

# Allopolyploid origin of *Cardamine silana* (Brassicaceae) from Calabria (southern Italy): karyological, morphological and molecular evidence

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Endemic *Cardamine silana* from Calabria (southern Italy) previously reported to be related to *C. raphanifolia* was found to be hexaploid. Morphological characters and AFLP data were analysed to evaluate the degree of differentiation of *C. silana* from closely related taxa and to find parental taxa of this polyploid. *Cardamine apennina* from the *C. pratensis* group was examined as one putative parent, as indicated in previous studies of nuclear ITS sequences, along with other related taxa based on both cpDNA and ITS sequences. Both multivariate morphometric analyses of quantitative characters and evaluation of qualitative morphological characters showed: (1) closest position of *C. silana* to two diploids: *C. acris* from the Balkan Peninsula and *C. apennina* from Central Italy; (2) good extent of morphological separation of *C. silana* from related taxa; and (3) within *C. acris* subspecies, *C. acris* ssp. *vardousiae* from Central Greece as closest to *C. silana*. Neighbour-joining tree and PCoA ordinations of AFLP data, as well as patterns of AFLP bands sharing, corroborated results of multivariate morphometrics. This evidence supports an allopolyploid origin of *C. silana*, with *C. apennina* and *C. acris* as parental taxa. Its origin may be dated to Pleistocene glacial events, because of the presumably wider geographical distributions of its parental taxa during more humid periods at that time. © 2005 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2005, 148, 101–116.

**ADDITIONAL KEYWORDS:** AFLPs – Apennine Peninsula – Balkans – biogeography – *Cardamine acris* – *Cardamine pratensis* – *Cardamine raphanifolia* – chromosome numbers – multivariate morphometrics – taxonomy.

## INTRODUCTION

The genus *Cardamine* L. comprises several species groups with a high proportion of polyploids. From section *Cardamine*, two of them, the *C. amara* and *C. pratensis* groups, have been frequently studied in their European ranges in the past 50 years (e.g. Lövkvist, 1956, 1957; Marhold, 1992, 1994, 1995, 1996, 1999; Urbanska *et al.*, 1997; Urbanska & Landolt, 1999; Franzke & Hurka, 2000; Lihová, Marhold &

Neuffer, 2000; Lihová, Marhold & Tribsch, 2003; Lihová *et al.*, 2004a, b). Little is known, however, about morphological variation patterns, relationships and evolution of taxa traditionally considered to be related to *C. raphanifolia* Pourr. A recent molecular phylogenetic study of diploid taxa of the *C. amara* and *C. pratensis* groups, and those around *C. raphanifolia* showed very close relationships among them, and indicated reticulate rather than branching patterns of evolution even at the diploid level (Marhold *et al.*, 2004).

Polyploidy plays one of the key roles in the evolution of vascular plants, with approximately 70% of angiosperms having polyploidy in their evolutionary history (Masterson, 1994). Despite their widespread

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occurrence, the origin of most polyploid taxa is unknown. The origin of polyploids especially in cases of allopolyploids is challenging (e.g. Widmer & Baltisberger, 1999; Hodkinson *et al.*, 2002). Molecular data have brought new and powerful insights for studies of polyploidy and helped to resolve the origin and evolution of polyploids, as well as phylogenetic relationships to allied taxa (reviewed in, e.g. Soltis & Soltis, 1999, 2000; Osborn *et al.*, 2003).

In this paper, we present an example of polyploid evolution in *Cardamine silana* Marhold & Perný (Brassicaceae), a hexaploid endemic from Calabria (southern Italy). Distribution of this taxon is restricted to a few localities in the Sila Mountains in Calabria, an area known as an important refugium during Pleistocene glaciations as well as a meeting point between Balkan and Apennine floras (Fineschi *et al.*, 2002). Not only does the parentage of this polyploid remain unresolved, but also its taxonomic position with respect to related taxa.

*Cardamine silana* has been always reported as being close to *C. raphanifolia* Pourr. (= *C. latifolia* Vahl), a taxon described from the Pyrénées (Jones & Akeroyd, 1993; Jalas & Suominen, 1994). Other taxa considered to be closely related include *C. gallaecica* (M. Laínz) Rivas Mart. & Izco (= *C. raphanifolia* ssp. *gallaecica* M. Laínz) from north-west Spain, *C. acris* Griseb. [= *C. raphanifolia* ssp. *acris* (Griseb.) O. E. Schulz] from the Balkan Peninsula, and *C. barbaraeoides* Halácsy [= *C. raphanifolia* ssp. *barbaraeoides* (Halácsy) Strid] from north-west Greece (Jones & Akeroyd, 1993; Rico, 1993; Jalas & Suominen, 1994). These taxa are octoploid (*C. raphanifolia*), hexaploid (*C. raphanifolia*, *C. gallaecica*), tetraploid (*C. gallaecica*, *C. barbaraeoides*) and diploid (*C. acris*) (Ančev, Marhold & Goranova, 1997; Perný, Tribsch & Ančev, 2004).

*Cardamine silana* was recognized as a distinct taxon as early as in 1821 by de Candolle, who, based on plants from Calabria, described the variety *C. latifolia* var. *calabrica* (de Candolle, 1821, see also de Candolle, 1824). This approach was followed by Tenore (1831) and Parlatore (1890), but later these populations were treated as *C. raphanifolia* (Pignatti, 1982), or even as *C. raphanifolia* ssp. *raphanifolia* (Zángheri, 1976). Often, Calabrian populations were also mentioned in a note within the accounts of *C. raphanifolia* with calls for detailed comparative studies to resolve their final taxonomic position (Pignatti, 1982; Jones & Akeroyd, 1993). Based on our preliminary results, the *nomen novum* *C. silana* was recently published to validate the species level name for these populations for the purposes of the Euro+Med PlantBase Project (Marhold, Perný & Kolník, 2003).

A recent study on nucleotide variation in ITS regions of nrDNA and noncoding regions of cpDNA

focusing mainly on the *C. amara* group, but also involving other lineages of Eurasian *Cardamine* sect. *Cardamine* (Lihová *et al.*, 2004a), also shed some light on *C. silana*. The ITS sequences of *C. silana* were very similar to those of the diploid central Italian endemic *C. apennina* Lihová & Marhold which belongs to the *C. pratensis* group (Lihová, Tribsch & Stuessy, 2004c); in fact, they differed by a single substitution. In both accessions of *C. silana* included in this study, five nucleotide sites showed within-individual polymorphism, indicating the presence of different ITS paralogues in their genomes. Four of the sites displayed an additive pattern, with an alternative base found exclusively in *C. apennina*. The ITS variation observed therefore strongly favours involvement of the central Italian diploid *C. apennina* in the origin of hexaploid *C. silana*. On the other hand, *trnL-trnF* cpDNA sequences of *C. silana* showed pronounced divergence from those of *C. apennina* (with several substitutions and indels recorded). This suggests that *C. silana* is of allopolyploid origin and *C. apennina* acted as the paternal donor (maternal plastid transmission occurs in Brassicaceae; Harris & Ingram, 1991).

In order to find the taxonomic position and to track the polyploid evolution of *C. silana*, we evaluated karyological, morphometric and AFLP-fingerprinting data from populations of this taxon together with its putative close relatives, namely *C. raphanifolia* and taxa considered to be related to this species (see above), and the already mentioned *C. apennina*. Combination of such methods has been already successfully used when addressing issues of taxonomic relationships, polyploidy and hybridization in previous studies of the genus *Cardamine* (Marhold *et al.*, 2002; Lihová *et al.*, 2003, 2004b, c), as well as other taxa (e.g. Hansen, Elven & Brochmann, 2000; Fjellheim, Elven & Brochmann, 2001; Ishida *et al.*, 2003). The aims of the study presented in this paper were: (1) to evaluate the taxonomic position of populations of *C. silana* and the degree of its morphological and molecular differentiation from closely related taxa; and (2) to explore the parentage of this allopolyploid taxon considering a potential impact of Pleistocene climatic changes on its evolution.

## MATERIAL AND METHODS

### PLANT MATERIAL

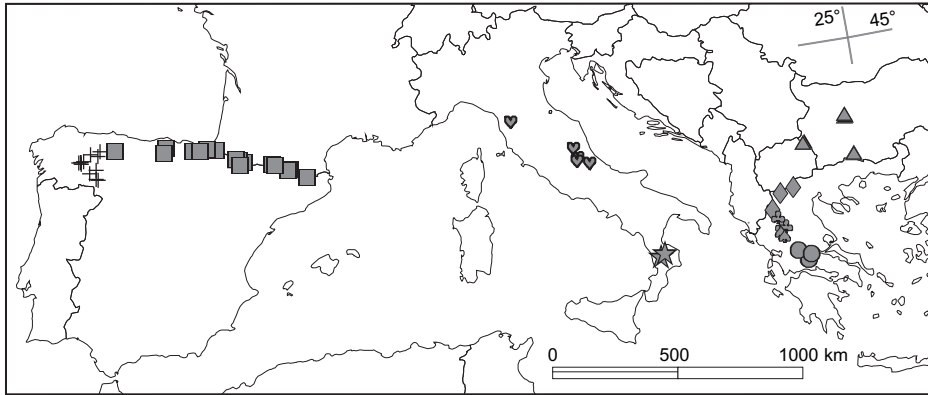
The origin of plant material studied is listed in Table 1 and illustrated in Figure 1. Sampling of all taxa covered most parts of their distribution areas. For karyological analyses, living plants were collected in the field and cultivated at the Institute of Botany, Slovak Academy of Sciences, Bratislava. Chromosome numbers were checked in two to four individuals per locality. For morphometric analyses mostly 20–30 plants

**Table 1.** List of populations and number of studied plants of *Cardamine silana*, *C. apennina*, *C. acris*, *C. barbaraeoides*, *C. raphanifolia* and *C. gallaecica* used for karyological, morphometric and AFLP analyses. The chromosome numbers ( $2n$ ) without a number in superscript represent new data, those with a superscript are taken from other studies: <sup>1</sup>Lihová *et al.* (2003), <sup>2</sup>Perný *et al.* (2004), <sup>3</sup>M. Perný, J. Lihová, A. Tribsch & K. Marhold (unpubl. data), <sup>4</sup>M. Perný, A. Tribsch, T. F. Stuessy, K. Marhold (unpubl. data). ‘–’ not used in the particular analysis

Code	Locality and collection data	$2n$	Morph	AFLP
<i>Cardamine silana</i> Marhold & Perný				
FOS	Italy, Calabria, Sila Grande Mts., Fossiateda, 1270 m, 12.vi.1999, <i>Marhold &amp; Passalacqua</i>	48	–	1
Si1	Italy, Calabria, Sila Grande Mts., Stazio Meteorologica 1750 m, 12.vi.1999, <i>Marhold &amp; Passalacqua</i>	48	32	2
Si2	Italy, Calabria, Sila Grande Mts., Monte Curcio, SE slope, 1680 m, 12.vi.1999, <i>Marhold &amp; Passalacqua</i>	48	28	2
Si3	Italy, Calabria, Sila Grande Mts., Macchianelo, 1640 m, 13.vi.1999, <i>Marhold &amp; Passalacqua</i>	48	30	1
Si4	Italy, Calabria, Sila Grande Mts., Macchia Sacra, 1660 m, 13.vi.1999, <i>Marhold &amp; Passalacqua</i>	48	32	1
<i>Cardamine apennina</i> Lihová & Marhold				
LG	Italy, Abruzzo, Lago di Campotosto, Le Serre, 1340 m, 27.v. 2000, 27.iv.2001, <i>Lihová et al.</i>	16 <sup>1</sup>	31	2
PC	Italy, Abruzzo, Gran Sasso, Sorgenti del Vomano, Passo delle Capannelle, 1257 m, 27.v.2000, 27.iv.2001, <i>Lihová et al.</i>	16 <sup>1</sup>	8	3
PG	Italy, Umbria, Monti Sibillini, Pian Grande, 1250 m, 26.v.2000, <i>Lihová et al.</i>	16 <sup>1</sup>	14	–
QS	Italy, Toscana, Stáffoli, 8 m, 26.iv.2001, <i>Lihová et al.</i>	16 <sup>1</sup>	18	3
VO	Italy, Abruzzo, Gran Sasso, Voltigno, under Mt. Fiore, 1360 m, 27.iv.2001, <i>Lihová et al.</i>	16 <sup>1</sup>	–	3
<i>Cardamine acris</i> Griseb. ssp. <i>acris</i> – Bulgaria				
AR	Bulgaria, Zapadni Rodopi, Pamporovo, Ardashla, 1450 m, 25.vi.2000, <i>Perný</i>	16 <sup>2</sup>	30	2
OG	Bulgaria, Osogovska planina, above Osogovo chalet, 28.vi.2000, <i>Perný &amp; Anchev</i>	16 <sup>2</sup>	22	–
RP	Bulgaria, Stara planina, Tetevenska planina, Ribarishki prokhoz, 1650 m, 22.vi.2000, <i>Perný &amp; Georgieva</i>	16 <sup>2</sup>	38	–
VN	Bulgaria, Stara planina, Tetevenska planina, NW of Vezhen (2198 m), 1850 m, 21.vi.2000, <i>Perný &amp; Georgieva</i>	16 <sup>2</sup>	25	1
ZA	Bulgaria, Stara planina, Tetevenska planina, NW of Vezhen (2198 m), Zavodna stream, 1500 m, 22.vi.2000, <i>Perný &amp; Georgieva</i>	16 <sup>2</sup>	–	1
GR	Bulgaria, Zapadni Rodopi, Pamporovo, Golyamata Reka, 1490 m, 25.vi.2000, <i>Perný</i>	16 <sup>2</sup>	–	1
GE	Bulgaria, Osogovska planina, W slopes from Gramadite (W of Trite Buky chalet) to the Ruen Hill, 1600 m, 28.vi.2000, <i>Perný &amp; Anchev</i>	16 <sup>2</sup>	–	2
OC	Bulgaria, Zapadni Rodopi, Pamporovo, Orlitsa, 1600 m, 25.vi.2000, <i>Perný</i>	16 <sup>2</sup>	–	1
<i>Cardamine acris</i> Griseb. ssp. <i>acris</i> – NW Greece and Montenegro				
MN	Montenegro, Mojkočač, Bjelasica Mts., Zekova glava Mt. 1900 m, 25.vii.2001, <i>Šida et al.</i>	16 <sup>2</sup>	–	1
AE	Greece, N Pindhos, Ioannina/Kastoria prov., Gramos Mts., Aetomilitsa, SE slope of the valley c. 5 km N of the village, 19.vi.2001, <i>Perný &amp; V. Kučera</i>	16 <sup>2</sup>	34	2
VR	Greece, North Central, Pella prov., Voras Mts., Voras Ski Resort, stream along the road near the resort, 21.vi.2001, <i>Perný &amp; V. Kučera</i>	16 <sup>2</sup>	23	1
2 PD	Greece, North Central, Florina prov., Varnous Mts. Pisoderi, 20.vi.2001, <i>Perný &amp; V. Kučera</i>	16 <sup>2</sup>	–	3
<i>Cardamine acris</i> ssp. <i>pindicola</i> Perný & Marhold				
KPN	Greece, N Pindhos, Ioannina prov., N Pindhos Mts., Metsovo, Katara Pass, c. 1.7 km S of Mt. Katara (1820 m), micropopulations: 1KPN, 2KPN, 3KPN, 18.vi.2001, <i>Perný &amp; V. Kučera</i>	16 <sup>2</sup>	–	3
2KP	Greece, N Pindhos, Ioannina prov., N Pindhos Mts., Metsovo, Katara Pass, c. 2.5 km S of Mt. Katara (1820 m), before crossroad to Haliki, 18.vi.2001, <i>Perný &amp; V. Kučera</i>	16 <sup>2</sup>	22	1
VSC	Greece, N Pindhos, Grevena/Ioannina prov., N Pindhos Mts., Vasilitsa Ski Resort, little stream below road, 18.vi.2001, <i>Perný &amp; V. Kučera</i>	16 <sup>2</sup>	–	2
<i>Cardamine acris</i> ssp. <i>vardousiae</i> Perný & Marhold				
TM	Greece, Sterea Ellas, Evritania prov., Timfristos Mts., Karpenisi Ski Resort, stream c. 2.5 km NW of the resort, 24.vi.2001, <i>Perný &amp; V. Kučera</i>	16 <sup>2</sup>	31	1
1VD	Greece, Sterea Ellas, Fokida prov., Vardousia Mts., c. 6 km WSW of Athanasios Diakos, pass between the hills 2340 m and 2495 m, 23.vi.2001, <i>Perný &amp; V. Kučera</i>	16 <sup>2</sup>	19	1

**Table 1.** *Continued*

Code	Locality and collection data	2n	Morph	AFLP
2VD	Greece, Sterea Ellas, Fokida prov., Vardousia Mts, c. 6 km WSW of Athanasios Diakos, stream c. 500 m of subpopulation VDA, 23.vi.2001, <i>Perný &amp; V. Kučera</i>	16 <sup>2</sup>	24	1
ITI	Greece, Sterea Ellas, Fthiotida prov., Iti Mts., c. 10 km E of Kastania, near the crossroads close to brick spring, 24.vi.2001, <i>Perný &amp; V. Kučera</i>	16 <sup>2</sup>	–	2
<i>Cardamine barbaraeoides</i> Halácsy				
02KA	Greece, S Pindhos, Ioannina prov., Lakmos Mts., c. 6 km N of Kalarites, in the stream above the road, 27.v.2002, <i>Perný</i>	32 <sup>3</sup>	42	–
3KA	Greece, S Pindhos, Ioannina prov., Lakmos Mts., c. 5 km N of Kalarites, in the stream above the road, 27.v.2002, <i>Perný</i>	32 <sup>3</sup>	14	–
<i>Cardamine raphanifolia</i> Pourr.				
CN	Spain, Catalonia, Can Nofre, 1200 m, 6.vi.2000, <i>Perný &amp; Vicens</i>	48 <sup>4</sup>	32	1
OR	Andorra, Pyrénées, Ordino, stream in the village, 1300 m, 8.vi.2000, <i>Perný</i>	48 <sup>4</sup>	–	1
SM	Andorra, Sispony, the Montaner stream, 8.vi.2000, <i>Perný</i>	48 <sup>4</sup>	26	1
GAU	Spain, Lérida, Val d'Aran, W of Vielha, along the stream Barranc de Casau, 1560 m, 22.vi.2002, <i>Lihová</i>	48 <sup>4</sup>	22	–
TRE	Spain, Lérida, Val d'Aran, S of Salardú, near Banhs de Tredós, Pónt dera Montanheta, Arriu d'Aiguamóg 1880 m, 26.vi.2002, <i>Lihová</i>	64 <sup>4</sup>	23	–
BOR	Spain, Huesca, Puerto de Portalet, Puento Brocuso, SE of the pass, 1700 m, 1.vi.2001, <i>Perný &amp; Sanz</i>	48 <sup>4</sup>	28	1
MCR	Spain, Huesca, Pyrénées, Selva de Oza, N slope of Monte Campanil, 30.v.2001, <i>Perný &amp; Villar</i>	48 <sup>4</sup>	–	1
VAR	Spain, Huesca, Vilanúa, under the bridge of the entrance to the village, 950 m, 31.v.2001, <i>Perný &amp; Villar</i>	48 <sup>4</sup>	–	1
LF	France, Vallée d'Aspe, Les Forges d'Abel S of Urδος, above N mouth of the Sampont Tunnel, 1200 m, 31.v.2001, <i>Perný &amp; Villar</i>	48 <sup>4</sup>	32	1
QRR	Spain, Navarra, Quinto Real, 2.2 km S of Irurito, 700 m, 8.v.2001, <i>Lihová &amp; Biurrun</i>	64 <sup>4</sup>	20	1
AM	Spain, Álava, Vittoria-Gasteiz, village Amarita, river Santa Engracia, 500 m, 22.v.2001, <i>Perný &amp; Biurrun</i>	64 <sup>4</sup>	–	1
VT	Spain, Álava, Zuya, Vitoriano, Río Ugalde, 600 m, 22.v.2001, <i>Perný &amp; Biurrun</i>	64 <sup>4</sup>	–	1
SG	Spain, Guipuzcoa, Otzaurte, 600 m, 22.v.2001, <i>Perný &amp; Biurrun</i>	48, 64 <sup>4</sup>	18	–
PL	Spain, Cantabria, Puerto de Los Tornos, 800 m, 21.v.2001, <i>Perný &amp; Herrera</i>	48 <sup>4</sup>	–	1
PO	Spain, Cantabria, Portillo de la Sía, 1000 m, 21.v.2001, <i>Perný &amp; Herrera</i>	48 <sup>4</sup>	36	1
AP	Spain, Asturias, Pajares, below Puerto Pajares, near the road, 24.v.2001, <i>Perný &amp; Llamas</i>	64 <sup>4</sup>	20	1
<i>Cardamine gallaecica</i> (M. Laínz) Rivas Mart. & Izco				
CS	Spain, Ourense, Serra de Mina Mts., Casaio, close to the village, 11.vi.2000, <i>Perný</i>	48 <sup>4</sup>	28	1
CB	Spain, León, Carbón del Sil, stream above the road c. 3 km of the village in the direction to Palacios del Sil, 27.v.2001, <i>Perný et al.</i>	48 <sup>4</sup>	32	1
LEG	Spain, León, Puerto de Leitariegos, NE slope, 1600 m, 25.v.2001, <i>Perný et al.</i>	48 <sup>4</sup>	–	1
LLA	Spain, Sierra Segundera, Barrios de Vigo (de Sanabria), along right tributary of Rio de la Forcadura, between localities Los Riegos a Marogates, 1250–1500 m, 27.vi.2002, <i>Marhold</i>	48 <sup>4</sup>	26	–
MD	Spain, Lugo, Serra do Courel Mts., Mirad, 14.vi.2000, <i>Perný</i>	32, 48 <sup>4</sup>	29	1
PA	Spain, Lugo, Serra do Courel Mts., Paderne, 14.vi.2000, <i>Perný</i>	32 <sup>4</sup>	44	–
PG	Spain, León, Peñalba de Santiago (S of Ponferrada), below the village, 27.v.2001, <i>Perný et al.</i>	48 <sup>4</sup>	34	1
PS	Spain, Lugo, Serra do Courel Mts., Pacios, 13.vi.2000, <i>Perný et al.</i>	48 <sup>4</sup>	35	1
PT	Spain, Lugo, Serra do Courel Mts., Piedrafita, 14.vi.2000, <i>Perný et al.</i>	48 <sup>4</sup>	–	1
RG	Spain, Lugo, Serra do Courel Mts., Rogueira, 13.vi.2000, <i>Perný et al.</i>	48 <sup>4</sup>	–	1
SAN	Spain, León, Sierra de Ancares Mts., Balouta, stream above the road c. 2 km before the village, 1300 m, 27.v.2001, <i>Perný et al.</i>	48 <sup>4</sup>	–	1
VIG	Spain, Sierra Segundera Mts., Vigo de Sanabria, along tributary of Rio de la Forcadura below locality Llamas del Campo, 1160–1300 m, 28.vi.2002, <i>Marhold</i>	48 <sup>4</sup>	29	–



**Figure 1.** Map of distribution of sample sites of *Cardamine silana* (stars), *C. acris* ssp. *acris* (Bulgaria – triangles; Greece – diamonds), *C. acris* ssp. *pindicola* (clubs), *C. acris* ssp. *vardousiae* (circles), *C. apennina* (hearts), *C. barbaraeoides* (spades), *C. raphanifolia* (squares), and *C. gallaecica* (crosses) (for sample site details see Table 1).

per population were collected in the field and preserved as herbarium specimens. For AFLPs more populations were sampled than for the morphometric study, but only one to three plants per population (altogether 67 plants) were analysed to examine variation among taxa represented by as many genotypes as possible instead of examining intrapopulation variability. Voucher specimens for all the analyses performed in this study are deposited in SAV.

#### CHROMOSOME NUMBERS

Chromosomes were counted from metaphase plates of meristematic cells from roots using a squash method employed in our previous studies (Marhold *et al.*, 2002) with minor modifications. After pretreatment in 0.002 mol/dm<sup>3</sup> aqueous solution of 8-hydroxyquinoline for 5 h at 4 °C, the root tips were fixed in a freshly prepared mixture of ethanol and acetic acid (3 : 1) for 3 h at room temperature, and then stored at –20 °C. Before squashing, the root tips were hydrolysed in a mixture of concentrated hydrochloric acid and ethanol (1 : 1) for 3 min and rinsed in water. Squashes were made in a drop of 45% acetic acid under a cellophane square (Murín, 1960) and stained in a 10% solution of Giemsa stock dye in Sørensen phosphate buffer for 1 h.

#### ANALYSES OF MORPHOLOGICAL CHARACTERS

Characters included in the morphometric analyses were those traditionally used for delimitation of taxa within *C. raphanifolia*, as well as those proven to be useful in the differentiation among taxa of related *C. amara* and *C. pratensis* groups. Seventeen vegetative and seven floral (all quantitative) characters were measured on each specimen, and 13 ratios were

derived (Table 2). Seven characters were used solely for computing ratios. Flower parts of one randomly chosen flower per individual were removed when fresh, attached by an adhesive tape to a paper, dried, and then scanned with a Microtek 9800XL scanner. Characters on flower organs were measured separately with the CARNOY 2.0 computer program (Schols *et al.*, 2002), which was calibrated by a scanned image of a ruler. Average values for each flower character per specimen were then computed. In order to measure pollen grains, anthers of up to three flower buds were removed from each specimen and pollen grains were stained using acetocarmine-jelly (Kearns & Inouye, 1993). Thirty pollen grains were measured from each plant and average values of pollen grain diameter were calculated. In some plants it was not possible to measure pollen grains (because of the late flowering stage and lack of pollen). In such cases, mean values per given population replaced missing values in the data matrix. In addition to quantitative characters, five binary ones were scored on herbarium specimens used in morphometric analyses and on plants under cultivation originating from the same populations; an additional three binary characters were scored on cultivated plants only (presence of pruina on stem, presence of adventitious buds and rosettes on basal leaves and shape of hairs on rachis of basal leaves) (see Table 2). As these characters could not be scored on each analysed specimen, they were not used in the morphometric evaluation, but evaluated and discussed separately.

Multivariate analyses were performed on three data sets (matrices) to provide insight on overall patterns of variation and to search for morphological discontinuities: matrix A, a set including *C. silana* ( $2n = 6x$ ; Calabria, southern Italy) and five related species: *C. acris* ( $2n = 2x$ ; Balkans), *C. barbaraeoides* ( $2n = 4x$ ; north-

**Table 2.** List of characters scored and measured for morphometric analyses.

VEGETATIVE CHARACTERS	
PR*	basal leaf-rosette present – 1, absent – 0
STE*	stem erect – 1, ascending – 0
STP*	stem pruinose – 1, not pruinose – 0
ROS*	adventitious buds and leaf-rosettes at base of terminal leaflets of basal leaves present – 1, absent – 0
WIS	width of stem (mm)
LSL	height of stem from the base to the base of the uppermost stem leaf (cm)
LSI	height of stem from the base to the base of the lowermost flower/fruit (cm)
NL	number of stem leaves
LC1	length of basal leaf (cm)
LC2†	length of middle stem leaf (cm)
LC3	length of the uppermost leaf (cm)
NFB	number of pairs of lateral leaflets of basal leaf
LTB and WTB	length and width of terminal leaflet of basal leaf (cm)
LLB and WLB	length and width of first (from the tip) lateral leaflet of basal leaf (cm)
LPB*	petiole of first lateral leaflet of basal leaf present – 1, absent – 0
HRA*	orientation of hairs on rachis of young basal leaves (if present) patent – 1, curved – 0
NFS†	number of pairs of lateral leaflets of the middle stem leaf
LTS† and WTS†	length and width of terminal leaflet of the middle stem leaf (cm)
LLS† and WLS†	length and width of first lateral leaflet of the middle stem leaf (cm)
LPS*	petiole of first lateral leaflet of the middle stem leaf present – 1, absent – 0
FLORAL CHARACTERS	
LSav and WSav	average length and width of sepals (mm)
LPav and WPav	average length and width of petals (mm)
LFLav and LFSav	average length of longer and shorter filaments (mm)
DPG‡	diameter of pollen grains (µm)
CP*	colour of petals: white – 0, pink to reddish-violet – 1
RATIOS OF CHARACTERS	
LSL/LSI, LC1/LSL, LC2/LSL, WTB/LTB, WLB/LLB, LLB/LTB, WLB/WTB, WTS/LTS, WLS/LLS, LLS/LTS, WLS/WTS, WPav/LPav, LFSav/LFLav	

\* characters scored and discussed in text, but not used in multivariate morphometric analyses; † the leaf closest to the midpoint of the leafy part of the stem (LSL/2 distance); ‡ average of 30 measured pollen grains per plant.

west Greece), *C. raphanifolia* ( $2n = 6x, 8x$ ; north Iberian Peninsula), and *C. gallaecica* ( $2n = 4x, 6x$ ; north-west Spain) [i.e. 985 plant individuals (38 populations)  $\times$  25 characters]; matrix B, including *C. silana*, *C. acris* and *C. apennina* only [i.e. 461 individuals (18 populations)  $\times$  37 characters]; and matrix C with *C. silana*, and *C. acris* classified to subspecies according to Perný *et al.* (2004), [i.e. 390 individuals (14 populations)  $\times$  37 characters]. *C. acris* ssp. *acris* was further divided based on its geographical distribution. The following groups of *C. acris* were then used: (1) *C. acris* ssp. *acris* – a group of populations from Bulgaria (2) *C. acris* ssp. *acris* – a group of populations from north-west Greece (3) *C. acris* ssp. *pindicola* from north Pindhos in north-west Greece, and (4) *C. acris* ssp. *vardousiae* from central Greece.

Hypotheses of morphological separation of Calabrian populations from other taxa and the relationships of this taxon to putative parents were tested by

several canonical discriminant analyses (CDA) and classificatory discriminant analyses based on individual plants as operational taxonomic units (OTUs) and with different groups (represented by individual species or groups of populations) defined above (Klecka, 1980; Krzanowski, 1990). CDA, which by weighting characters maximizes differences between groups, generally requires multivariate normality of characters and equality of the within-group covariance matrices, but has proven to be considerably robust to departures from these assumptions (Sneath & Sokal, 1973; Thorpe, 1976; Klecka, 1980). Moreover, multivariate normality is not strictly required when CDA is used as an ordination procedure and no statistical tests are carried out (Pimentel, 1981). To determine characters mostly contributing to the group separation in an ordination diagram, total canonical structure expressing correlation of the characters with the canonical axis was computed. The classificatory dis-

criminant analysis (Klecka, 1980), which assesses the percentage of OTUs classified correctly into predicted groups, discriminant function was determined by the cross-validation procedure using *k*-nearest-neighbours. In this procedure, classification criterion is based on  $N-1$  individuals ( $N$  – total number of individuals) and then applied to classify the individual left out. Non-parametric classificatory discriminant analysis was chosen in this study as characters used in the analyses more or less deviated from the normal distribution. (1) First, the CDA was computed based on matrix A with six species as groups in order to examine the degree of separation of *C. silana* from other taxa considered to be related to *C. raphanifolia* and from *C. apennina* and their mutual position in a morphological characters space. (2) In the second step, detailed separation and relationships of morphologically close (as shown from the first step) *C. apennina*, *C. acris*, and *C. silana* were examined. For this purpose CDA and a nonparametric *k*-nearest-neighbours ( $k = 7$ ) classificatory discriminant analysis were performed using matrix B and three taxa as groups. (3) Finally, to resolve also the relationships of *C. silana* to accessions of *C. acris* from different geographical areas, another CDA was computed. In this analysis, matrix C with five groups (*C. silana* and *C. acris* divided into four groups of populations – see above) was used.

Exploratory data analysis including means, standard deviations, and percentiles was computed for all quantitative characters.

All morphometric analyses were performed using the SAS 8.2 package (SAS Institute, 2000).

#### AFLP FINGERPRINTING

Total genomic DNA was extracted from silica gel-dried leaves following the CTAB extraction protocol (Doyle & Doyle, 1987) with minor modifications (see Schönswetter *et al.*, 2004). The AFLP procedure (Vos *et al.*, 1995) followed the general protocol by Applied Biosystems (PE Applied Biosystems, 1996) with a few modifications, described in detail in Schönswetter *et al.* (2004). PCR conditions for preselective and selective amplification were the same as described by Lihová *et al.* (2004c). On a basis of a primer test with 15 different primer combinations, three primer pairs were chosen for selective amplification: *EcoRI*–AAG–(HEX), *MseI*–CTG; *EcoRI*–ATC–(6FAM), *MseI*–CAG; *EcoRI*–AGC–(NED), *MseI*–CTG. The amplified AFLP fragments were electrophoresed and detected in 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 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/>). A neighbour-joining tree based on the Nei & Li (1979) genetic distance was constructed using PAUP\* (version 4.0b10, Swofford, 2003), with the following settings: Unweighted Least Squares was chosen as the Objective Function; constraint branch lengths were set to be non-negative and branches of effectively zero length when searching were set to collapse. The tree was rooted at midpoint. Group support was assessed with the same program by repeated bootstrap analyses with 5000 replications. Principal coordinate analysis (PCoA; Krzanowski, 1990) using Jaccard's coefficient was computed to recover non-hierarchical structure of AFLP data. Non-hierarchical analysis of data represented by such ordination techniques unlike a hierarchical one represented by tree-building methods may better resolve relationships in groups affected by reticulating pattern of evolution, e.g. groups with allopolyploids (see also Bachmann, 2000). Two different PCoAs using the SYN-TAX 2000 program (Podani, 2001) were computed: (1) on a matrix of all 67 samples analysed, representing five species; and (2) on a matrix of samples of *C. acris* (three subspecies from four geographical areas included) and *C. silana*, with an aim to examine relationships in a poorly resolved cluster from the first analysis. *Cardamine barbaraeoides* was not included in the AFLP analysis as, in respect to this marker, it stands close to *C. amara*, well outside the group of taxa examined in this study, and will be evaluated separately elsewhere (M. Perný, J. Lihová, A. Tribsch & K. Marhold, unpubl. data). For each taxon (or a group of populations in the case of *C. acris*) the total number of AFLP fragments (bands), mean number of fragments per individual ( $\pm$  standard deviation), number of exclusive (present in a given taxon only, but not necessarily in all its samples) and diagnostic (present in all samples of a taxon and absent from all other taxa) fragments were calculated. Furthermore, AFLP fragment sharing among particular pairs or groups of taxa and populations was evaluated.

## RESULTS

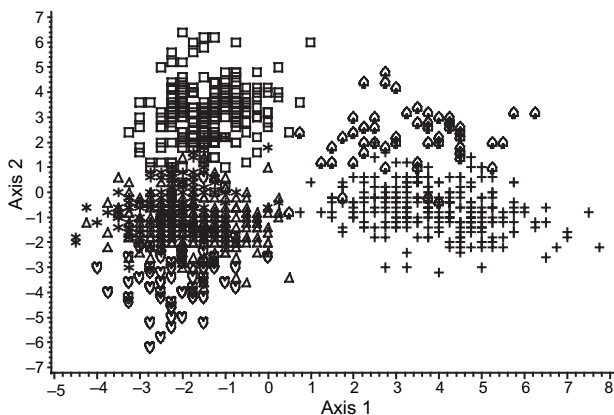
### CHROMOSOME NUMBERS

Data on chromosome numbers are presented in Table 1. The hexaploid chromosome level ( $2n = 6x = 48$ ) was confirmed for all five examined populations of *C. silana*.

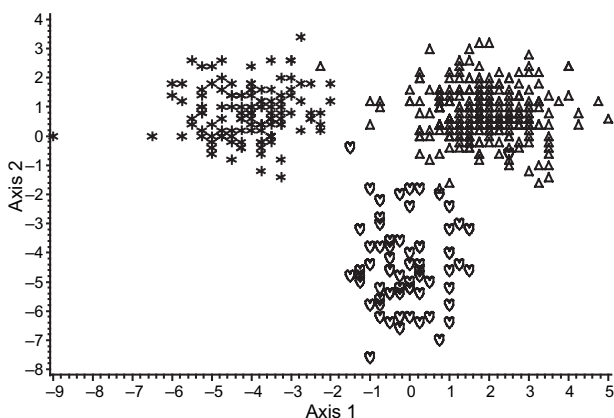
### ANALYSES OF MORPHOLOGICAL CHARACTERS

Canonical discriminant analysis of six species (matrix A) showed a group of overlapping individuals of *C. silana*, *C. acris* and *C. apennina*, separated from

*C. barbaraeoides* and *C. gallaecica* along the first axis, and, with slight overlap, from *C. raphanifolia* along the second axis (Fig. 2). As seen from canonical correlation coefficients (Table 3A) two characters, number of stem leaves (NL) and ratio LLS/LTS, were the most important for the separation of the individuals along the first axis, while length of sepals (LSav), length of filaments (LFLav, LFSav) and ratio WLS/LLS contributed mostly to the separation along the second axis. This pattern showed morphological affinity of *C. silana* to *C. acris* and *C. apennina*, while the other taxa included in the analysis appeared as morphologically more distant.



**Figure 2.** Canonical discriminant analysis based on 25 morphological characters and individuals as OTUs, with six species as groups: *Cardamine silana* (asterisks), *C. apennina* (hearts), *C. acris* (triangles), *C. barbaraeoides* (spades), *C. gallaecica* (crosses), and *C. raphanifolia* (squares). First two axes explain 48.7 and 27.1% of the variation among groups.

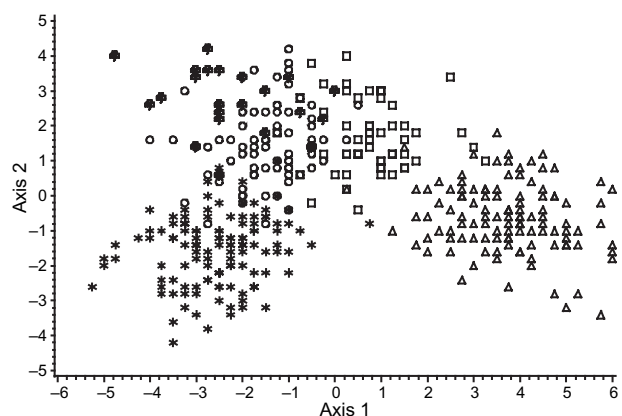


**Figure 3.** Canonical discriminant analysis based on 37 morphological characters and individuals as OTUs, with three species as groups: *Cardamine silana* (asterisks), *C. apennina* (hearts), and *C. acris* (triangles). The two axes explain 64.6 and 35.4% of the variation among groups.

CDA using *C. silana*, *C. acris* and *C. apennina* as groups (matrix B) revealed the separation of the first two species along the first axis, and both of them from *C. apennina* along the second one (Fig. 3). Diameter of pollen grains (DPG) was the most important character contributing to the separation of the taxa along the first axis, together with three other characters [length and width of petals (LPav and WPav), and length of longer filaments (LFLav), see Table 3B]. Ratio WTS/LTS was the best-separating character of *C. apennina* from the remaining two taxa along the second axis. Classificatory discriminant analysis also showed a good extent of separation, as more than 98.5% of the specimens of each taxon were correctly classified. When we examined relationships of *C. silana* to populations of *C. acris* from different geographical areas using CDA and matrix C, central Greek plants of *C. acris* ssp. *vardousiae* appeared closest to *C. silana*, while Bulgarian *C. acris* ssp. *acris* appeared in the most distant position (Fig. 4).

A summary of the most important characters differentiating *C. silana* from *C. apennina* and *C. acris*, showing 5 and 95 percentiles and median values, is shown in Table 4.

Examination of several qualitative characters not included in the morphometric analyses showed that most plants of *C. silana* were similar to *C. acris* and *C. apennina* in the presence of basal leaf-rosette and erect stem. *Cardamine raphanifolia*, *C. gallaecica* and *C. barbaraeoides* plants, on the other hand, never developed basal leaf-rosette, neither in natural habitats nor under cultivation, and stems of these species were usually ascending. All *C. silana* plants both in



**Figure 4.** Canonical discriminant analysis based on 36 morphological characters and individuals as OTUs, with the following groups: *Cardamine silana* (asterisks), *C. acris* ssp. *acris* from Bulgaria (triangles), *C. acris* ssp. *acris* from north-west Greece (squares), *C. acris* ssp. *pindicola* (clubs), and *C. acris* ssp. *vardousiae* (circles). The first two axes explain 59.9 and 17.7% of the variation among groups.

**Table 3.** Canonical discriminant analysis (CDA). Correlation coefficients of morphological characters and canonical axes (CAN1, CAN2) are presented; those exceeding 0.6 are marked in bold. A, CDA of *Cardamine silana*, *C. apennina*, *C. acris*, *C. barbaraeoides*, *C. raphanifolia*, and *C. gallaecica* (see Fig. 2). B, CDA of *C. silana*, *C. acris*, and *C. apennina* (see Fig. 3). C, CDA of *C. silana*, *C. acris* ssp. *acris* from Bulgaria, *C. acris* ssp. *acris* from north-west Greece, *C. acris* ssp. *pindicola* and *C. acris* ssp. *vardousiae* (see Fig. 4). For character abbreviations see Table 2

Character	A		B		C	
	CAN1	CAN2	CAN1	CAN2	CAN1	CAN2
WIS	0.198	0.120	0.391	0.408	<b>0.728</b>	-0.117
LSL	0.259	-0.169	0.318	0.373	<b>0.807</b>	-0.287
LSI	0.241	-0.178	0.314	0.320	<b>0.778</b>	-0.273
NL	<b>0.817</b>	-0.169	0.322	0.487	<b>0.716</b>	-0.172
LC1	-	-	0.127	0.253	0.503	-0.363
LC2	0.558	0.230	0.306	0.353	0.592	-0.143
LC3	0.247	0.437	0.315	0.125	0.160	0.279
NFB	-	-	0.584	0.455	<b>0.831</b>	0.117
LTB	-	-	0.184	0.436	0.410	-0.138
WTB	-	-	0.153	0.435	0.358	-0.140
LLB	-	-	0.220	0.281	0.443	-0.121
WLB	-	-	0.238	0.300	0.418	-0.053
NFS	0.416	-0.453	0.388	0.060	<b>0.621</b>	-0.046
LTS	0.263	0.443	0.306	0.380	0.485	-0.036
WTS	0.055	0.587	0.321	0.504	0.410	0.082
LLS	0.543	0.267	0.297	0.276	0.565	-0.123
WLS	0.323	0.522	0.327	0.413	0.386	0.112
LPav	-0.346	0.490	<b>-0.659</b>	-0.019	<b>-0.726</b>	-0.366
WPav	-0.549	0.393	<b>-0.674</b>	0.132	<b>-0.857</b>	-0.198
LSav	-0.102	<b>0.650</b>	-0.572	0.107	<b>-0.699</b>	-0.261
WSav	-0.177	0.384	-0.194	0.125	-0.565	0.306
LFLav	-0.182	<b>0.739</b>	<b>-0.679</b>	0.307	-0.590	-0.552
LFSav	0.041	<b>0.749</b>	-0.546	0.251	-0.485	-0.460
DPG	-	-	<b>-0.922</b>	-0.034	-	-
LSL/LSI	0.309	0.022	0.167	0.561	<b>0.643</b>	-0.264
LC1/LSL	-	-	-0.193	-0.169	-0.381	-0.101
LC2/LSL	0.369	0.485	0.064	0.049	-0.215	0.207
WTB/LTB	-	-	-0.124	0.082	-0.131	-0.087
WLB/LLB	-	-	0.101	0.010	-0.064	0.303
LLB/LTB	-	-	0.129	-0.307	0.278	-0.064
WLB/WTB	-	-	0.213	-0.310	0.261	0.148
WTS/LTS	-0.211	0.500	0.123	<b>0.647</b>	-0.022	0.270
WLS/LLS	-0.130	<b>0.610</b>	0.174	0.482	-0.163	0.438
LLS/LTS	<b>0.688</b>	-0.084	0.092	-0.312	0.442	-0.227
WLS/WTS	0.570	0.068	0.096	-0.396	0.168	0.092
WPav/LPav	-0.523	0.056	-0.348	0.204	<b>-0.636</b>	0.131
LFSav/LFLav	0.412	0.363	-0.016	0.016	-0.029	-0.049

the field and in cultivation had pruinose stems. In this character they resembled plants of *C. acris* and *C. apennina*, while *C. raphanifolia*, *C. gallaecica* and *C. barbaraeoides* lacked pruinose stems. We also observed a tendency of *C. raphanifolia* to develop petioles of lateral leaflets, most remarkably on basal leaves, contrary to *C. silana*, *C. acris* and *C. apennina* which have sessile leaflets (or even pinnatisect leaves). We also observed differences in presence and

orientation of hairs on the rachis of young basal leaves. While *C. silana* had always appressed hairs, they were either absent, patent or curved in *C. acris*, and completely absent in *C. apennina*. A unique qualitative character of *C. silana*, in comparison with most populations of the remaining taxa, is a tendency to develop small leaf-rosettes at the base of terminal leaflets of rosette leaves, which seems to be a common mode of vegetative reproduction of these plants. The

**Table 4.** Morphological characters distinguishing *Cardamine acris* (Balkan Peninsula), *C. silana* from Calabria (southern Italy), and *C. apennina* (Central Apennines, Italy). For quantitative characters, median values with 5th and 95th percentiles are given in parentheses

Character	<i>C. acris</i>	<i>C. silana</i>	<i>C. apennina</i>
<b>STEM</b>			
width of stem (mm)	(1.8–) 3.9 (–8.0)	(1.5–) 2.5 (–4.0)	(1.0–) 1.8 (–2.8)
<b>LEAVES</b>			
shape/division of stem leaves	mostly pinnate	pinnatisect or pinnate	pinnatisect
shape of terminal leaflet of stem leaves	oblong to orbicular	ovate or oblong	linear to narrowly obovate
presence of hairs on rachis of basal leaves	absent, patent or curved	appressed	absent
number of leaflets of basal leaves	(2–) 4 (–7)	(1–) 2 (–4)	(1–) 2 (–4)
number of leaflets of middle stem leaves	(2–) 3 (–5)	(1–) 2 (–3)	(2–) 3 (–4)
<b>FLOWERS</b>			
length of petals (mm)	(7.3–) 9.2 (–11.6)	(9.9–) 11.5 (–13.2)	(7.7–) 10.1 (–11.8)
length of longer filaments (mm)	(4.1–) 5.2 (–6.1)	(5.4–) 6.5 (–7.7)	(3.8–) 5.0 (–5.8)
diameter of pollen grains (µm)	(24.3–) 26.6 (–28.7)	(29.4–) 32.8 (–33.4)	(25.4–) 28.5 (–30.0)

only exception among populations of other taxa studied were plants of *C. acris* from Aetomilitsa in north Greece (AE, Table 1), on which we also regularly noticed similar small leaf-rosettes on basal leaf blades under cultivation. We have not observed this mode of reproduction in *C. apennina*, but this might be caused by the small amount of plants in cultivation, as this mode of reproduction is generally well developed in the *C. pratensis* group (Salisbury, 1965).

#### AFLP FINGERPRINTING

The AFLP analysis of Calabrian *C. silana*, Apennine *C. apennina*, Balkan *C. acris*, and Iberian *C. raphanifolia* and *C. gallaecica* with three selective primer pairs resulted in a total of 333 scorable bands (Table 5A). Ten bands (3%) were monomorphic and 50 bands (15%) were restricted to a single individual. The average number of bands per individual was 66.34, with the lowest values for diploid taxa *C. apennina* and *C. acris*, and the highest for hexa- and octoploid *C. raphanifolia* and *C. silana*. *C. gallaecica* had an intermediate number of bands. *Cardamine silana* had similar low intersample variability as diploid *C. apennina*.

Comparison of AFLP bands shared by *C. silana* and other taxa or groups of populations (Table 5B) indicated its close relationships to *C. acris* and *C. apennina* (six bands shared by these three taxa, 22 by *C. silana*

and *C. acris*, and four by *C. silana* and *C. apennina*; in contrast to two bands shared by *C. silana* with *C. raphanifolia* and one band shared with *C. gallaecica*). Closer examination of the sharing pattern of AFLP bands of *C. silana* to samples of *C. acris* from different geographical regions revealed that the highest number of shared unique bands was with populations of *C. acris* ssp. *acris* from north-west Greece and Montenegro (6), while only two bands were shared with *C. acris* ssp. *vardousiae* from central Greece and one band with *C. acris* ssp. *pindicola* from the northern Pindhos Mountains in north-west Greece. While *C. silana* shared 20 bands with Greek populations of all subspecies of *C. acris*, only two unique bands were shared with Bulgarian *C. acris* ssp. *acris*.

A PCoA ordination diagram of AFLP markers of all accessions (Fig. 5) showed a group of *C. silana* and *C. acris* clearly separated from *C. raphanifolia* and *C. gallaecica* along the first axis and from *C. apennina* along the second one. PCoA including only *C. acris* and *C. silana* (Fig. 6) showed plants of the latter taxon well separated along the first axis from *C. acris* accessions from different geographical regions. In the closest position to *C. silana* were populations of south Greek *C. acris* ssp. *vardousiae*. *C. acris* ssp. *pindicola* was in a similar position to *C. silana* along the first axis but separated along the second. Accessions of *C. acris* ssp. *acris* formed a single grouping in a most distant position from *C. silana*.

**Table 5.** Number of AFLP bands scored for the analysed *Cardamine* taxa. A, band total (BT) – total number of bands present in a given taxon or group; bands average (BA) – mean number  $\pm$  standard deviation of bands present per individual; exclusive bands (EB) – bands present in a given taxon/group, but absent in other taxa/groups; EB-A – band present in only one individual of a given taxon/group, but absent in other taxa/groups; diagnostic bands (DB) – bands present in all samples of a given taxon/group, but absent in other taxa/groups. B, Bands shared by a particular group or a pair of the taxa/groups, but absent in other taxa/groups. Number of individuals of *C. silana* is seven, those of other taxa/groups are given in parentheses

A

Taxon/group ( <i>N</i> )	BT	BA	EB	EB-A	DB
<i>C. acris</i> ssp. <i>acris</i> Bulgaria (8)	110	61.13 $\pm$ 2.90	14	4	0
<i>C. acris</i> ssp. <i>acris</i> north-west Greece and Montenegro (8)	119	55.25 $\pm$ 6.73	20	15	0
<i>C. acris</i> ssp. <i>pindicola</i> north-west Greece (5)	86	63.20 $\pm$ 2.59	7	4	1
<i>C. acris</i> ssp. <i>vardousiae</i> central Greece (7)	98	60.29 $\pm$ 8.77	10	5	0
<i>C. acris</i> (28)	193	59.61 $\pm$ 6.39	79	28	0
<i>C. silana</i> (7)	91	69.71 $\pm$ 2.75	8	1	4
<i>C. apennina</i> (11)	94	57.36 $\pm$ 3.23	20	4	3
<i>C. gallaecica</i> (9)	110	71.11 $\pm$ 5.13	15	4	7
<i>C. raphanifolia</i> (12)	153	84.75 $\pm$ 5.33	45	13	3
Total	333	66.34 $\pm$ 11.20	–	–	–

B

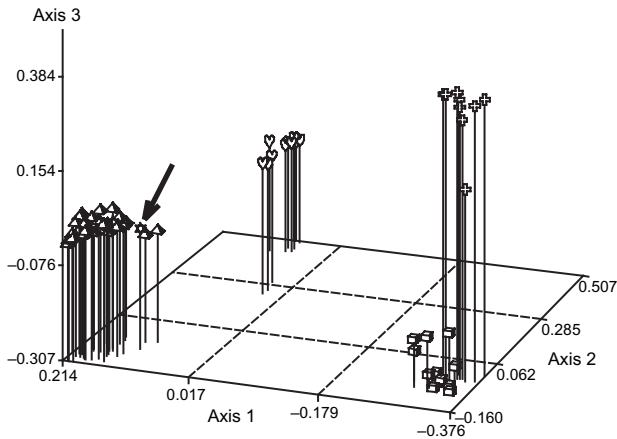
Taxon/group ( <i>N</i> )	No. of shared bands
<i>C. silana</i> + <i>C. acris</i> (28) + <i>C. apennina</i> (11)	6
<i>C. silana</i> + <i>C. acris</i> (28)	22
<i>C. silana</i> + <i>C. acris</i> ssp. <i>acris</i> from Bulgaria (8)	2
<i>C. silana</i> + <i>C. acris</i> from Greece and Montenegro (20)	20
<i>C. silana</i> + <i>C. acris</i> ssp. <i>acris</i> from north-west Greece and Montenegro (8)	6
<i>C. silana</i> + <i>C. acris</i> ssp. <i>pindicola</i> from north-west Greece (5)	1
<i>C. silana</i> + <i>C. acris</i> ssp. <i>vardousiae</i> from central Greece (7)	2
<i>C. silana</i> + <i>C. apennina</i> (11)	4
<i>C. silana</i> + <i>C. raphanifolia</i> (12)	3
<i>C. silana</i> + <i>C. gallaecica</i> (9)	1

In a neighbour-joining tree (Fig. 7), clusters roughly corresponded to the pattern observed in PCoA. *Cardamine silana* samples appeared with 100% bootstrap support in a separate cluster, connected (89% bootstrap) with a cluster of *C. acris* supported by < 50% bootstrap. Most *C. acris* ssp. *acris* accessions were in more distant clusters from *C. silana*, except accessions from north-west Greece from population AE and from Montenegro. *Cardamine apennina* formed a well supported (100% bootstrap) cluster; its grouping with *C. silana* and *C. acris* received 100% bootstrap support. Iberian *C. raphanifolia* and *C. gallaecica* formed another well supported clusters.

## DISCUSSION

*Cardamine silana*, the Calabrian local endemic of restricted distribution in the Sila Mountains (see

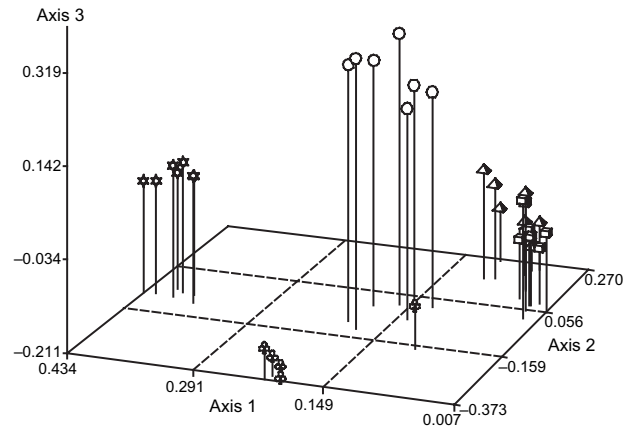
Appendix), is a hexaploid taxon. As already mentioned, Lihová et al. (2004a) showed ITS sequences of two accessions of *C. silana*, included in their study, as being very close to diploid *C. apennina* Lihová & Marhold (of the *C. pratensis* group), differing only by a single substitution. In addition, the additive polymorphic pattern detected in *C. silana* strongly favoured involvement of *C. apennina* in the origin of this polyploid. On the other hand, divergence of trnL-trnF cpDNA haplotypes of *C. silana* from those of *C. apennina* suggested *C. apennina* as the paternal donor. Indeed, morphological and AFLP data presented in this paper also indicate *C. apennina* as one of the taxa which gave origin to *C. silana*. This is also supported by the geographical proximity of *C. apennina* and *C. silana*. Other evidence presented in this paper strongly suggests as another parental species the diploid *C. acris* from the Balkan Peninsula. *Cardamine*



**Figure 5.** Principal coordinate analysis of AFLP data of *Cardamine silana* (stars, see arrow), *C. acris* (pyramids), *C. apennina* (hearts), *C. raphanifolia* (cubes), *C. gallaecica* (crosses). The first three coordinates explain 16.1, 11.5 and 8.8% of the total variation.

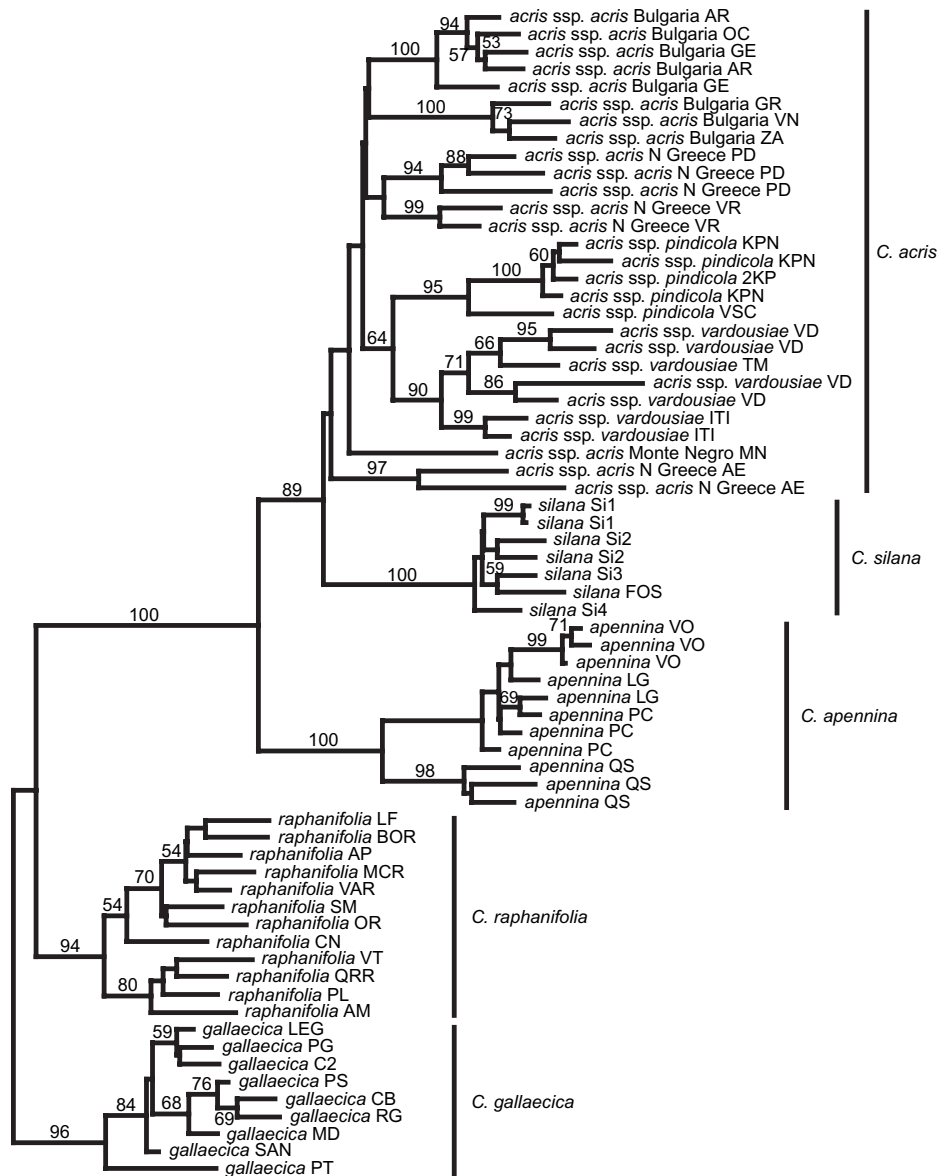
*acris* is not a homogeneous species, it is diversified into three geographically separated subspecies (Perný *et al.*, 2004). The results of morphometric evaluation and AFLP data presented in this paper suggest a more close relationship of *C. silana* to Greek populations of *C. acris* than to Bulgarian ones. While in morphometric analyses and in the PCoA diagram of AFLP data (Figs 4, 6) *C. acris* ssp. *vardousiae* appeared as closest to *C. silana*, more bands were shared between *C. silana* and *C. acris* ssp. *acris* from north-west Greece and Montenegro (Table 5). It is not clear, however, if the differentiation of *C. acris* predates the origin of *C. silana* or not.

Thus all lines of evidence support close relationships of *C. silana* to *C. acris* from the Balkan Peninsula and *C. apennina* from central Italy. This contradicts not only the opinion of de Candolle (1821), who described this taxon as a variety, *C. latifolia* var. *calabrica* (*C. latifolia* being a synonym of *C. raphanifolia*), but also the treatments in recent Italian or European Floras, where populations from Calabria were treated under *C. raphanifolia*, and thus at least implicitly assumed to be related to Iberian *C. raphanifolia* (Zángheri, 1976; Pignatti, 1982; Jones & Akeroyd, 1993). Jones & Akeroyd (1993) also speculated about the intermediate position of Calabrian plants between two Balkan taxa, classified here as *C. acris* and *C. barbaraeoides*. However, the latter species was revealed to be closer to the *C. amara* group by both AFLP data (M. Perný, J. Lihová, A. Tribsch & K. Marhold, unpubl. data) and ITS sequences (Lihová *et al.*, 2004a), and was proven to be well differentiated from *C. silana* based on morphological data presented in this paper.



**Figure 6.** Principal coordinate analysis of AFLP data of *Cardamine silana* (stars), *C. acris* ssp. *acris* from Bulgaria (pyramids), *C. acris* ssp. *acris* from north-west Greece (cubes), *C. acris* ssp. *pindicola* (clubs), and *C. acris* ssp. *vardousiae* (circles). The first three coordinates explain 16.5, 11.1 and 8.1% of the total variation.

At the moment we can only speculate about the time and place of the allopolyploid origin of *C. silana*. Its present distribution is strictly localized to the Sila Mountains, an area rich in endemic and relic taxa (e.g. *Astragalus parnassi* ssp. *calabrus* Chater, *Luzula calabra* Ten., *Soldanella calabrella* Kress, *Doronicum hungaricum* (Sadler) Rechb., *Lathyrus laxiflorus* (Desf.) Kuntze, *Ranunculus serbicus* Vis.; N. Passalacqua, unpubl. data). Moreover, currently *C. silana* is geographically separated from its likely diploid progenitors and their current or recent contact and/or gene flow can be safely excluded. *Cardamine apennina*, based on molecular data (Lihová *et al.*, 2004c), is an old taxon representing one of the basal lineages of the *C. pratensis* group. Although at present it occurs in wet habitats in a few localities in central Italy, a much wider distribution in the past, e.g. under glacial conditions, can be supposed. As discussed by Lihová *et al.* (2004c), judging from the ecology of *C. apennina*, it seems possible that this species was successful during the Quaternary glaciation when such habitats were widely available in the Mediterranean, facilitating its wider distribution. Thus it could be hypothesized that during Pleistocene climatic oscillations the distribution of this taxon reached to the south as far as to the area of Calabria, which represented an important refugium at that time (Fineschi *et al.*, 2002). The second likely progenitor of *C. silana*, *C. acris*, is currently separated from this species not only by the terrestrial disjunction, but also by the Channel of Otranto, a slightly more than 70 km-wide strip of the sea. It should be noted, however, that during the last glacial maximum the sea level was considerable lower and there was



**Figure 7.** Neighbour-joining analysis of AFLP data of *Cardamine silana*, *C. acris* from different geographical areas, *C. apennina*, *C. raphanifolia*, and *C. gallaecica*. Bootstrap values above 50% are shown. For population codes see Table 1.

contact of the Apennine and Balkan flora in northern part of the Adriatic region (Frenzel, Pésci & Velichko, 1992; Adams, 1997). There is considerable evidence for past migration and gene flow between the Apennine and Balkan peninsulas, e.g. in the recent phylogeographical study of the genus *Quercus* in Italy (Fineschi *et al.*, 2002). Further evidence supporting close contacts between the Apennine and Balkan floras is provided by a number of taxa with distributions restricted to Italy and the Balkans, in Brassicaceae, e.g. *Barbarea bracteosa* Guss. and *Aubrieta columnae* Guss., or even with distributions restricted to the southern part of Italy (including Calabria) and the

Balkans, e.g. *Cardamine glauca* Spreng., *Arabis surculosa* N. Terracc., *Aubrieta deltoidea* (L.) DC., and possibly also *Erysimum crassistylum* C. Presl (Jalas & Suominen, 1994). The close relationship between Calabrian and Balkan flora has also been recently exemplified in the study of two sibling species from Campanulaceae, *Asyneuma pichleri* (Vis) D. Lakušić & F. Conti (Balkan endemic) and *A. trichocalycinum* (Ten.) K. Malý (southern Italian endemic; Lakušić & Conti, 2004). Thus, *Cardamine silana* may be another example of a taxon which originated at the time of Pleistocene glacial events. It could be hypothesized that this species was never really widespread, and

that it originated more or less in the same area as its present distribution, although other scenarios for its origin cannot be excluded.

*Cardamine silana*, *C. acris* and *C. apennina* grow in similar habitats, typically on wet meadows and pastures, along springs, streams, or on wet places in forests. Generally, all taxa related to *C. raphanifolia* and those of the *C. pratensis* and *C. amara* groups are confined to such various types of wet habitats, and often grow in sympatry (e.g. Marhold *et al.*, 2002; Lihová *et al.*, 2004a). No clear ecological differentiation is observed currently among the proposed parental species and *C. silana*. Despite this, even minor ecological niche differentiation (considered often as crucial for the establishment of new allopolyploids, for review see Levin, 2002: 121–124) together with the higher fitness of *C. silana* might have played an important role in successful establishment of this allopolyploid.

It is noteworthy that *C. silana* is not the only endemic species of the genus *Cardamine* in Calabria. Recently, a new high polyploid ( $2n = c. 160$ ) species, *C. battagliae*, was described by Cesca & Peruzzi (2002), considered to be a polyploid derivative from *C. heptaphylla* (Vill.) O.E. Schulz ( $\equiv$  *Dentaria heptaphylla* Vill.;  $2n = 48$ ). The latter species is distributed in south-west Europe (Spain, France, southern Germany, Switzerland) extending to Italy, where it occurs as far south as the province of Campania, but does not reach Calabria. Cesca & Peruzzi (2002) suggest *C. heptaphylla* as the only progenitor of the newly described autopolyploid species. If these assumptions are correct, it seems that, unlike *C. silana*, *C. battagliae* originated without any influence of taxa from the Balkan peninsula. Molecular evidence, however, is lacking in this case.

The likely allopolyploid origin of *C. silana* proposed above provides further evidence for reticulate evolution and close relationships of taxa which were formerly considered to be subspecies of widely conceived *C. raphanifolia* and those from the *C. pratensis* group. Marhold *et al.* (2004) showed that neither data from ITS sequences, nor AFLPs provide support for the recognition of the *C. pratensis* group on one hand, and diploid taxa formerly assigned to *C. raphanifolia* on the other. Both diploid taxa of the *C. pratensis* group and accessions of *C. acris* appeared in an unresolved polytomy both in the neighbour-joining tree based on AFLP data and in the consensus tree based on most parsimonious trees of ITS sequences (Marhold *et al.*, 2004).

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#### REFERENCES

- Adams JM. 1997.** *Global land environments since the last interglacial*. <http://www.esd.ornl.gov/ern/qen/nerc.html> Oak Ridge, Tennessee: Oak Ridge National Laboratory.
- Ančev ME, Marhold K, Goranova V. 1997.** Report 873. *Cardamine acris* Griseb. In: Kamari G, Felber F, Garbari F, eds. Mediterranean chromosome number reports 7. *Flora Mediterranea* **7**: 258.
- Bachmann K. 2000.** Molecules, morphology and maps: new directions in evolutionary genetics. *Plant Species Biology* **15**: 197–210.
- de Candolle AP. 1821.** *Regni vegetabilis systema naturale* 2. Paris, Strasbourg, London.
- de Candolle AP. 1824.** *Prodromus systematis naturalis regni vegetabilis* 1. Paris, Strasbourg, London.
- Cesca G, Peruzzi L. 2002.** A new species of *Cardamine* subg. *Dentaria* (Cruciferae), apocentric in Calabria (Southern Italy). *Plant Biosystems* **136**: 313–320.
- 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.
- Fineschi S, Turchini D, Grossoni P, Petit RJ, Vendramin GG. 2002.** Chloroplast DNA variation of white oaks in Italy. *Forest Ecology and Management* **156**: 103–114.
- Fjellheim S, Elven R, Brochmann C. 2001.** Molecules and morphology in concert. II. The *Festuca brachyphylla* complex (Poaceae) in Svalbard. *American Journal of Botany* **88**: 869–882.
- Franzke A, Hurka H. 2000.** Molecular systematics and biogeography of the *Cardamine pratensis* complex (Brassicaceae). *Plant Systematics and Evolution* **224**: 213–234.
- Frenzel B, Pécsi B, Velichko AA, eds. 1992.** *Atlas of palaeoclimates and palaeoenvironments of the Northern Hemisphere*. Budapest, Stuttgart: Geographical Research Institute, Hungarian Academy of Sciences, Gustav Fisher.
- Hansen KT, Elven R, Brochmann C. 2000.** Molecules and morphology in concert: tests of some hypotheses in Arctic *Potentilla* (Rosaceae). *American Journal of Botany* **87**: 1466–1479.
- Harris SA, Ingram R. 1991.** Chloroplast DNA and biosystem-

- atics: the effect of intraspecific diversity and plastid transmission. *Taxon* **40**: 393–412.
- Hodkinson TR, Chase MW, Takahashi C, Leitch IJ, Bennet 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.
- Ishida TA, Hattori K, Sato H, Kimura MT. 2003.** Differentiation and hybridization between *Quercus crispula* and *Q. dentata* (Fagaceae): insights from morphological traits, amplified fragment length polymorphism markers, and leaf-miner composition. *American Journal of Botany* **90**: 769–776.
- Jalas J, Suominen J. 1994.** *Atlas florae europaeae 10*. Helsinki: The Committee for Mapping the Flora of Europe and Societas Biologica Fennica Vanamo.
- Jones BMG, Akeroyd JR. 1993.** *Cardamine* L. In: Tutin TG, Burges NA, Chater AO, Edmondson JR, Heywood VH, Moore DM, Valentine DH, Walters SM, Webb DA, eds. *Flora Europaea 1*, ed. 2. Cambridge: Cambridge University Press, 346–351.
- Kearns CA, Inouye DW. 1993.** *Techniques for pollination biologists*. Colorado: University Press of Colorado.
- Klecka WR. 1980.** *Discriminant analysis*. Sage University Papers, Series: Quantitative applications in the social sciences, no. 19. Sage, Beverly Hills, London: Sage Publications Inc.
- Krzanowski WJ. 1990.** *Principles of multivariate analysis*. Oxford: Clarendon Press.
- Lakušić D, Conti F. 2004.** *Asyneuma pichleri* (Campanulaceae), a neglected species of the Balkan Peninsula. *Plant Systematics and Evolution* **247**: 23–36.
- Levin DA. 2002.** *The role of chromosomal change in plant evolution*. Oxford: Oxford University Press.
- Lihová J, Fuertes Aguilar J, Marhold K, Nieto Feliner G. 2004a.** Origin of the disjunct tetraploid *Cardamine amporitana* (Brassicaceae) assessed with nuclear and chloroplast DNA sequence data. *American Journal of Botany* **91**: 1231–1242.
- Lihová J, Marhold K, Neuffer B. 2000.** Taxonomy of *Cardamine amara* (Brassicaceae) in the Iberian Peninsula. *Taxon* **49**: 747–763.
- Lihová J, Marhold K, Tribsch A. 2003.** The *Cardamine pratensis* (Brassicaceae) group in the Iberian Peninsula: taxonomy, polyploidy and distribution. *Taxon* **52**: 783–802.
- Lihová J, Marhold K, Tribsch A, Stuessy TF. 2004b.** Morphometric and AFLP re-evaluation of tetraploid *Cardamine amara* (Brassicaceae) in the Western Mediterranean. *Systematic Botany* **29**: 134–146.
- Lihová J, Tribsch A, Stuessy TF. 2004c.** *Cardamine apennina*: a new endemic diploid species of the *C. pratensis* group (Brassicaceae) from Italy. *Plant Systematics and Evolution* **245**: 69–92.
- Lövkvist B. 1956.** The *Cardamine pratensis* complex. Outline of its cytogenetics and taxonomy. *Symbolae Botanicae Upsalienses* **14/2**: 1–131.
- Lövkvist B. 1957.** Experimental studies in *Cardamine amara*. *Botaniska Notiser* **110**: 423–441.
- 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. 1994.** Taxonomy of the genus *Cardamine* L. (Cruciferae) in the Carpathians and Pannonia I. *Cardamine pratensis* group. *Folia Geobotanica & 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 & Phytotaxonomica* **30**: 63–88.
- Marhold K. 1996.** Multivariate morphometric study of the *Cardamine pratensis* group (Cruciferae) in the Carpathian and Pannonian area. *Plant Systematics and Evolution* **200**: 141–159.
- 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, Lihová J, Perný M, Bleeker W. 2004.** Comparative ITS and AFLP analysis of diploid *Cardamine* (Brassicaceae) taxa from closely related polyploid complexes. *Annals of Botany (Oxford)* **93**: 507–520.
- Marhold K, Lihová J, Perný M, Gruppe R, Neuffer B. 2002.** 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.
- Masterson J. 1994.** Stomatal size in fossil plants – evidence for polyploidy in majority of angiosperms. *Science* **264**: 421–424.
- Murín A. 1960.** Substitution of cellophane for glass covers to facilitate preparation of permanent squashes and smears. *Stain Technology* **35**: 351–353.
- Nei M, Li W-H. 1979.** Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences, USA* **76**: 5269–5273.
- Osborn TC, Pires JC, Birchler JA, Auger DL, Chen ZJ, Lee H-S, Comai L, Madlung A, Doerge RW, Colot V, Martienssen RA. 2003.** Understanding mechanisms of novel gene expression in polyploids. *Trends in Ecology and Evolution* **19**: 141–147.
- Parlatore F. 1890.** *Flora Italiana 9*. Firenze.
- PE Applied Biosystems. 1996.** *AFLP™ Plant Mapping Protocol*. Foster City: PE Applied Biosystems, A Division of Perkin-Elmer.
- Perný M, Tribsch A, Anchev ME. 2004.** Intraspecific differentiation in the Balkan diploid *Cardamine acris* (Brassicaceae): molecular and morphological evidence. *Folia Geobotanica* **39**: 405–429.
- Pignatti S. 1982.** *Flora D'italia 1*. Bologna: Edagricole.
- Pimentel RA. 1981.** A comparative study of data and ordination techniques based on a hybrid swarm of sand verbenas *Abronia* Juss. *Systematic Zoology* **30**: 250–267.

- Podani J. 2001.** SYN-TAX 2000. *Computer programs for data analysis in ecology and systematics. User's manual.* Budapest: Scientia Publishing.
- Rico E. 1993.** *Cardamine* L. In: Castroviejo S, Aedo C, Gómez Campo C, Laínz M, Montserrat P, Morales R, Muñoz Garmedia F, Nieto Feliner G, Rico E, Talavera S, Villar L, eds. *Flora Iberica 4.* Madrid: Real Jardín Botánico, CSIC, 119–133.
- Salisbury E. 1965.** The reproduction of *Cardamine pratensis* L. & *Cardamine palustris* Peterman particularly in relation to their specialized foliar vivipary, and its deflexion of the constraints of natural selection. *Proceedings of the Royal Society of London B* **163**: 321–342.
- SAS Institute. 2000.** *SAS OnlineDoc, Version 8 (available online).* Cary: SAS Institute.
- Schols P, Dessein S, D'hondt C, Huysmans S, Smets E. 2002.** Carnoy: a new digital measurement tool for palynology. *Grana* **41**: 124–126.
- 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.
- Sneath PAH, Sokal RR. 1973.** *Numerical taxonomy.* San Francisco: W. H. Freeman.
- Soltis DE, Soltis PS. 1999.** Polyploidy: recurrent formation and genome evolution. *Trends in Ecology and Systematics* **14**: 348–352.
- Soltis DE, Soltis PS. 2000.** The role of genetic and genomic attributes in the success of polyploids. *Proceedings of the National Academy of Sciences, USA* **97**: 7015–7057.
- Swofford DL. 2003.** *PAUP\*: phylogenetic analysis using parsimony (\*and other methods).* Version 4. Sunderland: Sinauer Associates.
- Tenore M. 1831.** *Sylloge plantarum vascularium florae neapolitanae.* Neapoli.
- Thorpe RS. 1976.** Biometric analysis of geographic variation and racial varieties. *Biological Reviews* **51**: 407–452.
- 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 KM, Landolt E. 1999.** Patterns and processes of man-influenced hybridization in *Cardamine* L. In: van Raamsdonk LWD, den Nijs JCM, eds. *Plant evolution in man-made habitats* Amsterdam: Hugo de Vries Laboratory, Institute for Systematics and Population Biology, University of Amsterdam, 29–47.
- 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.
- Widmer A, Baltisberger M. 1999.** Molecular evidence for allopolyploid speciation and a single origin of the narrow endemic *Draba ladina* (Brassicaceae). *American Journal of Botany* **86**: 1282–1289.
- Zángheri P. 1976.** *Flora Italica*, Vol. 1. Padova: Cedam–Casa Editrice Dott. Antonio Milani.

## APPENDIX

## TAXONOMIC ACCOUNT

*Cardamine silana* Marhold & Perný, Willdenowia 33: 69, 2003.

≡ *Cardamine latifolia* var. *calabrica* DC., Syst. Nat. 2: 262, 1821.

Holotype: 'Des ruiss[e]aux de la Sila. Thomas 1818' (G-DC)

≡ *Cardamine raphanifolia* 'proles' *calabrica* (DC.) O. E. Schulz, Bot. Jahrb. Syst. 33: 513, 1903.

Perennial herb (26–) 29–57 (–62) cm tall; rhizome prostrate to ascending; stem usually erect, simple or with 1–5 (–7) branches, glabrous and pruinose, up to (1.3–) 1.5–4.0 (–4.5) mm wide. Leaves forming a basal rosette, rarely only congested near the stem base. Rosette leaves glabrous or pubescent, later becoming glabrous, pinnate (3.2–) 5.3–14.0 (–14.9) cm long, with 1–4 pairs of lateral leaflets, their terminal leaflet much larger than the lateral ones, orbicular (1.2–) 1.3–3.2 (–3.4) cm long (1.2–) 1.5–3.3 (–3.7) cm wide, lateral leaflets sessile, ovate to orbicular (0.3–) 0.4–1.5 (–1.8) cm long (0.3–) 0.4–1.4 (–1.7) cm wide. Stem leaves (3–) 4–10 (–11), mostly in the upper half of the stem, pinnate, similar to the basal leaves, glabrous; middle cauline leaves (1.6–) 2.0–6.8 (–8.2) cm long, with 1–3 (–4) pairs of sessile lateral leaflets/segments; terminal leaflet/segment orbicular to obovate (0.8–) 1.0–2.8 (–3.0) cm long (0.6–) 0.8–2.2 (–2.6) cm wide, lateral leaflets/segments elliptic (0.3–) 0.4–1.5 (–1.9) cm long (0.1–) 0.2–0.9 (–1.1) cm wide. Inflorescence racemose, peduncles glabrous. Sepals ovate-lanceolate with membranous margins (3.1–) 3.5–4.9 (–5.3) mm long and (1.2–) 1.4–2.5 (–2.8) mm wide. Petals reddish-violet, obovate (9.2–) 9.9–13.2 (–13.8) mm long and (5.4–) 5.7–8.2 (–9.0) mm wide, with short claw, apex truncate to emarginate, glabrous. Stamens six, tetradynamous, shorter filaments (2.5–) 2.9–4.8 (–5.1) mm long, longer filaments (4.9–) 5.4–7.7 (–8.2) mm long; anthers yellow. Stigma wider than style.

## SPECIMENS SEEN

Sila, s.d., Tenore (NAP). – Magna Sila (Calabria): stazioni umide a botte Donato, c. 1700 m, 16.viii.1909, *Cavara e Grande* (BM 000758500). – Calabria, Sila Grande, Lorica, torrente presso l'impianto di risa lita per M.te Botte Donato, 1400 m, 1.vi.1996, *Bernardo* (CLU). – Calabria, Sila Grande, Lorica, Pedace, Torrente presso il Lago Arvo, 1.vi.1996, *Bernardo* (CLU). – Sila Grande, c. 13.5 km ENE of Campigliatello Silano, Macchialonga, 39°21'59"N, 16°36'11"E, 1510–1560 m, 11.vi.1997, *Partecipanti VIII Iter Mediterraneo 1001/04* (CLU, RNG, W). – Basilicata, Prov. di Potenza, Massiccio del Pollino, c. 11 km north of Castrovillari, Piani di Pollino, 39°55'N, 16°12'E 1835 m, *OPTIMA iter VIII 1860*, 19.vi.1997 (RNG). – Calabria, Sila Grande, Macchialonga, 39°22'N, 16°36'E, 1510 m, 11.vi.1997, *Jury 17323* (RNG).