2000, Perný & Georgieva | | 1 |
| VN – Bulgaria, Stara planina Mts, Tetevenska planina, NW of Vezhen (2198 m), 1850 m, 21 June 2000, Perný & Georgieva | AY246003<br>AY246033 | 1 |
| NEG/MN – Montenegro, Mojkovač, Bjelasica Mts, Zekova glava Mt., 1900 m, 25 July 2001, Šída, Štěpánek *et al.* | AY245977<br>AY246007 | 1 |
| AE – Greece, prov. Ioannina/Kastoria, Gramos Mts, Aetomilitsa, approx. 5 km N of the village, 19 June 2001, Perný & Kučera | | 1 |
| 2PD – Greece, prov. Florina, Varnous Mts, Pisoderi Ski Resort, 20 June 2001, Perný & Kučera | | 1 |
| VRA – Greece, prov. Pella, Voras Mts (Kaimaktsalan), close to the Voras Ski Resort, 21 June 2001, Perný & Kučera | AY246001<br>AY246031 | 1 |
| VSC – Greece, prov. Grevena, North Pindos Mts, close to the Vasilitsa Ski Resort, 18 June 2001, Perný & Kučera | | 1 |
| 1KPN – Greece, prov. Ioannina, North Pindos Mts, Metsovo, Katara Pass, approx. 1·7 km S of Mt. Katara, micropopulation 1, 18 June 2001, Perný & Kučera | | 1 |
| 2KPN – Greece, prov. Ioannina, North Pindos Mts, Metsovo, Katara Pass, approx. 1·7 km S of Mt. Katara, micropopulation 2, 18 June 2001, Perný & Kučera | AY246002<br>AY246032 | 1 |
| TM – Greece, prov. Evritania, Timfristos Mts, Karpenisi Ski Resort, approx. 2·5 km NW of the ski resort, 24 June 2001, Perný & Kučera | AY245989<br>AY246019 | 1 |
| 1ITI, 2ITI – Greece, prov. Fthiotida, Iti Mts, approx. 10 km E of Kastania, 24 June 2001, Perný & Kučera | | 1 |
| VDA – Greece, prov. Fokida, Vardousia Mts, approx. 6 km WSW of Athanasios Diakos, 23 June 2001, Perný & Kučera | | 1 |
| *Cardamine seidlitziana* | | |
| BES – Russia, Krasnodarskii krai, Sochinskii raion, W Greater Caucasus, Mzymta valley, Krasnaya Polyana, Beshenka stream, approx. 700 m, 22 April 2001, Perný | AY245979<br>AY246009 | 1 |
| *Cardamine tenera* | | |
| TAL – Azerbaijan, the Talysh Mts, 135 m, 26 May 2001, Lihová | AY245991<br>AY246021 | 1 |
| CF – Russia, distr. Krasnodarskii krai, Sochinskii raion, Sochi, part Adler, 50 m, 20 April 2001, Perný & Tuniev | | 1 |
| GK – Russia, distr. Krasnodarskii krai, Goryachii Klyuch, camping approx. 10 km E of the town, 150 m, 25 April 2001, Perný | AY245992<br>AY246022 | 1 |
| GZT – Russia, Republika Adigeya, W Greater Caucasus, Kavkazskii zapovednik, Guzeripl', near the Museum of 'Kavkazskii zapovednik', 850 m, 29 April 2001, Perný | | 1 |
| KH – Russia, Krasnodarskii krai, Sochinskii raion, Sochi, part Khosta, 50 m, 21 April 2001, Perný *et al.* | | 1 |
| KU – Russia, Republika Adigeya, Kurdzhipskaya near Maikop, S of the village, 250 m, 28 April 2001, Perný | | 1 |
| KS – Russia, W Greater Caucasus, Mzymta valley, Krasnaya skala, 120 m, 21 April 2001, Perný & Tuniev | AY245980<br>AY246010 | – |
| NM – Russia, Krasnodarskii krai, Sochinskii raion: Sochi, part Novaya Macesta, 30 m, 24 April 2001, Perný *et al.* | | 1 |
| *Cardamine uliginosa* | | |
| TUR – Turkey, Bolu Mts, lake Abant Gölü, 1997, Sádlo | AY245990<br>AY246020 | 1 |
| BAK1 – Georgia, Minor Caucasus Mts, Bakuriani, 1700 m, 20 May 2001, Lihová | | 1 |
| DJ – Georgia, Khevi province, Kazbegi, E of village Djuta, Sakhizno, 2350 m, 8 July 2001, Marhold | | 1 |
| GTU – Georgia, Khevi province, N of Kazbegi, Gveleti, Tibaitsskali rivulet, 1480 m, 7 July 2001, Marhold | | 1 |
| KGU – Georgia, Khevi province, Kazbegi, along the Tergi (Terek) river, 1732 m, 10 July 2001, Marhold | | 1 |
| KHR1 – Georgia, Minor Caucasus Mts, 'Khrats' karos' ughelt' pass, S of Bakuriani, 2150 m, 22 May 2001, Lihová | AY246004<br>AY246034 | 1 |
| KPU – Georgia, Khevi province, Cross pass (Djvris ugeltekhili), 2394 m, 5 July 2001, Marhold | AY245981<br>AY246011 | 1 |
| TE – Georgia, Khevi province, Kazbegi, Truso Gorge, between the villages of Ketrisi and Zemo-Okrokavam along the Tergi (Terek) river, 2115 m, 7 July 2001, Marhold | | 1 |

TABLE 1. *Continued*

| Population code, origin and collection data | ITS1, ITS2 GenBank accession numbers | AFLP no. of indiv. |
|---|---|---|
| *Cardamine amara* group | | |
| *Cardamine amara* subsp. *amara* | | |
| ROZ – Czech Republic, Pardubice, Rozhrna fishpond, 220 m, 8 Aug. 2000, Marhold | AY245998 AY246028 | 1 |
| BDA – Slovakia, Vtáčnik Mts, Gepniarova dolina valley, 500 m, July 2001, Perný | AY245985 AY246015 | 1 |
| ZS – Slovakia, Bratislava, Železná studnička, 200 m, 16 Sept. 2001, Lihová | | 1 |
| LAI – Italy, Piemont, Val di Susa, N of Condove, Laietto, 1000 m, 17 May 2002, Lihová & Marhold | AY246015 AY246016 | – |
| *Cardamine amara* subsp. *balcanica* | | |
| GR – Bulgaria, Zapadni Rodopi Mts, Pamporovo, Goljamata Reka, 1490 m, 25 June 2000, Perný | | 1 |
| AN – Bulgaria, Vitosha Mts, Aleko, 1800 m, July 2001, Anchev | AY245976 AY246006 | 1 |
| VN – Bulgaria, Stara Planina, Tetevenska planina, Vezhen, 1850 m, 21 June 2000, Perný & Georgieva | | 1 |
| VH – Bulgaria, Vitosha Mts, north-eastern slopes, approx. 1600 m, 24 June 2000, Perný *et al.* | | 1 |
| RP – Bulgaria, Stara planina Mts, Tetevenska planina, the 'Ribarishki prokhod' pass, 1650 m, 22 June 2000, Perný & Georgieva | AY245975 AY246005 | – |
| MB – Bulgaria, Pirin Mts, Demyanitsa Valley, near chalet Demyanitsa, 1850 m, 29 June 1995, Anchev & Marhold | AF265184* AF265204* | – |
| *Cardamine amara* subsp. *opicii* | | |
| IDS – Slovakia, Slovenské rudohorie Mts, Idčianske sedlo, 900 m, 9 June 2001, Lihová | AY245999 AY246029 | 2 |
| ZP – Slovakia, Vysoké Tatry Mts, Zelený potok valley, 1440 m, 2 Sept. 2001, Lihová | | 1 |
| MSD – Slovakia, Vysoké Tatry Mts, Malá Studená dolina valley, 1595 m, 3 Sept. 2001, Lihová | AY245988 AY246018 | 1 |
| *Cardamine amara* subsp. *pyrenaea* | | |
| RMP – Spain, E Pyrenees, Planell de les Eugues, 1975 m, 30 June 2001, Lihová | | 1 |
| MRG3 – Spain, E Pyrenees, Meranges, 2080 m, 1 July 2001, Lihová | AY245987 AY246017 | 2 |
| NU6 – Spain, E Pyrenees, Núria, Torrent de Finestreles, 2000–2100 m, 28 June 2001, Lihová | | 1 |
| NU2 – Spain, E Pyrenees, Núria, Coma de Noufonts, 2600 m, 27 June 2001, Lihová | | 1 |
| NU – Spain, E Pyrenees, near Núria, Coma de Noucreus, 2290–2310, 6 July 1997, Marhold | AF265183* AF265203* | – |
| *Cardamine wiedemanniana* | | |
| GZL – Russia, W Greater Caucasus, Kavkazskii zapovednik, Guzeripl', 700 m, 29 April 2001, Perný | AY246000 AY246030 | 2 |
| BEL – Russia, W Greater Caucasus, Mzymta valley, Krasnaya Polyana, the Beshenka stream, 680–720 m, 21 April 2001, Perný | | 1 |
| GAL – Russia, W Greater Caucasus, Mzymta valley, Galitsino, 200 m, 21 April 2001, Perný & Tuniev | AY245982 AY246012 | 1 |
| MO – Russia, W Greater Caucasus, Mzymta valley, Monastyr, 450 m, 21 April 2001, Perný & Tuniev | | 1 |
| *Cardamine pratensis* group | | |
| *Cardamine pratensis* s. str. | | |
| MAG – Spain, Cantabria, Pto. de la Magdalena, 780 m, 7 May 2001, Lihová & Herrera | AY245996 AY246026 | 1 |
| AVE – Portugal, Beira Litoral, Fermentelos, near Aveiro, 6 m, 10 April 2000, Lihová *et al.* | AY245995 AY246025 | – |
| *Cardamine matthioli* | | |
| BR – Slovakia, Slovenské rudohorie Mts, Brezno, 1996, Franzke | AF077985‡ AF077986‡ | – |
| SVI – Slovakia, Považský Inovec Mts, S of Trenčianske Jastrabie, Patrovec, 320–340 m, 6 Oct. 2001, Perný | | 1 |
| TV – Slovenia, Subpanonsko območje, Trnovska vas, 250 m, 20 April 2001, Lihová & Frajman | | 1 |
| SJ – Slovenia, Predalpsko območje, vicinity of Celje, Šmartinsko jezero lake, 260 m, 20 April 2001, Lihová & Frajman | | 1 |
| PO – Slovenia, Subpanonsko območje, Slovenske Konjice, between Tepanje and Pobrež, 280 m, 20 April 2001, Lihová & Frajman | | 1 |
| *Cardamine castellana* | | |
| LEP – Spain, Asturias, near Puerto de Leitariegos, 1470 m, 25 May 2001, Perný *et al.* | AY245994 AY246024 | – |
| HOY – Spain, Ávila, Hoyos del Espino, Plataforma de Gredos, 1460 m, 10 May 2001, Lihová | | 1 |
| PDH – Spain, Ávila, Santiago del Collado, on the way to Puerto de Peña Negra, 1700 m, 10 May 2001, Lihová | | 1 |
| COLL – Spain, Ávila, Hoyos del Espino, near Hoyos del Collado, 1500 m, 10 May 2001, Lihová | | 1 |

TABLE 1. *Continued*

| Population code, origin and collection data | ITS1, ITS2 GenBank accession numbers | AFLP no. of indiv. |
|---|---|---|
| AGU – Spain, Teruel, Sierra de Albarracín, W of Noguera, Barranco de la Peña Aguda, 1400 m, 14 May 2001, Lihová & Nieto Feliner | | 1 |
| ALF – Spain, Guadalajara, Sierra de Albarracín, SW of Alustante, Fuente de los Valles, 1500 m, 14 May 2001, Lihová & Nieto Feliner | | 1 |
| HOZ – Spain, Guadalajara, Sierra de Albarracín, S of Orea, Hoz Seca, 1500 m, 14 May 2001, Lihová & Nieto Feliner | | 2 |
| *Cardamine crassifolia* | | |
| NU – Spain, Gerona, E Pyrenees, Núria, 2100 m, 1996, Franzke | AF265180*<br>AF265200* | – |
| HO – Spain, Gerona, E Pyrenees, Setcases, Pla dels Hospitalets, 1750 m, 23 Aug. 1996, Marhold & Vicens | AF265674*<br>AF265680* | – |
| NU1 – Spain, E Pyrenees, Núria valley, torrent de Noufonts, 2000 m, 27 June 2001, Lihová | | 2 |
| NU3 – Spain, E Pyrenees, Núria valley, torrent de Noufonts, 2200–2300 m, 27 June 2001, Lihová | | 2 |
| NU4 – Spain, E Pyrenees, Núria valley, torrent de Fontalba, 2100 m, 28 June 2001, Lihová | | 1 |
| NU5 – Spain, E Pyrenees, Núria valley, torrent de Finestreles, 2200 m, 28 June 2001, Lihová | | 1 |
| RMC – Spain, E Pyrenees, east of Núria valley, Refugi Manelic, 2000 m, 30 June 2001, Lihová | | 1 |
| MRG1 – Spain, E Pyrenees, N of Meranges, Estany de Malmiu, 2260 m, 1 July 2001, Lihová | | 2 |
| MRG4 – Spain, E Pyrenees, NW of Meranges, Riu Duran, near Refugi J. Folchi Girona, 2320 m, 2 July 2001, Lihová | | 1 |
| *Cardamine granulosa* | | |
| POM – Italy, Piemont, Giaveno, Pomeri, 17 May 2002, Lihová *et al.* | AY245997<br>AY246027 | 2 |
| FRA – Italy, Piemont, San Francesco su Avigliana, 620 m, 17 May 2002, Lihová *et al.* | AY245983<br>AY246013 | 2 |
| *Cardamine apennina* | | |
| LG – Italy, Abruzzo, Lago di Campotosto, Le Serre, 1340 m, 27 April 2001, Lihová *et al.* | | 2 |
| QS – Italy, Toscana, Stáffoli, 8 m, 26 April 2001, Lihová *et al.* | AY245978<br>AY246008 | 2 |
| PAS – Italy, Abruzzo, Gran Sasso, Sorgenti del Vomano, Passo delle Capannelle, 1257 m, 27 April 2001, Lihová *et al.* | AY245993<br>AY246023 | 2 |
| VO – Italy, Abruzzo, Gran Sasso, Voltigno, under Mt. Fiore, 1360 m, 27 April 2001, Lihová *et al.* | AY245984<br>AY246014 | – |
| *Cardamine penzesii* | | |
| JP – Bulgaria, Black Sea cost, N of the road Yasna Polyana – Primorsko, approx. 6 km of Yasna Polyana, 50 m, 21 April 1996, Anchev & Marhold | AF265182*<br>AF265202* | – |
| VE – Bulgaria, Black Sea cost, Natural Park Strandzha, Sinemorets, flood-plain forest near the Veleka river, 21 April 1997, Marhold & Vassilev | AF265675*<br>AF265681* | – |
| *Cardamine rivularis* | | |
| VA – Romania, Mtii Făgăraşului, Valea Arpaşului, 1800 m, 8 July 1989, Marhold | AF077981‡<br>AF077982‡ | – |
| Outgroups | | |
| *Cardamine debilis* | AY047614†<br>AY047625† | |
| *Cardamine corymbosa* | AY047613†<br>AY047624† | |

Sequences obtained by *Franzke and Hurka (2000); †Bleeker *et al*. (2002); ‡Franzke *et al*. (1998) are marked.

Farris (1989) expressing the proportion of similarities on a tree interpreted as synapomorphies, are presented. Equally parsimonious trees were summarized by the strict consensus method. Bootstrap analyses (1000 replications) were performed with the fast-heuristic search as implemented in PAUP* ('fast' stepwise-addition) to assess the relative support of the clades (Felsenstein, 1985).

*AFLP data analyses*

From the AFLP binary data, a neighbour-joining tree (Saitou and Nei, 1987) based on Nei and Li (1979) genetic distance was generated using TREECON (version 1.3b; Van de Peer and De Wachter, 1994) with bootstrap option (2000 replications). For each taxon, the total number of AFLP fragments (bands), mean number of fragments per individual (± standard deviation), number of exclusive (present in a given taxon only, but not necessarily in all its samples) and diagnostic fragments (present in all samples of a taxon and absent from all other taxa), number of bands shared by particular groups of taxa (those present in all samples of all taxa of a group, those present in each taxon of a group, but not necessarily in all samples, and those shared by at least two taxa from a group) were calculated.

## RESULTS

### *ITS sequence analysis*

The ITS1 and ITS2 alignment of 38 sequences of the analysed *Cardamine* taxa (including two outgroup species) was 458 bp in length (269 bp for ITS1, 189 bp for ITS2). Nucleotide substitutions were found at 83 sites, out of which 52 were potentially phylogenetically informative and 31 autapomorphic. Parsimony analysis of the informative characters resulted in 46 742 most-parsimonious trees of 99 steps in length. The consistency index (CI) was 0·92 (0·88 excluding autapomorphies) and the retention index 0·97. The large number of equally parsimonious trees was caused mainly by within-individual polymorphisms detected in some taxa. The strict consensus tree and one of the most-parsimonious (fundamental) trees indicating the number of substitutions supporting each clade, based on 46 742 trees from the first analysis (with 100 replicates), are depicted in Figs 1 and 2, respectively, the former including the bootstrap values. A heuristic search with 1000 replicates and a limit of 1000 trees saved per replicate resulted in 46 266 most-parsimonious trees of the same length, CI and RI as in the above-mentioned search with 100 replicates. The strict consensus tree based on 46 266 trees from the second analysis (not shown) was of the same topology as that shown in Fig. 1, with only negligible differences in bootstrap values.

Two main well-supported clades were resolved in the ITS strict consensus tree: one formed by the *C. amara* group, i.e. including four diploid subspecies of the species *C. amara*, and *C. wiedemanniana*, and the other composed of members of the *C. pratensis* group, Balkan *C. acris* and Caucasian *C. tenera*, *C. seidlitziana* and *C. uliginosa* (*C. tenera* group). Resolution within both clades was low, with large polytomies formed by a few small subclades and single terminals (Fig. 1). Within the *C. amara* clade, only six synapomorphies were recorded for *C. wiedemanniana* and a single synapomorphy was shared by two samples of *C. amara* subsp. *balcanica*. Within the second main clade more nucleotide variation was found, but conflicting topologies of fundamental trees collapsed into the polytomy in the strict consensus tree. Only six subclades were found in this polytomy (Fig. 1). Two Balkan taxa, *C. rivularis* and *C. penzesii*, shared one synapomorphy, and two Iberian diploids, *C. crassifolia* and *C. castellana*, shared four synapomorphies (Fig. 2). The other four clades were each formed by samples belonging to one taxon. The placement of two samples of northern Italian *C. granulosa* in a subclade distinct from that of central Italian plants (*C. apennina*), previously also ascribed to the same species, is noteworthy. Relatively high levels of nucleotide diversity were observed in the Caucasian species, *C. uliginosa* and *C. tenera*, each represented by three plants from geographically distant locations. Much higher numbers of within-individual polymorphisms were found in these samples when compared with other taxa, and the distribution of substitutions did not correspond to taxon assignment (Table 2). No synapomorphies were recorded for either of these species.

### *AFLP analyses*

The neighbour-joining tree (Fig. 3) is composed of two main well-supported clusters. As in the ITS-based tree, one of the clusters comprises taxa of the *C. amara* group and the other is formed by taxa of the *C. pratensis* group, *C. acris* and three Caucasian diploids (the *C. tenera* group). Within the former cluster, the highest bootstrap support was received by the subcluster composed of *C. wiedemanniana* (100 %). With the exception of the typical subspecies, the other subspecies of *C. amara* were each placed in corresponding subclusters with bootstrap values higher than 75 %. Clustering among them was only weakly to moderately supported, indicating a possible close affinity between *C. amara* subsp. *pyrenaea* and subsp. *opicii* (only 64 % bootstrap). Within the second main cluster, *C. tenera*, *C. uliginosa* and *C. seidlitziana* were placed in a single weakly supported subcluster (51 %), but further clustering was not consistent with the three morphologically separated taxa. Although the diploids of the *C. pratensis* group and *C. acris* formed two separate subclusters, both had bootstrap support < 50 %. Among these taxa, a closer relationship is suggested for Iberian species *C. crassifolia* and *C. castellana* (94 % bootstrap), but no other well-supported higher-level groupings of the subclusters consisting of single species of the *C. pratensis* group were revealed. For the *C. acris* cluster higher bootstrap values were observed only in branches close to the terminals.

AFLP fingerprinting produced 358 well-scorable informative bands, with an average number of 43·75 bands per individual. Despite the uneven sampling of individual taxa, and apparent association between number of individuals and total number of bands scored per taxon, certain differences can be seen among the taxa analysed, e.g. in average numbers of bands per individual, and numbers of unique and diagnostic bands (Table 3A). The average number of bands per individual ranged in most taxa from 40 to 50, but was only 34 in *C. tenera* and almost 52 in *C. amara* subsp. *balcanica*. The highest number of unique bands (those present in at least one sample of a given taxon, but not in other taxa) was found in *C. acris*, here represented by the largest number of samples (16) and total number of bands (113), but no diagnostic bands (those restricted to the given taxon, found in all its individuals) were recorded. The highest number of diagnostic bands was found in *C. wiedemanniana*, *C. crassifolia*, *C. granulosa* (four bands in each) and *C. apennina* (three bands). AFLP-band sharing in particular taxon groupings is shown in Table 3B. The highest number of bands shared exclusively by taxa of a group was recorded in the *C. amara* group. A lower number of shared bands was found in the *C. pratensis* group, but this number increased if *C. acris* from the *C. tenera* group was included, as there were several bands in common between *C. acris* and taxa of the *C. pratensis* group. On the other hand, low support, if any, is given to the *C. tenera* group (including *C. acris*, *C. tenera*, *C. uliginosa* and *C. seidlitziana*). After excluding *C. acris*, the remaining grouping of three Caucasian taxa is much better supported (13 exclusively shared bands, out of which one was present in all samples; see Table 3B).

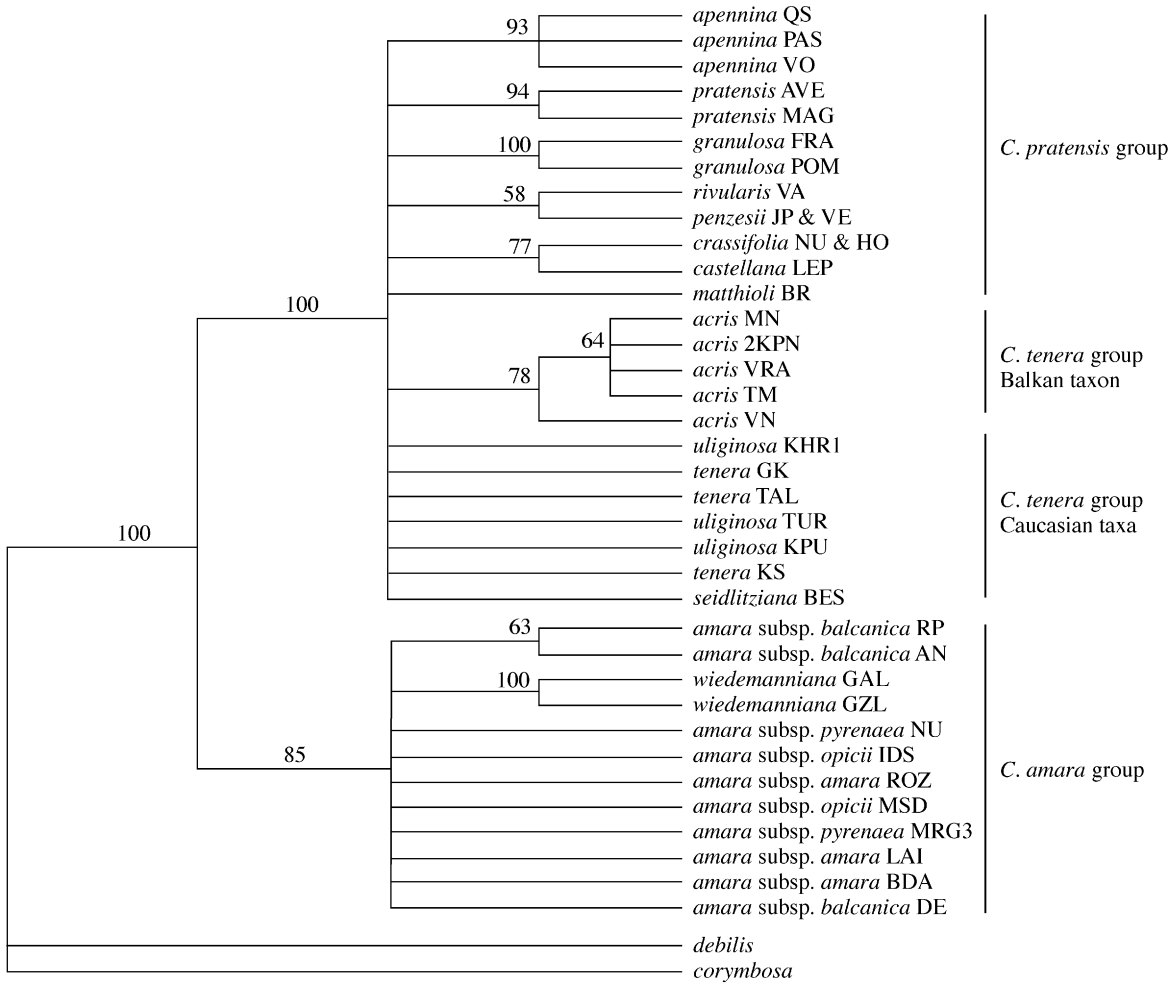


FIG. 1. Strict consensus tree of ITS1 and ITS2 data. Bootstrap values above 50 % are shown. For sample codes see Table 1.

## DISCUSSION

Both AFLP and ITS data presented here are congruent in resolving two main lineages within the diploid *Cardamine* taxa studied. The monophyly of the *C. amara* group at the diploid level, is well supported, including four diploid subspecies of *C. amara* and *C. wiedemanniana*, a diploid distributed in the western Transcaucasia and Turkey. The close association of *C. wiedemanniana* with the Eurasian species *C. amara* was first proposed by Spasskaya (1978) who treated both as subsection *Amarae* Spasskaja of section *Cardamine*. Although the morphology of *C. wiedemanniana* has not been comparatively studied in more detail, the overall morphological resemblance of these two species favours their affinities, as supported also by AFLP analyses in our recent study of all taxa of the *C. amara* group, including also tetraploids (Lihová *et al*., 2004*a*). In the much broader sampling analysed here, *C. wiedemanniana* is closely allied to *C. amara* (Fig. 3), and additional evidence is provided by ITS sequences. Given the overall low ITS nucleotide variation observed in the group, the six synapomorphies shared by the two accessions of *C. wiedemanniana* (Fig. 2) indicate its clear differentiation from the subspecies of *C. amara*. Except for two accessions (out of three analysed) of *C. amara* subsp. *balcanica* sharing a single nucleotide substitution, the ITS sequences provided no further resolution within the *C. amara* group. The AFLP data provided more information in this respect; besides clustering together samples belonging to the same subspecies (except subsp. *amara*), they also indicate relationships among the subspecies (although support for such indications is not high; Fig. 3). The typical subspecies appears to be less homogeneous than the other subspecies, a finding also obtained from isozyme analyses of the *C. amara* group (Marhold *et al*., 2002*a*). This might be attributable to its wide distribution area, in contrast to the restricted ranges of the other subspecies.

Neither AFLP nor ITS data support current taxonomic separation of remaining diploid taxa into two groups, *C. pratensis* and *C. tenera*. They were all placed in a single clade/cluster with poorly resolved relationships among the taxa and no evidence for the monophyly of either of the two

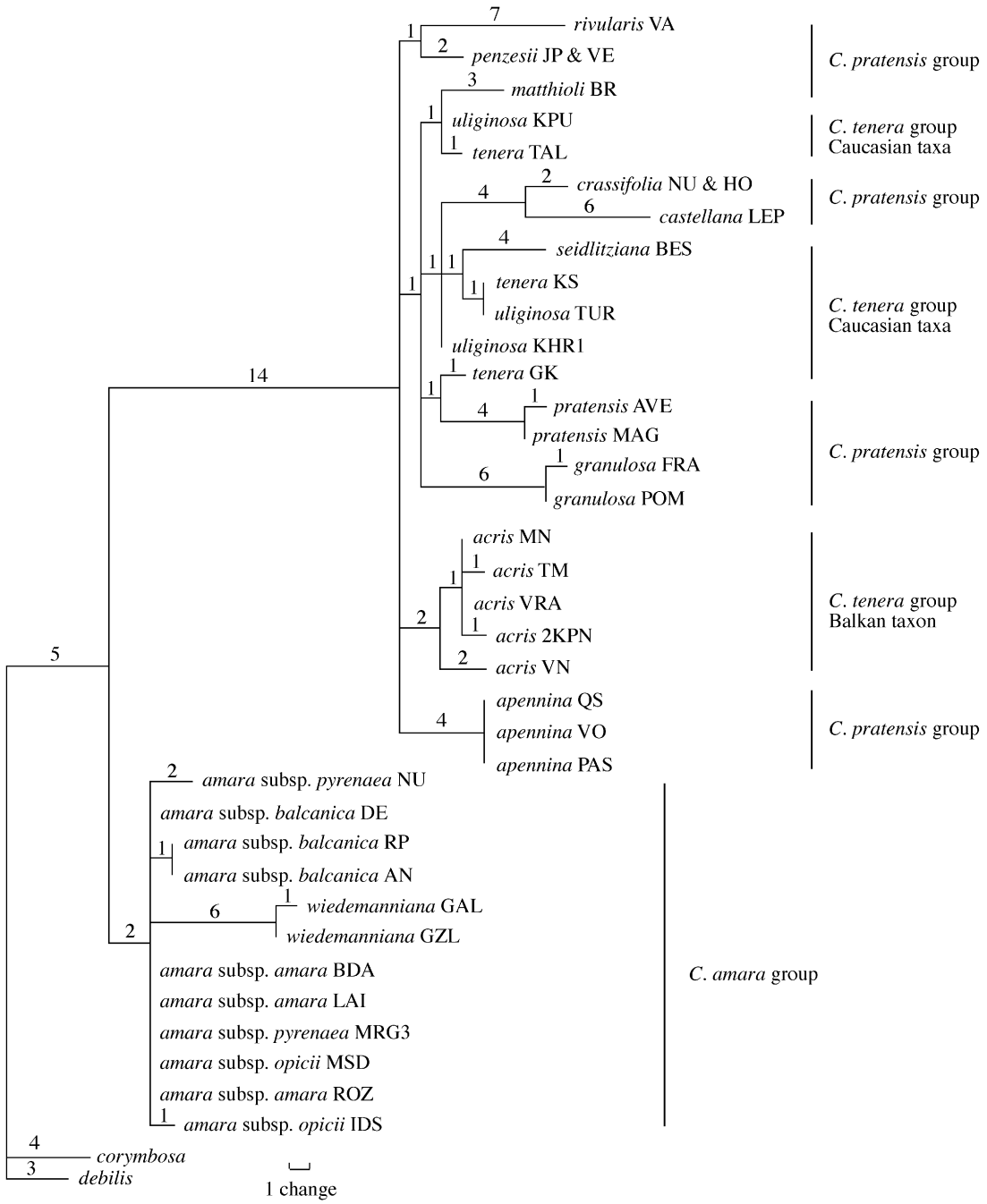


FIG. 2. One of the most-parsimonious trees of ITS1 and ITS2 data. Numbers above branches indicate number of mutational steps. For sample codes see Table 1.

groups (Fig. 1). Especially in the traditionally recognized *C. pratensis* group (see Lövkvist, 1956; Urbanska-Worytkiewicz and Landolt, 1974; Marhold, 1993, 1994; Franzke and Hurka, 2000), such a pattern was not expected. We hypothesize that the pattern shown may result from ancient hybridization and introgression among the taxa (even at diploid level) that might obscure genetic differentiation between the groups. Alternative explanations may be that there is retained polymorphism (in ITS data) or that neither group is monophyletic, but together they form a single group with a common ancestor.

Three hierarchical patterns within this large grouping (*C. pratensis* and *C. tenera* groups) received some support: *C. rivularis* and *C. penzesii* from the Balkans (ITS data, Fig. 1), *C. crassifolia* and *C. castellana* from the Iberian Peninsula (both ITS and AFLP data, Figs 1 and 3), and three Caucasian taxa *C. tenera*, *C. seidlitziana* and *C. uliginosa* (AFLP data, Fig. 3 and Table 2). Associations between the

TABLE 2. *Nucleotide site variation in the ITS1 and ITS2 regions in the Caucasian diploids* Cardamine tenera, C. uliginosa *and* C. seidlitziana

| Taxon/position | 23. | 50. | 56. | 73. | 82. | 87. | 116. | 132. | 202. | 219. | 244. | 293. | 300. | 315. | 335. | 363. | 424. | 432. | 456. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *tenera*/KS | T | T | G/T | A | C | G | C | C/T | A | C | C/T | T | G/T | G | C/T | C/T | C | A | C |
| *tenera*/GK | T | T | G/T | A | C | G | C | C | A | C/T | C | T | T | G | C | C/T | C | A | C |
| *tenera*/TAL | C/T | A | G | A | C | G | C | C | A | C | C | T | A | G | C | C | T | A | C |
| *uliginosa*/KPU | T | A/T | G | A/G | C | G | C | C/T | A/T | C | C | T | G | G | C | C | T | A | C |
| *uliginosa*/TUR | T | T | G | A | C/T | G | C | T | A | C/T | C | T | G | G | C | T | C | A | C/T |
| *uliginosa*/KHR1 | T | A/T | G | A | C | A/G | C/T | C/T | A | C | C | T | G | G | C | C/T | C | A | C |
| *seidlitziana* | T | T | G | A | C | G | C | T | A | C | C | A | G | A | C | C | C | T | C |
| Other taxa | T | A, T | G, A | A | C, T | G | C | C, T | A | C, T | C | T | G | G | C | C | C, T, A | T, A | C |

Two nucleotides at the same position refer to single-site polymorphisms within individuals.
Numbers refer to the positions in the aligned matrix.

two species pairs (*C. rivularis* + *C. penzesii* and *C. crassifolia* + *C. castellana*) were also shown in the previous study by Franzke and Hurka (2000) who examined evolutionary and biogeographic history of the *C. pratensis* group. On the basis of pronounced genetic differentiation, *C. rivularis* and *C. penzesii* were considered to form a 'basal' lineage of this group. Populations of *C. castellana* were classified by them as *C. crassifolia*, following previous, widely accepted taxonomic treatments (see, for example, Rico, 1993; Jalas and Suominen, 1994). However, in our recent study we argue that *C. crassifolia* is a Pyrenean endemic, whereas populations from central Iberian mountains represent a different entity, which we classify as a separate species, *C. castellana* (Lihová *et al*., 2003). Such a conclusion is supported also here; although both taxa are undoubtedly related to each other, they represent two well-differentiated lineages.

Confusing patterns of molecular variation found in Caucasian taxa *C. tenera* and *C. uliginosa* are worthy of discussion. These two species can be easily distinguished morphologically from each other, e.g. by the presence of long leafy stolons, a much larger terminal leaflet and lower number of lateral leaflets in *C. tenera*. In addition, they occur allopatrically and are ecologically differentiated: *C. tenera* is found at lower elevations to sub-montane deciduous wet forests in Crimea and the Western Greater Caucasus and in a second isolated area in the Talysh Mountains in Azerbaijan. *Cardamine uliginosa* is a mainly alpine or sub-alpine species distributed from Asia Minor across the Caucasus to Iraq and Iran. It grows mainly on sunny, wet alpine meadows and along streams. The presence of several polymorphic sites within and between sequences of both species indicates co-occurrence of paralogous ITS copies. Although the ITS repeat units are usually considered to be rapidly homogenized by concerted evolution (Baldwin *et al*., 1995; Franzke and Mummenhoff, 1999; Graur and Li, 1999), as has been shown experimentally (e.g. Fuertes Aguilar *et al*., 1999), there are also an increasing number of studies suggesting the occurrence of intraspecific and intra-individual ITS polymorphism. This has often been attributed to interspecific hybridization and polyploidy, as a result of combining sequences contributed by parental genomes (Sang *et al*., 1995; Hodkinson *et al*., 2002; Hughes *et al*., 2002; Fuertes Aguilar and Nieto Feliner, 2003; Koch *et al*., 2003). Given the geographic and habitat separation between *C. tenera* and *C. uliginosa*, gene-flow between them can hardly be expected to occur at present. The most likely explanation for the pattern found is that they have come into contact in the past and hybridized. In several phylogeographic studies evidence for glacial-induced migrations has been provided (Comes and Kadereit, 1998; Taberlet *et al*., 1998), and most recently it was demonstrated that, especially in southern European regions, distribution changes did not necessarily take place in large geographical areas, but may have been restricted to altitudinal shifts in a single mountain range (Gutiérrez Larena *et al*., 2002). Populations previously (and also currently) isolated may have met as they ascended and descended mountain slopes. We assume that such processes also occurred within the Caucasian area and could have resulted in the patterns observed in *C. tenera* and *C. uliginosa*. Another possibility, which cannot be excluded, is that the present pattern is a result of retained ancestral polymorphism due to recent isolation or speciation. However, this alternative scenario appears less probable, taking into account the same pattern (lack of support for either *C. tenera* or *C. uliginosa*) observed in the AFLP data (Fig. 3), derived from multiple loci across the genome, rather than from a single sequence. In any case, it seems that concerted evolution failed to homogenize the ITS repeat units in these taxa, in contrast to the case of *C.* ×*insueta* Urbanska and *C. schulzii* Urbanska reported by Franzke and Mummenhoff (1999).

Another Caucasian diploid from the *C. tenera* group, *C. seidlitziana*, occurs in western Transcaucasia and differs from *C. tenera* in the absence of stolons at the base of ascending stem and more stem leaves, and from *C. uliginosa* by a lower number of lateral leaflets on stem leaves. Although only a single plant was examined here, it showed little affinity with *C. acris*, with which it was considered conspecific (see Introduction; Khatri, 1988). Rather, a closer position to *C. uliginosa* is indicated.

From the results presented in this study, we summarize the following:

Both molecular markers used showed congruence in arrangement of the taxa into two major clades/clusters.

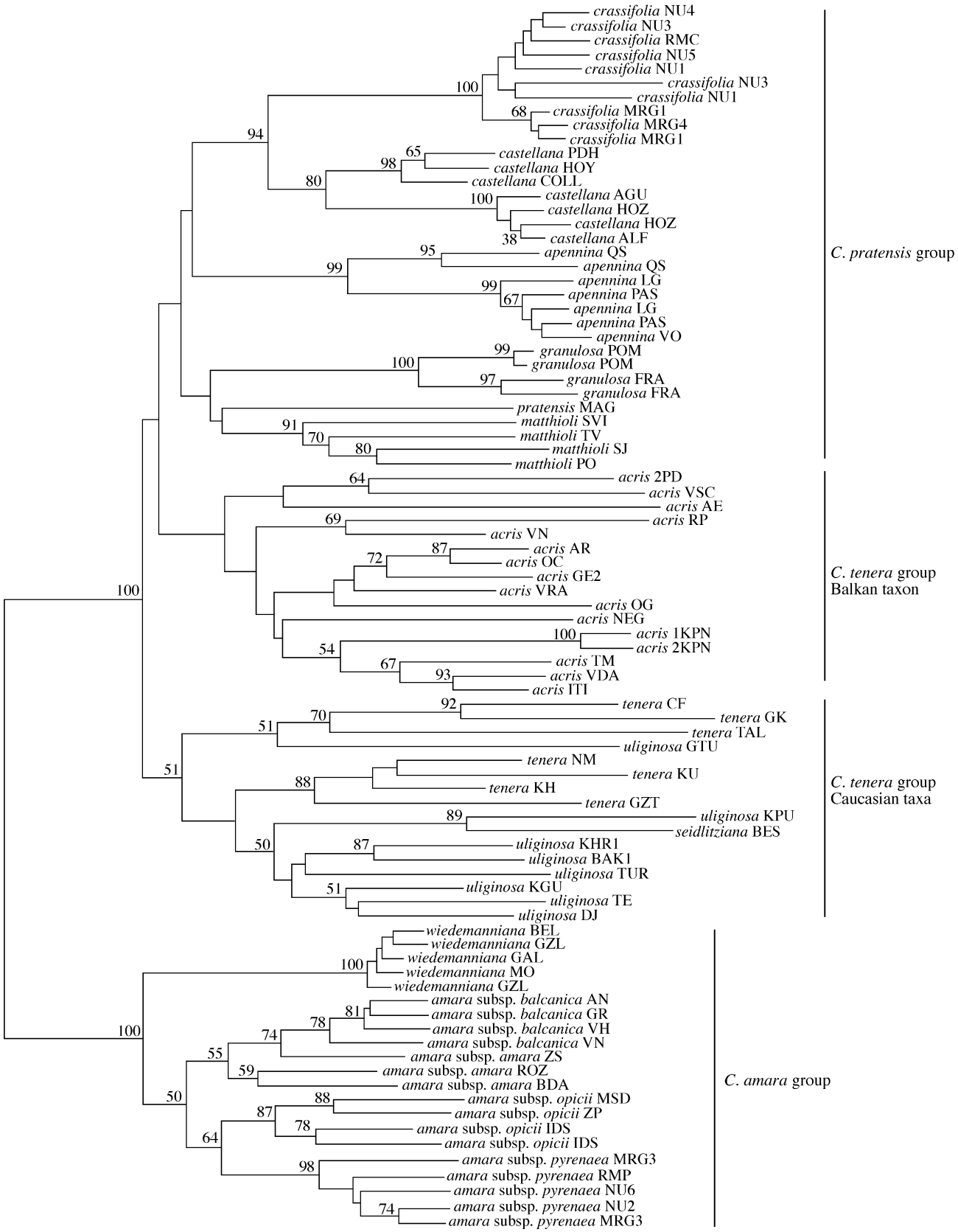


FIG. 3. Neighbour-joining tree of AFLP data. Bootstrap values above 50 % are shown. For sample codes see Table 1.

TABLE 3. *Number of scored AFLP bands and their distribution across the analysed* Cardamine *taxa (A)*

| Taxon (*n*) | Number of bands | | | |
|---|---|---|---|---|
| | Total | Mean | Exclusive | Diagnostic |
| *amara* subsp. *amara* (3) | 59 | 45·00 ± 2·65 | 2 | 0 |
| *amara* subsp. *opicii* (4) | 71 | 49·75 ± 5·56 | 12 | 0 |
| *amara* subsp. *balcanica* (4) | 62 | 51·75 ± 3·30 | 6 | 0 |
| *amara* subsp. *pyrenaea* (5) | 58 | 45·00 ± 2·74 | 7 | 2 |
| *wiedemanniana* (5) | 47 | 42·80 ± 1·48 | 6 | 4 |
| *matthioli* (4) | 71 | 46·00 ± 5·10 | 9 | 0 |
| *pratensis* (1) | 32 | – | 1 | 1 |
| *crassifolia* (10) | 69 | 49·30 ± 3·62 | 15 | 4 |
| *castellana* (7) | 63 | 43·71 ± 3·25 | 17 | 1 |
| *apennina* (7) | 69 | 46·86 ± 5·34 | 15 | 3 |
| *granulosa* (4) | 57 | 44·00 ± 2·71 | 9 | 4 |
| *acris* (16) | 113 | 40·06 ± 9·12 | 51 | 0 |
| *seidlitziana* (1) | 27 | – | 4 | 4 |
| *tenera* (7) | 76 | 33·86 ± 11·2 | 15 | 0 |
| *uliginosa* (8) | 84 | 41·25 ± 9·32 | 20 | 0 |
| Total | 358 | – | 166 | 18 |

(B) Band sharing

| | Some taxa | Each taxon | All samples |
|---|---|---|---|
| *C. amara* group | 23 | 4 | 1 |
| *C. pratensis* group | 16 | 0 | 0 |
| *C. tenera* group | 5 | 0 | 0 |
| *C. acris* + *C. pratensis* group | 20 | 1 | 0 |
| *C. seidlitziana*, *C. tenera*, *C. uliginosa* | 13 | 1 | 1 |
| *C. pratensis* + *C. tenera* groups | 75 | 3 | 0 |

In (A) *n* = number of individuals; total = total number of bands present in a given taxon; mean = mean number ± standard deviation of bands present in samples of a given taxon; exclusive = bands present in at least one sample of a given taxon, but absent in other taxa; diagnostic = number of bands present in all samples of a given taxon, but absent in other taxa.

(B) Number of bands shared by a particular group of taxa, but absent in other taxa. Some taxa = bands shared by at least two taxa from a group; each taxon = bands present in each taxon of a group; all samples = bands present in all samples of all taxa of a group.

AFLP as a fingerprinting method provided higher resolution, but additional clusters, not found in the ITS phylogenetic tree, received only weak support.

*Cardamine amara* is a well-supported monophyletic group (at least at the diploid level), but low levels of ITS variation preclude exploration of subspecific differentiation within this species.

Due to the lack of supported hierarchical structure within the second main lineage, monophyletic origins of the *C. tenera* group or the diploid taxa from the *C. pratensis* group could not be supported nor rejected. A predominance of reticulate *vs*. divergent evolutionary history might have brought about the pattern observed. The question arises whether standard phylogenetic analyses, even when using a combination of markers, can adequately reconstruct relationships among the closely related species and subspecies studied here.

The present study has several implications for further investigation of polyploid complexes of *C. amara*, *C. pratensis* and *C. raphanifolia*. Care is required in interpretation of results derived from studies restricted to only one of these complexes. When tracing origins of polyploid taxa, a much more complex scenario should be expected, taking into account all relatives as potential parents, irrespective of the group in which they are classified.

## ACKNOWLEDGEMENTS

The authors are grateful to Tod F. Stuessy and Andreas Tribsch for useful comments on the manuscript, to those listed in Table 1 for their assistance in the field, and to Michael Barfuss for preparing polyacrylamide gels in AFLP analyses. The study was supported by an IAPT Research Grant to J.L., by the Grant Agency VEGA, Bratislava, Slovak Republic (grant no. 3042) and by project no. 1131-4 from the Ministry of Education, Youth and Sports of the Czech Republic, both to K.M.

## LITERATURE CITED

**Al-Shehbaz IA. 1988.** The genera of *Arabideae* (Cruciferae, Brassicaceae) in the Southeastern United States. *Journal of the Arnold Arboretum* **69**: 85–166.

**Bachmann K. 2000.** Molecules, morphology and maps: new directions in evolutionary genetics. *Plant Species Biology* **15**: 197–210.

**Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF,**

**Campbell ChS, Donoghue MJ. 1995.** The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Annals of the Missouri Botanical Garden* **82**: 247–277.

**Bleeker W, Franzke A, Pollmann K, Brown AHD, Hurka H. 2002.** Phylogeny and biogeography of Southern Hemisphere high-mountain *Cardamine* species. *Australian Systematic Botany* **15**: 575–581.

**Chase MW, Hills HG. 1991.** Silica gel: an ideal material for field preservation of leaf samples for DNA studies. *Taxon* **40**: 215–220.

**Comes HP, Kadereit JW. 1998.** The effect of Quaternary climatic changes on plant distribution and evolution. *Trends in Plant Science* **3**: 432–438.

**Cullen J. 1965.** *Cardamine* L. In: Davis PH, ed. *Flora of Turkey and East Aegean Islands. Vol. 1*. Edinburgh: Edinburgh University Press, 438–444.

**Doyle JJ, Doyle JL. 1987.** A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin Botanical Society of America* **19**: 11–15.

**Farris JS. 1989.** The retention index and the rescaled consistency index. *Cladistics* **5**: 417–419.

**Felsenstein J. 1985.** Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.

**Franzke A, Hurka H. 2000.** Molecular systematics and biogeography of the *Cardamine pratensis* complex (Brassicaceae). *Plant Systematics and Evolution* **224**: 213–234.

**Franzke A, Mummenhoff K. 1999.** Recent hybrid speciation in *Cardamine* (Brassicaceae) – conversion of nuclear ribosomal ITS sequences in statu nascendi. *Theoretical and Applied Genetics* **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 Geobotanica* **33**: 225–240.

**Fuertes Aguilar J, Nieto Feliner G. 2003.** Additive polymorphisms and reticulation in an ITS phylogeny of thrifts (*Armeria*, Plumbaginaceae). *Molecular Phylogenetics and Evolution* **28**: 430–447.

**Fuertes Aguilar J, Roselló JA, Nieto Feliner G. 1999.** Nuclear ribosomal DNA (nrDNA) concerted evolution in natural and artificial hybrids of *Armeria* (Plumbaginaceae). *Molecular Ecology* **8**: 1341–1346.

**Graur D, Li W-H. 1999.** *Fundamentals of molecular evolution*. Sunderland, MA: Sinauer Associates.

**Gutiérrez Larena B, Fuertes Aguilar J, Nieto Feliner G. 2002.** Glacial-induced altitudinal migrations in *Armeria* (Plumbaginaceae) inferred from patterns of chloroplast DNA haplotype sharing. *Molecular Ecology* **11**: 1965–1974.

**Hodkinson TR, Chase MW, Takahashi C, Leitch IJ, Bennett MD, Renvoize SA. 2002.** The use of DNA sequencing (ITS and *trnL-F*), AFLP, and fluorescent *in situ* hybridization to study allopolyploid *Miscanthus* (Poaceae). *American Journal of Botany* **89**: 279–286.

**Hughes CE, Bailey CD, Harris SA. 2002.** Divergent and reticulate species relationships in *Leucaena* (Fabaceae) inferred from multiple data sources: insights into polyploid origins and nrDNA polymorphism. *American Journal of Botany* **89**: 1057–1073.

**Jalas J, Suominen J, eds. 1994.** *Atlas florae europaeae. Vol. 10*. Helsinki: The Committee for Mapping the Flora of Europe and Societas Biologica Fennica Vanamo.

**Jones BMG, Akeroyd J. 1993.** *Cardamine* L. In: Tutin TG *et al*., eds. *Flora Europaea, Vol. 1, 2nd edn*. Cambridge: Cambridge University Press, 346–351.

**Kerner A. 1870.** Novae plantarum species Tirolia, Venetiae, Carnioliae, Styriae et Austriae. *Zeitschrift des Ferdinandeum für Tirol und Vorarlberg* **15**: 247–292.

**Khatri KS. 1988.** Studies in the Caucasian species of *Cardamine* L. (Cruciferae). *Phyton (Horn, Austria)* **28**: 55–80.

**Kluge AG, Farris JS. 1969.** Quantitative phyletics and the evolution of anurans. *Systematic Zoology* **18**: 1–32.

**Koch M, Dobeš C, Mitchell-Olds T. 2003.** Multiple hybrid formation in natural populations: concerted evolution of the internal transcribed spacer of nuclear ribosomal DNA (ITS) in North American *Arabis divaricarpa* (Brassicaceae). *Molecular Biology and Evolution* **20**: 338–350.

**Koch M, Haubold B, Mitchell-Olds T. 2001.** Molecular systematics of the Brassicaceae: evidence from coding plastidic *matK* and nuclear *Chs* sequences. *American Journal of Botany* **88**: 534–544.

**Lihová J, Marhold K, Neuffer B. 2000.** Taxonomy of *Cardamine amara* (Cruciferae) in the Iberian Peninsula. *Taxon* **49**: 747–763.

**Lihová J, Marhold K, Tribsch A, Stuessy TF. 2004*a*.** Morphometric and AFLP re-evaluation of tetraploid *Cardamine amara* (Brassicaceae) in the Mediterranean. *Systematic Botany* **29**: 134–146.

**Lihová J, Tribsch A, Marhold K. 2003.** The *Cardamine pratensis* (Brassicaceae) group in the Iberian Peninsula: taxonomy, polyploidy and distribution. *Taxon* **52**: 783–802.

**Lihová J, Tribsch A, Stuessy TF. 2004*b*.** *Cardamine apennina*: a new endemic diploid species of the *C. pratensis* group (Brassicaceae) from Italy. *Plant Systematics and Evolution* (in press).

**Lövkvist B. 1956.** The *Cardamine pratensis* complex. Outlines of its cytogenetics and taxonomy. *Symbolae Botanicae Upsalienses* **14/2**: 1–131.

**Marhold K. 1992.** A multivariate morphometric study of the *Cardamine amara* group (Cruciferae) in the Carpathian and Sudeten mountains. *Botanical Journal of the Linnean Society* **110**: 121–135.

**Marhold K. 1993.** *Cardamine amara* L. & *C. pratensis* L. In: Tutin TG *et al*., eds. *Flora Europaea, Vol. 1, 2nd. edn*. Cambridge: Cambridge University Press, 348–350.

**Marhold K. 1994.** Taxonomy of the genus *Cardamine* L. (Cruciferae) in the Carpathians and Pannonia. I. *Cardamine pratensis* group. *Folia Geobotanica et Phytotaxonomica* **29**: 335–374.

**Marhold K. 1995.** Taxonomy of the genus *Cardamine* L. (Cruciferae) in the Carpathians and Pannonia. II. *Cardamine amara* L. *Folia Geobotanica et Phytotaxonomica* **30**: 63–80.

**Marhold K. 1998.** Morphometric comparison of diploid populations of *Cardamine amara* (Brassicaceae) from Central Europe and the Balkan Peninsula. *Thaiszia – Journal of Botany* **8**: 19–32.

**Marhold K. 1999.** Taxonomic evaluation of the tetraploid populations of *Cardamine amara* (Brassicaceae) from the Eastern Alps and adjacent areas. *Botanica Helvetica* **109**: 67–84.

**Marhold K, Ančev ME, Kit Tan 1996.** A new subspecies of *Cardamine amara* (Brassicaceae) from Bulgaria and Greece. *Annales Botanici Fennici* **33**: 199–204.

**Marhold K, Huthmann M, Hurka H. 2002*a*.** Evolutionary history of the polyploid complex of *Cardamine amara* (Brassicaceae): isozyme evidence. *Plant Systematics and Evolution* **233**: 15–28.

**Marhold K, Lihová J, Perný M, Grupe R, Neuffer B. 2002*b*.** Natural hybridization in *Cardamine* (Brassicaceae) in the Pyrenees: evidence from morphological and molecular data. *Botanical Journal of the Linnean Society* **139**: 275–294.

**Marhold K, Perný M, Kolník M. 2003.** Miscellaneous validations in Cruciferae and Crassulaceae. *Willdenowia* **33**: 69–70.

**Nei M, Li W-H. 1979.** Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Acadademy of Sciences of the USA* **76**: 5269–5273.

**PE Applied Biosystems 1996.** *AFLP™ Plant Mapping. Protocol*. Foster City, CA: PE Applied Biosystems.

**Rico E. 1993.** 22. *Cardamine* L. In: Castroviejo S *et al*., eds. *Flora Iberica, Vol. 4*. Madrid: Real Jardín Botánico, CSIC, 119–133.

**Saitou N, Nei M. 1987.** The neighbor-joining method, a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406–425.

**Sang T, Crawford DJ, Stuessy TF. 1995.** Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: implications for biogeography and concerted evolution. *Proceedings of the National Academy of the USA* **92**: 6813–6817.

**Schönswetter P, Tribsch A, Stehlik I, Niklfeld H. 2004.** Glacial history of high alpine *Ranunculus glacialis* (Ranunculaceae) in the European Alps in a comparative phylogeographical context. *Biological Journal of the Linnean Society* **81**: 183–195.

**Schulz OE. 1903.** Monographie der Gattung *Cardamine*. *Botanische Jahrbücher für Systematik, Pflanzengeschichte und Pflanzengeographie* **32**: 280–623.

**Spasskaya NA. 1978.** Konspekt sistemy roda *Cardamine* L. dlya evropeiskoi chasti Sovetskogo soyuza. *Vestnik Leningradskogo Universiteta, Biologiya* **15**: 59–67.

**Strid A. 1986.** *Mountain flora of Greece, Vol. 1*. Cambridge: Cambridge University Press.

**Sweeney PW, Price RA. 2000.** Polyphyly of the genus *Dentaria* (Brassicaceae): evidence from trnL intron and ndhF sequence data. *Systematic Botany* **25**: 468–478.

**Swofford DL. 2001.** *PAUP*: Phylogenetic analysis using parsimony (* and other methods)*. Sunderland, MA: Sinauer Associates.

**Taberlet P, Fumagalli L, Wust-Saucy AG, Cosson JF. 1998.** Comparative phylogeography and postglacial colonization routes in Europe. *Molecular Ecology* **7**: 453–464.

**Urbanska KM, Hurka H, Landolt E, Neuffer B, Mummenhoff K. 1997.** Hybridization and evolution in *Cardamine* (Brassicaceae) at Urnerboden, Central Switzerland: biosystematic and molecular evidence. *Plant Systematics and Evolution* **204**: 233–256.

**Urbanska-Worytkiewicz K, Landolt E. 1974.** Biosystematic investigations in *Cardamine pratensis* L. s.l. I. Diploid taxa in central Europe and their fertility relationships. *Berichte des Geobotanischen Institutes Eidg. Techn. Hochschule Stiftung Rübel* **42**: 42–139.

**Van de Peer Y, de Wachter R. 1994.** TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Computer Applications in the Biosciences* **10**: 569–570.

**Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M. 1995.** AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407–4414.

**White TJ, Bruns T, Lee S, Taylor J. 1990.** Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M, Gelfand D, Sninsky JJ, White TJ, eds. *A guide to methods and application*. San Diego: Academic Press, 315–322

**Yang Y-W, Lai K-N, Tai P-Y, Ma D-P, Li W-H. 1999.** Molecular phylogenetic studies of *Brassica*, *Rorippa*, *Arabidopsis* and allied genera based on internal transcribed spacer region of 18S–25S rDNA. *Molecular Phylogenetics and Evolution* **13**: 455–462.