

Evolutionary history of the polyploid complex of *Cardamine amara* (Brassicaceae): isozyme evidence

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Abstract. In samples from 56 populations of *Cardamine amara*, representing four diploid subspecies (subsp. *amara*, *opicii*, *balcanica*, and *pyrenaea*) and two tetraploid subspecies (subsp. *austriaca* and *olotensis*) from different parts of the European distribution area, four enzyme systems with 23 alleles were studied. These data, together with previous morphological and karyological data, suggest that the distribution and variation pattern within the species was strongly influenced by the last glacial period and postglacial migrations. *Cardamine amara* subsp. *pyrenaea* is monomorphic for a unique allele, and subsp. *balcanica* has a unique allele, too which, however, is not fixed in all populations of the taxon. Both taxa seem to be relic ones, although otherwise subsp. *balcanica* in respect of allelic spectrum much resembles subsp. *amara*. The other two diploid subspecies, subsp. *amara* and subsp. *opicii*, are not characterised by presence of unique alleles but differ in allele frequencies. The two tetraploid subspecies have different evolutionary histories. *C. amara* subsp. *austriaca* seems to be an autopolyploid derivative of subsp. *amara* which colonised open space offered by retreating glaciers in the Eastern Alps. *C. amara* subsp. *olotensis* from the Iberian Peninsula represents most probably a polyploid of preglacial time.

Key words: *Cardamine amara*, Large Bitter-cress, isozymes, polyploidy, glaciation, relic taxa.

Introduction

Cardamine L. comprises several polyploid complexes in its European distribution area, e.g. the *C. pratensis* group, *C. amara* L. and the *C. raphanifolia* group. Until now detailed attention has been paid mainly to the *C. pratensis* complex, which consists of several diploid taxa and higher polyploids up to dodecaploid level, including dysploids and aneuploids (e.g. Lövkvist 1956; Urbanska-Worytkiewicz and Landolt 1974; Marhold 1994a,b, 1996; Marhold and Ančev 1999; Franzke and Hurka 2000).

Recent studies of populations of *C. amara* in various parts of Europe revealed an interesting pattern of karyological and morphological variation classified at the subspecific level (Lihová et al. 2000; Marhold 1992, 1999; Marhold et al. 1996). Four diploid subspecies are currently recognised within *C. amara*: subsp. *amara*, widespread in most of Europe

extending to Asia, except some European mountain ranges where it is replaced by other subspecies; subsp. *opicii* (J. Presl et C. Presl) Čelak. occurring at the higher altitudes of the Sudety Mts. and Carpathians (Marhold 1992) (for the distribution of all subspecies in studied part of Europe see Fig. 1); subsp. *balcanica* Marhold, Ančev et Kit Tan, occurring in the mountains of SW Bulgaria and NE Greece (Marhold et al. 1996); and subsp. *pyrenaea* Sennen, a recently “rediscovered” taxon of the Eastern Pyrenees (Lihová et al. 2000). There are two tetraploid subspecies in *C. amara*. They occupy different restricted areas: subsp. *austriaca* Marhold occurs in the Eastern Alps

and neighbouring areas (Marhold 1999), while subsp. *olotensis* occupies a small area in Catalonia (Lihová et al. 2000). The results of random amplified polymorphic DNA (RAPD) analyses of all six subspecies of *Cardamine amara* (Lihová et al. 2000) based on a restricted amount of material supported the above-mentioned taxonomic treatment except that diploid subsp. *amara* and tetraploid subsp. *austriaca* were not differentiated from each other. This can be explained by the possible autotetraploid origin of subsp. *austriaca* from subsp. *amara*. *C. amara* subsp. *pyrenaea*, subsp. *opicii*, subsp. *balcanica*, and especially subsp. *olotensis* formed well-defined groups in

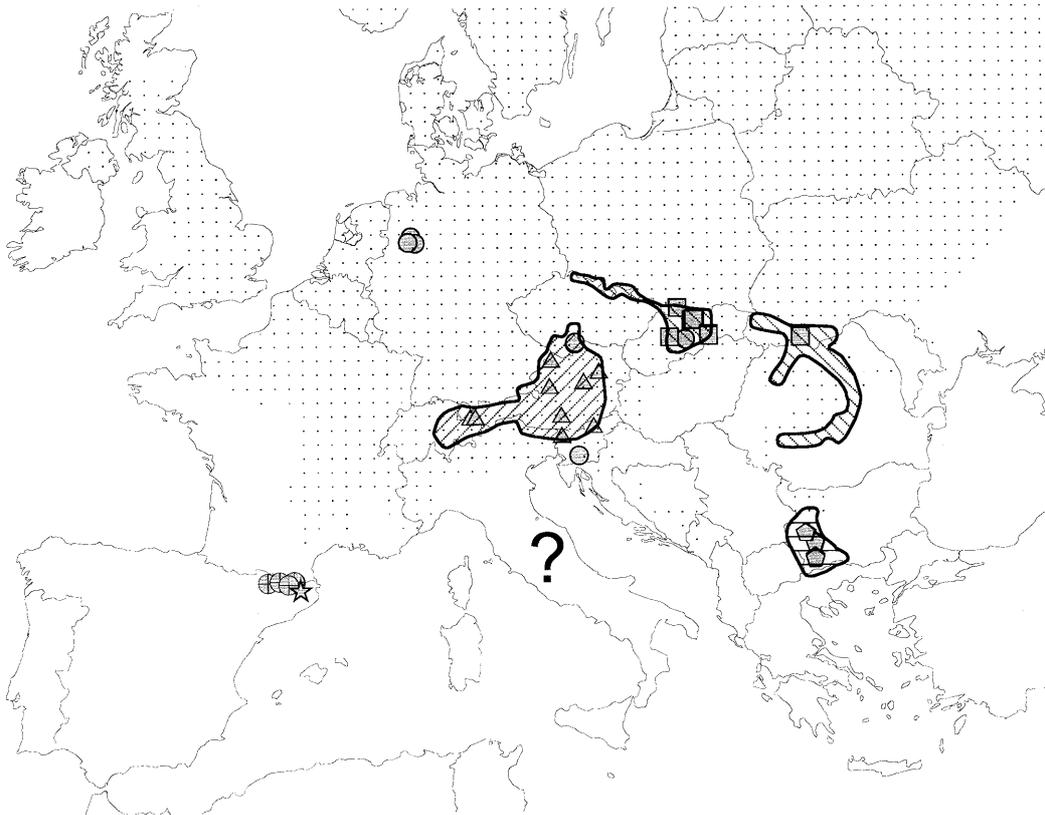


Fig. 1. Map of distribution of sample sites, and distribution areas, of *Cardamine amara* subsp. *amara* (circle ○, ◻, representing the approximate distribution area, which further extends to the east to Asia, in some parts of the Balkan Peninsula the detailed distribution is still poorly known), *C. amara* subsp. *opicii* (square ◻, ▨), *C. amara* subsp. *austriaca* (triangle △, ▨, only marginally overlapping with the area of subsp. *amara*), *C. amara* subsp. *balcanica* (pentagon ◑, ▨), *C. amara* subsp. *olotensis* (star ☆), and *C. amara* subsp. *pyrenaea* (crossed circle ⊕) (distribution areas of the latter two subspecies coincide with distribution of sample sites). The taxonomic status of the central Italian populations is uncertain and it is being studied now

neighbour joining-distance analysis as well as in principal coordinate analysis of the RAPD data.

The distribution area of *C. amara* subsp. *austriaca* is clearly influenced by the migrations and evolutionary processes connected with Pleistocene glaciation. Its occurrence is centred in the area covered by the glacier during the last glaciation and for most of this area it represents the only representative of this species; its establishment was very likely connected with the quick occupation of these areas after retreat of the glacier. The distribution of taxa influenced by glaciation and postglacial migrations is documented in recent literature, especially in connection with the use of various kinds of molecular methods (e.g. Taberlet et al. 1998; Hewitt 1999, 2000).

Franzke and Hurka (2000) analysed isozymes, internal transcribed spacer (ITS), non-coding chloroplast DNA, and RAPDs to elucidate phylogenetic relationships and historical biogeography of the *Cardamine pratensis* complex, closely related to the group studied in the present paper.

The aim of the present paper is to analyse isozyme variation in *Cardamine amara* for phylogenetic and historical biogeographic signals.

Material and methods

Plant material. Living plants of *Cardamine amara* L. comprising all the currently recognised six subspecies were collected in the field and transferred to the greenhouse of the University of Osnabrück, Germany, where they were grown under uniform conditions (for list of localities see Table 1 and for their distribution Fig. 1). Young leaves were harvested and stored at -80°C . Samples from 56 populations or sub-populations (altogether 351 plants, see Table 1) of *C. amara* were studied. Voucher specimens are deposited in the herbarium SAV.

Chromosome counts. Chromosome numbers of the studied populations are presented in Table 1. In most cases they are previously published counts (Lihová et al. 2000; Marhold 1994a, 1999; Marhold et al. 1996). In a few cases they represent new data

by Marion Huthmann counted by the procedure described in Koch et al. (1996: 588), on at least ten plants from each locality. Voucher specimens for these counts are deposited in WHB.

Isozyme analysis. The following enzyme systems were assayed: aspartate aminotransferase (AAT, EC 2.6.1.1); glutamate dehydrogenase (GDH, EC 1.4.1.2-4); leucine aminopeptidase (LAP, EC 3.4.11.1); phosphoglucomutase (PGM, EC 2.7.5.1). Extracts were prepared from 0.5 g frozen leaves of single plants in 1 ml ice-cold extraction buffer (0.165 M Tris-HCl, pH 8.0, and 0.107 M glycine), and 200 mg Amberlite IRA-401 (Serva, Heidelberg) were added. The extract was filtered through four layers of mull and centrifuged at 4°C for 30 min at 20,000 rpm. The supernatant was stored at -80°C . Electrophoresis was performed in a continuous system on 5.5% (w/v, GDH) and 7.5% (w/v, AAT, LAP, PGM) polyacrylamide gel using different buffer systems and staining procedures (see Franzke and Hurka (2000) for AAT, GDH, and LAP; Mummenhoff and Hurka (1995) for PGM). The allozymes were characterised by their R_f values relative to an internal standard with a migration distance of 100 (=allele 1 of the respective locus). The term allozyme is used in the present paper also for tetraploids for simplicity, although no genetic analysis of inheritance patterns has been carried out.

Data analyses. For the samples from the diploid populations (subsp. *amara*, subsp. *opicii*, subsp. *balcanica*, and subsp. *pyrenaea*), allele frequencies within populations were determined, and genetic (chord) distances were calculated according to Cavalli-Sforza and Edwards (1967) (for the arguments in favor of this distance measure compared to distance according to Nei (1972, 1978) see Koch and Hurka (1999) and references therein). For the tetraploid taxa (subsp. *austriaca* and subsp. *olotensis*) frequency data presented in Results section reflect presence/absence of alleles in individuals rather than actual allelic frequencies as it was hard to evaluate the number of alleles in particular polyploid individuals based on intensity of bands. Therefore only the presence and absence of alleles was used for numeric evaluation of tetraploid populations. Matrix of frequencies for diploid populations, and a matrix of presence/absence data for diploid and tetraploid populations were subjected to principal component analysis.

Table 1. List of populations of *Cardamine amara* examined for isozyme analyses. Data on chromosome numbers with references are taken from previous papers, the other ones represent new data by M. Huthmann (voucher specimens in SAV and WHB)

Locality code	Locality	Collector	Number of analysed plants	Chromosome number (2n)
<i>Cardamine amara</i> subsp. <i>amara</i>				
A-83-DRB	Slovak Republic, Slovenské rudohorie Mts, Drábsko, 980 m a.s.l.	K. Marhold	9	16 (Lihová et al. 2000)
A-02-CBL	Czech Republic, Třeboňská pánev, Červené blato, 470 m a.s.l	K. Marhold	5	16 (Marhold 1999: 80)
A-12-RY1	Czech Republic, Třeboňská pánev, Chlum u Třeboně, nearby fishpond	K. Marhold	4	16 (Marhold 1999: 80)
A-54-RY2	Czech Republic, Třeboňská pánev, Podsedek, 430 m a.s.l. (microlocality 1)	K. Marhold	3	16 (Marhold 1999: 80)
A-29-RY3	Czech Republic, Třeboňská pánev, Chlum u Třeboně, nearby fishpond	K. Marhold	3	16 (Marhold 1999: 80)
A-53-RY4	Czech Republic, Třeboňská pánev, Podsedek, 430 m a.s.l. (microlocality 2)	K. Marhold	4	16 (Marhold 1999: 80)
A-06-RY7	Czech Republic, Třeboňská pánev, Chlum u Třeboně, nearby fishpond	K. Marhold	3	16 (Marhold 1999: 80)
A-20-PUB	Slovenia, Notranjsko, Loška dolina, river banks of Veliki Obrh, W of the village of Pudob, 570 m a.s.l.	K. Marhold & N. Jogan	5	16 (Marhold 1999: 30)
A-MÜ	Germany, Lower Saxony, Vehrte, Venner Mühlenbach, 90 m a.s.l.	M. Huthmann & M. Koch	19	16
A-SCH	Germany, Lower Saxony, Schelenburg near Osnabrück, 95 m a.s.l.	M. Huthmann & M. Koch	9	16
A-GS	Germany, Lower Saxony, Gaste-Hasbergen, Leedner Mühlbach, 85 m a.s.l.	M. Huthmann & M. Koch	17	16

Table 1 (continued)

<i>Cardamine amara</i> subsp. <i>austriaca</i>					
T-10-NEN	Austria, Vorarlberg, Klösterle, Nenzingast Tal, 1300 m a.s.l.	P. Mráz	5		32 (Marhold 1999: 81)
T-57-AIG	Austria, Upper Austria, Mühlviertel, ca. 6 km ENE of village of Aigen, area called Bayerische Au, ca. 1 km of Zollhaus, 740 m a.s.l.	E. Vitek	3		32 (Marhold 1999: 79)
T-65-ATS	Austria, Upper Austria, Salzkammergut, E of Attersee, valley N of Weißenbachtal, 580 m a.s.l.	E. Vitek	3		32 (Marhold 1999: 80)
T-78-ST5	Austria, North Tyrol, St. Anton, Steißbach Tal, 1800 m a.s.l.	G. Brandstätter, J. Chrték & P. Mráz	5		32 (Marhold 1999: 80)
T-LI	Austria, Lower Austria, Lunz am See, Eisenwurzen, 700 m a.s.l.	M. Huthmann & M. Koch	8		32
T-ZE	Austria, Lower Austria, Zetterrain near Lunz am See, Eisenwurzen, 850 m a.s.l.	M. Huthmann & M. Koch	7		32
T-MI	Austria, Lower Austria, Westl. Wienerwald, Michelbach, 450 m a.s.l.	M. Huthmann & M. Koch	7		32
T-OB	Austria, Lower Austria, Lunz am See, Obersee, 1100 m a.s.l.	M. Huthmann & M. Koch	5		32
T-LU	Austria, Lower Austria, Lunz am See, creek south of the lake, 610 m a.s.l.	M. Huthmann & M. Koch	6		32
T-DI	Austria, Styria, Nockberge, Eisenhut, Dieslingsee, 2150 m a.s.l.	M. Koch	5		32
T-FA	Austria, Carinthia, Nockberge, Falkert, Falkertsee, 2000 m a.s.l.	M. Koch	10		32 (Marhold 1999: 80)
T-50-ANT	Slovenia, Štajerska, Pohorje, between Vuhred and Ribnica na Pohorju, 530 m a.s.l.	K. Marhold & N. Jogan	3		32 (Marhold 1999: 81)
<i>Cardamine amara</i> subsp. <i>balcanica</i>					
B-01-BO	Bulgaria, Rila Mts, Borovets, 1300 m a.s.l.	K. Marhold & M. Anchev	8		16 (Marhold et al. 1996: 203)
B-80-SIT	Bulgaria, Rila Mts, between Borovets and Sitnyakov, 1600 m a.s.l.	K. Marhold & M. Anchev	17		16 (Marhold et al. 1996: 203)

Table 1 (continued)

Locality code	Locality	Collector	Number of analysed plants	Chromosome number (2n)
B-73-AKO	Bulgaria, Vitosha Mts, Aleko, 1800 m a.s.l.	K. Marhold & M. Anchev	11	16 (Marhold et al. 1996: 203)
B-74-ZLZ	Bulgaria, Vitosha Mts, Zhelezmitsa, 1100-1150 m a.s.l.	K. Marhold & M. Anchev	6	16 (Lihová et al. 2000)
B-79-BAN	Bulgaria, Pirin Mts, Bansko, near rivulet Glazne, 1050 m a.s.l.	K. Marhold & M. Anchev	5	not analysed
B-41-DMJ	Bulgaria, Pirin Mts, near Demyanitza chalet, 1850 m a.s.l.	K. Marhold & M. Anchev	6	16 (Marhold et al. 1996: 203)
B-81-DMP	Bulgaria, Pirin Mts, Demyanishka polyana, 1620 m a.s.l.	K. Marhold & M. Anchev	5	16 (Marhold et al. 1996: 203)
<i>Cardamine amara</i> subsp. <i>olotensis</i>				
L-66-OL1	Spain, Catalonia, Olot, Parc Nou	K. Marhold, C. Benedí & J. Vicens	8	32 (Lihová et al. 2000)
L-86-OL2	Spain, Catalonia, Olot, Paratges de la Deu	K. Marhold, C. Benedí & J. Vicens	11	32 (Lihová et al. 2000)
<i>Cardamine amara</i> subsp. <i>opicii</i>				
O-03-MS1	Slovak Republic, Vysoké Tatry Mts, Malá Studená dolina Valley, 1595 m a.s.l.	K. Marhold	4	16 (Lihová et al. 2000)
O-49-MS2	Slovak Republic, Vysoké Tatry Mts, Malá Studená dolina Valley, 1630 m a.s.l.	K. Marhold	4	16 (Lihová et al. 2000)
O-22-BAB	Slovak Republic, Západné Beskydy Mts, Mt. Babia hora, S slope, 1450 m a.s.l.	K. Janovicová, J. Somogyi & P. Mráz	5	16 (Lihová et al. 2000)
O-26-ML1	Slovak Republic, Vysoké Tatry Mts, Mlynická dolina Valley, 1640-1660 m a.s.l. (microlocality 1)	K. Marhold	4	16 (Marhold 1994: 30)
O-52-ML2	Slovak Republic, Vysoké Tatry Mts, Mlynická dolina Valley, 1640-1660 m a.s.l. (microlocality 2)	K. Marhold	5	16 (Marhold 1994: 30)

Table 1 (continued)

O-42-VSD	Slovak Republic, Vysoké Tatry Mts, Veľká Studená dolina Valley, 1560 m a.s.l.	K. Marhold	3	16 (Marhold 1994: 30)
O-44-VEH	Slovak Republic, Vysoké Tatry Mts, Veľická dolina Valley, 1950 m a.s.l.	K. Marhold	5	16 (Marhold 1994: 30)
O-48-VED	Slovak Republic, Vysoké Tatry Mts, Veľická dolina Valley, 1805 m a.s.l.	K. Marhold	5	16 (Marhold 1994: 30)
O-59-PIP	Slovak Republic, Slovenské rudohorie Mts, S of the village of Úhorná, between the mountains of Malá Pipitka and Pipitka, 875 m a.s.l.	P. Mráz	5	16 (Lihová et al. 2000)
O-64-KRE	Slovak Republic, Kremnické vrchy Mts, above the Kordické sedlo Saddle, 1130 m a.s.l.	K. Marhold	5	16 (Marhold 1994: 29)
O-75-HEK	Slovak Republic, Slovenské rudohorie Mts, source of the rivulet Starovodský potok, slopes of Mt. Hekeřová, 1180 m a.s.l.	P. & R. Mráz	4	16 (Lihová et al. 2000)
O-99-HOV	Ukraine, Chornogora Mts, Mt. Hoverla, W slope, 1740 m a.s.l.	P. Mráz	5	16 (Marhold 1994: 30)
<i>Cardamine amara</i> subsp. <i>pyrenaica</i>				
S-15-MNL	Spain, Catalonia, Ripollès, Querolbs, Planell de les Eugenes, nearby Refugi Mannelic, near the rivulet el Freser, 1975 m a.s.l.	K. Marhold	8	16 (Lihová et al. 2000)
S-23-VAM	Spain, Catalonia, Ripollès, Querolbs, Coma de Freser, near the bridge over the Torrent de Freser, 1560 m a.s.l.	K. Marhold	5	16 (Lihová et al. 2000)
S-27-COE	Spain, Catalonia, Ripollès, Querolbs, Núria, Coma d'Eina, 2065-2105 m a.s.l.	K. Marhold	5	16 (Lihová et al. 2000)
S-35-NUR	Spain, Catalonia, Ripollès, Querolbs, Núria, 2000 m a.s.l.	K. Marhold	5	16 (Lihová et al. 2000)
S-56-NCR	Spain, Catalonia, Ripollès, Querolbs, Núria, Coma de Noucreus, 2290-2310 m a.s.l.	K. Marhold	5	16 (Lihová et al. 2000)

Table 1 (continued)

Locality code	Locality	Collector	Number of analysed plants	Chromosome number (2n)
S-93-NOF	Spain, Catalonia, Ripollès, Querolbs, Núria, Coma de Noufonts, 2440 m a.s.l.	K. Marhold	12	16 (Lihová et al. 2000)
S-91-FIN	Spain, Catalonia, Ripollès, Querolbs, Torrent de Finestrelles, 2140 m a.s.l.	K. Marhold	4	16 (Lihová et al. 2000)
S-16-ULD	Spain, Catalonia, Ripollès, Setcases, nearby Refugi d'Ulldeter, NW of Pla dels Hospitalets, 2130 m a.s.l.	K. Marhold & J. Vicens	11	16 (Lihová et al. 2000)
S-94-ERM	Spain, Catalonia, Ripollès, Setcases, Pla dels Hospitalets, near the curve of the road, near the rivulet Clot de Coma Ermada, 1925 m a.s.l.	K. Marhold & J. Vicens	5	16 (Lihová et al. 2000)
S-97-MEI	Spain, Catalonia, Ripollès, near the mountain road from Tregura de Dalt (near Vilallonga de Ter) to Ribesaltes (near Ribes de Freser), near the rivulet Torrent de Meianell, 1985 m a.s.l.	K. Marhold & J. Vicens	4	16 (Lihová et al. 2000)
S-92-PMI	Spain, Catalonia, Cerdanya, Martinet, Aranser, Estació d'esquí nordic d'Aranser, Prat Miró, 1950 m a.s.l.	K. Marhold & J. Vicens	5	16 (Lihová et al. 2000)
S-96-SAT	Spain, Catalonia, Cerdanya, nearby the road from Ribes de Freser to Puigcerda, ca. 0.75 km E of Casilla de Saltèguet, nearby the crossroad to Baga de Saltèguet, 1725 m a.s.l.	K. Marhold & J. Vicens	3	16 (Lihová et al. 2000)

Table 3. Allele frequencies for the polymorphic enzyme loci *Aat1*, *Aat2*, *Pgm1*, *Lap2*, *Lap3*, *Gdh1* and *Gdh2* in the diploid and tetraploid subspecies of *Cardamine amara*. In the case of tetraploids these frequencies reflect presence or absence of alleles in individual plants

Locus/ Alleles	subsp. <i>amara</i>	subsp. <i>opicii</i>	subsp. <i>balcanica</i>	subsp. <i>pyrenaica</i>	subsp. <i>austriaca</i>	subsp. <i>olotensis</i>
<i>Aat1</i>	81 ind./11 pop.	54 ind./12 pop.	58 ind./7 pop.	72 ind./12 pop.	56 ind./12 pop.	19 ind./2 pop.
<i>Aat1-1</i>	0.512	0.870	0.853	1.000	0.875	0.842
<i>Aat1-2</i>	0.488	0.046	0.009	–	0.116	–
<i>Aat1-5</i>	–	0.083	0.138	–	0.009	0.158
<i>Aat2</i>	81 ind./11 pop.	54 ind./12 pop.	58 ind./7 pop.	72 ind./12 pop.	56 ind./12 pop.	19 ind./2 pop.
<i>Aat2-1</i>	1.000	1.000	0.586	–	1.000	1.000
<i>Aat2-4</i>	–	–	0.414	–	–	–
<i>Aat2-7</i>	–	–	–	1.000	–	–
<i>Pgm1</i>	81 ind./11 pop.	54 ind./12 pop.	58 ind./7 pop.	59 ind./12 pop.	61 ind./12 pop.	19 ind./2 pop.
<i>Pgm1-1</i>	0.123	–	–	–	0.246	0.500
<i>Pgm1-2</i>	–	–	0.026	0.771	–	0.079
<i>Pgm1-3</i>	0.877	1.000	0.974	0.229	0.754	0.421
<i>Pgm2</i>	81 ind./11 pop.	54 ind./12 pop.	58 ind./7 pop.	61 ind./12 pop.	61 ind./12 pop.	19 ind./2 pop.
<i>Pgm2-1</i>	–	–	–	–	–	1.000
<i>Pgm2-2</i>	0.605	1.000	1.000	0.918	0.967	–
<i>Pgm2-8</i>	0.395	–	–	0.082	0.033	–
<i>Lap2</i>	81 ind./11 pop.	54 ind./12 pop.	58 ind./7 pop.	72 ind./12 pop.	54 ind./12 pop.	19 ind./2 pop.
<i>Lap2-1</i>	0.617	–	–	0.132	0.546	0.553
<i>Lap2-2</i>	0.383	1.000	1.000	0.868	0.454	0.447
<i>Lap3</i>	81 ind./11 pop.	54 ind./12 pop.	58 ind./7 pop.	72 ind./12 pop.	54 ind./12 pop.	19 ind./2 pop.
<i>Lap3-1</i>	0.284	0.528	0.534	0.278	0.324	0.289
<i>Lap3-2</i>	0.093	0.130	0.250	0.438	0.120	0.237
<i>Lap3-3</i>	0.623	0.343	0.216	0.285	0.556	0.474
<i>Gdh1</i>	81 ind./11 pop.	54 ind./12 pop.	56 ind./7 pop.	35 ind./12 pop.	24 ind./12 pop.	19 ind./2 pop.
<i>Gdh1-1</i>	0.117	–	0.714	–	–	0.789
<i>Gdh1-2</i>	0.327	1.000	0.286	1.000	1.000	0.211
<i>Gdh1-6</i>	0.556	–	–	–	–	–
<i>Gdh2</i>	81 ind./11 pop.	54 ind./12 pop.	56 ind./7 pop.	35 ind./12 pop.	24 ind./12 pop.	19 ind./2 pop.
<i>Gdh2-1</i>	0.173	–	0.054	–	0.593	–
<i>Gdh2-2</i>	–	1.000	–	1.000	0.375	0.789
<i>Gdh2-4</i>	0.827	–	0.946	–	0.042	0.211

austriaca, *Gdh2-1* was nearly fixed, whereas it was detected only in three of the 11 populations surveyed in subsp. *amara*. These populations were within or close to the range of subsp. *austriaca*. The allele *Gdh2-1* was also found in one population of subsp. *balcanica*, but neither recorded from subsp. *pyrenaea* nor from subsp. *olotensis* (Tables 2 and 3). The allele *Gdh2-2* was fixed in subsp. *opicii* and subsp. *pyrenaea* and was also recorded from subsp. *olotensis* and subsp. *austriaca*.

Principal component analyses (PCA). Ordination diagrams based on allelic frequencies (diploid taxa only, not shown) and on presence/absence data (all taxa, Fig. 2) showed similar patterns. *C. amara* subsp. *pyrenaea* appeared in a clearly separated position along the first component axis, while subsp. *amara*, subsp. *opicii* and subsp. *balcanica* formed only partly overlapping groupings. The two groups

of tetraploids occupied different positions on the ordination diagram. While subsp. *olotensis* was separated from the rest of material along the third component axis, samples of subsp. *austriaca* overlapped to a large extent with those of subsp. *amara*.

Principal coordinate analyses (PCO). PCO of the diploid populations (Fig. 3) based on chord distances of Cavalli-Sforza and Edwards (1967) revealed *C. amara* subsp. *pyrenaea* in a clearly separated position along the first axis and subsp. *opicii* along the first and second axes. *C. amara* subsp. *amara* and subsp. *balcanica* partly overlapped, but this overlap was less apparent in the diagram of PCO (not shown) where subsp. *pyrenaea* was omitted (in order to get a better resolution on the first few axes).

Non-parametric classificatory discriminant analysis. All accessions of *C. amara* subsp.

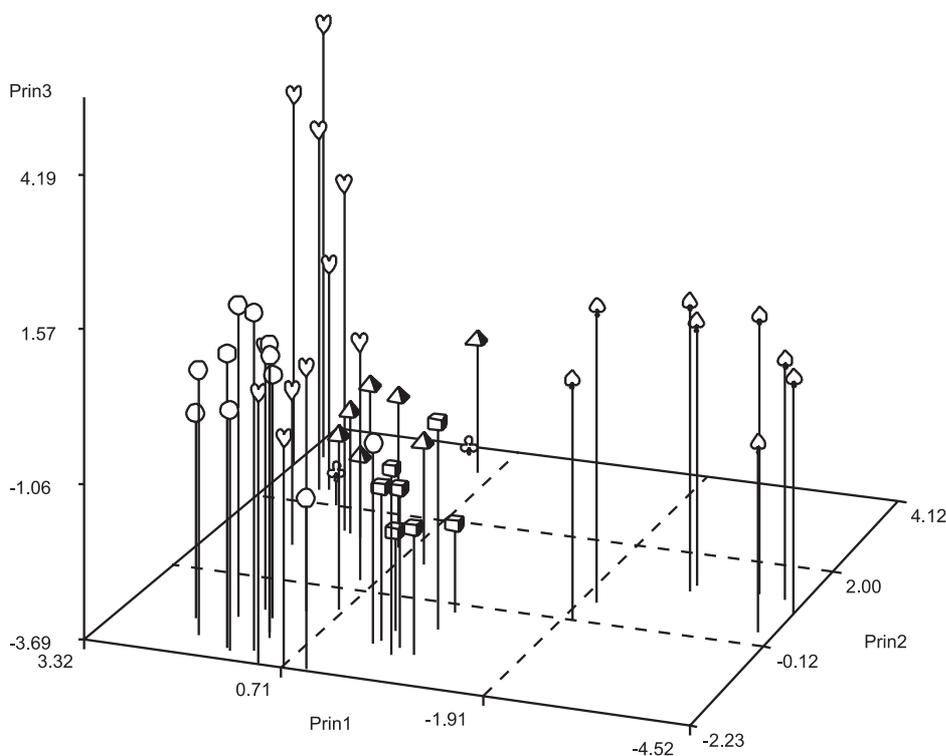


Fig. 2. Principal component analysis of diploid and tetraploid populations of *Cardamine amara* based on presence/absence of alleles (heart – subsp. *amara*, pyramid – subsp. *balcanica*, cube – subsp. *opicii*, spade – subsp. *pyrenaea*, ball – subsp. *austriaca*, club – subsp. *olotensis*). The first three component axes account for 22.2%, 13.4% and 10.6% of variation respectively

opicii, subsp. *balcanica*, subsp. *pyrenaea* and subsp. *olotensis* were classified by the classificatory discriminant analysis according to their taxonomic position. Two samples (out of 11) of subsp. *amara* were incorrectly classified into subsp. *austriaca* and one sample of subsp. *austriaca* (out of 12) was incorrectly classified into subsp. *opicii*.

Cluster analysis. Cluster analysis of the diploid populations (not shown), based on chord distances of Cavalli-Sforza and Edwards (1967) performed by UPGMA clustering method showed only two compact subspecies clusters, namely those composed of subsp. *pyrenaea* and *opicii*. Neither populations of subsp. *balcanica* nor those of subsp. *amara* formed compact clusters in this analysis. This might be interpreted as a consequence of the wide variation of subsp. *amara* (corresponding to its large distribution area compared with other subspecies) as well as indicating a close position of subsp. *amara* and *balcanica*.

Discussion

Differentiation at the isozyme level within *C. amara* reflects to certain extent the morphologically and geographically based subspecies concept. Presence/absence data and differences in allele frequencies characterise most of the infraspecific taxa. There was enough information in the isozyme data (presence/absence of alleles) to discriminate subspecies as was demonstrated by the classificatory discriminant analysis. It should be stressed, however, that this analysis gives more weight to the characters variable among predefined groups (in our case subspecies) than to those with larger within-group variation. The results of PCA, which gives to all characters equal weight, based on the same data provided more overlapping groupings, but still the tendency of populations for a given subspecies to group together was apparent. The same grouping pattern was apparent also in the analysis of the diploid taxa using

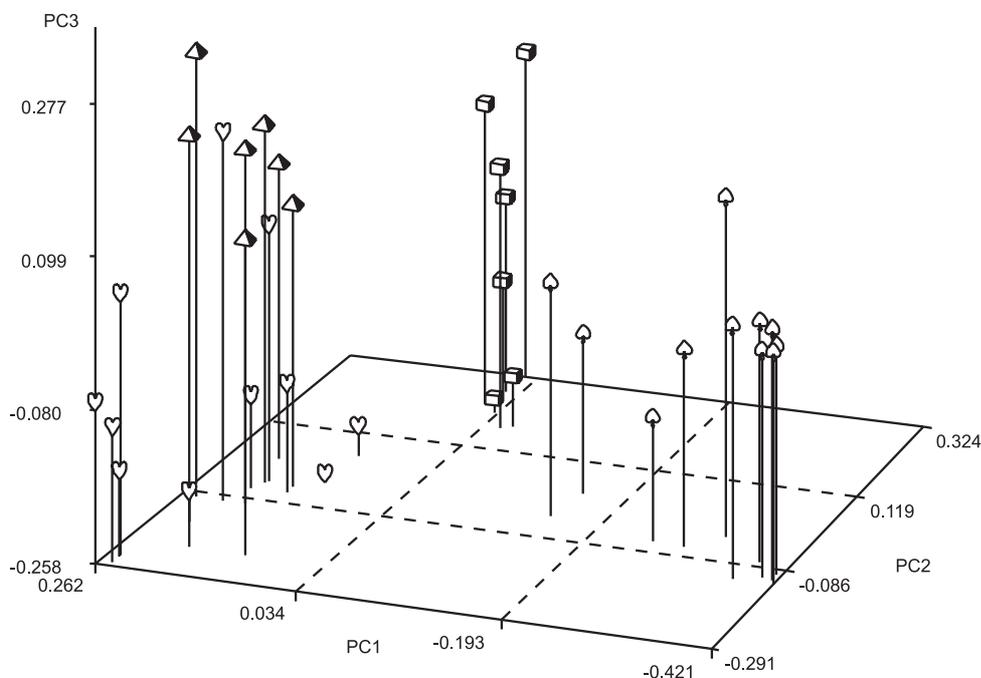


Fig. 3. Principal coordinate analysis of diploid populations of *Cardamine amara* based on chord distances of Cavalli-Sforza and Edwards (1967) (heart – subsp. *amara*, pyramid – subsp. *balcanica*, cube – subsp. *opicii*, spade – subsp. *pyrenaea*). The first three coordinate axes account for 21.8%, 13.7% and 8.2% of variation, respectively

chord distance and PCO and partly also in the cluster analysis.

From among the diploid taxa, *Cardamine amara* subsp. *pyrenaea* was monomorphic for a unique allele (*Aat2-7*), and subsp. *balcanica* had a unique allele, too (*Aat2-4*) which, however, was not fixed in the subspecies. This allele was also detected in *C. flexuosa* (Hurka, unpubl.). Both subspecies, *pyrenaea* and *balcanica*, occur on the margin of the entire distribution range of *C. amara* in areas, which are known as refuge areas during the last glaciation periods (e.g. Hewitt 1999). This coincidence (unique alleles and present distribution area) argues for a relic character and considerable age of these two diploid subspecies. This view is supported by the RAPD data where groupings of subsp. *pyrenaea* and subsp. *balcanica* had high bootstrap values (Lihová et al. 2000). The other two diploid subspecies, subsp. *amara* and subsp. *opicii*, were not characterised by presence of unique alleles but differed in allele frequencies. The three diploid subspecies *C. amara* subsp. *amara*, subsp. *opicii* and subsp. *balcanica*, although partly differentiated at isozyme loci, still seem to be closely related. Their distribution areas either overlap (subsp. *amara* vs. subsp. *opicii*) or they occur in close neighbourhood (subsp. *amara* vs. subsp. *balcanica*) and there is probably gene flow between these taxa.

The two tetraploid taxa strongly differ in isozyme pattern which is most probably the result of their different evolutionary histories. *C. amara* subsp. *olotensis* is well separated from all other subspecies by the unique allele (*Pgm2-1*) and to a certain extent by the frequency of other alleles. On the other hand, tetraploid subsp. *austriaca* differs only slightly from the diploid subsp. *amara*. The same pattern was found while evaluating the RAPD data. While subsp. *olotensis* was clearly different from the rest of the material of the species, subsp. *amara* and subsp. *austriaca* did not form separate groupings or clusters in any analysis (Lihová et al. 2000). This is also in accordance with the morphological data and distribution of these taxa. *C. amara* subsp. *olotensis* differs from all

other subspecies of *C. amara* by the yellow anthers, which is an important and stable character in this genus, as well as in quantitative characters (Lihová et al. 2000). The tetraploid *C. amara* subsp. *austriaca* is morphologically very similar to its most likely diploid progenitor, subsp. *amara*. The only completely reliable character is the size of pollen grains, while other characters, such as the number of leaves, leaflets, and size of some flower parts overlap. Such poor morphological differentiation of this taxon favours a hypothesis of its relatively recent origin. The tetraploid taxa also occupy very different distribution areas. The distribution area of subsp. *austriaca* coincides with an area heavily affected by the Pleistocene glaciation which lead Marhold (1999) to hypothesise its origin during the last interglacial or in the early postglacial periods. *C. amara* subsp. *olotensis* occupies a small relic area in Catalonia, which may indicate its preglacial origin.

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References

- Cavalli-Sforza L. L., Edwards A. W. F. (1967): Phylogenetic analysis: models and estimation procedures. *Evolution* 21: 550–570.
- Franzke A., Hurka H. (2000) Molecular systematics and biogeography of the *Cardamine pratensis* complex (Brassicaceae). *Plant Syst. Evol.* 224: 213–234.
- Hewitt G. (1999) Post-glacial re-colonization of European biota. *Biol. J. Linn. Soc.* 68: 7–112.
- Hewitt G. (2000) The genetic legacy of the Quaternary ice ages. *Nature* 405: 907–913.
- Klecka W. R. (1980) Discriminant analysis. Sage University Papers, Series: Quantitative applications in the social sciences, no. 19, Beverly Hills and London.

- Koch M., Hurka H. (2000) Isozyme analysis in the polyploid complex *Microthlaspi perfoliatum* (L.) F. K. Meyer: Morphology, biogeography and evolutionary history. *Flora* 194: 33–48.
- Koch M., Hurka H., Mummenhoff K. (1996) Chloroplast DNA restriction site variation and RAPD-analyses in *Cochlearia* (Brassicaceae): Biosystematics and speciation. *Nord. J. Bot.* 16: 595–603.
- Lihová J., Marhold K., Neuffer B. (2000) Taxonomy of *Cardamine amara* (Cruciferae) in the Iberian Peninsula. *Taxon* 49: 747–763.
- Lövkvist B. (1956) The *Cardamine pratensis* complex. Outlines of its cytogenetics and taxonomy. *Symb. Bot. Upsal.* 14/2: 1–131.
- Marhold K. (1992) A multivariate morphometric study of the *Cardamine amara* group (Cruciferae) in the Carpathian and Sudeten mountains. *Bot. J. Linn. Soc.* 110: 121–135.
- Marhold K. (1994a) Chromosome numbers of the genus *Cardamine* L. (Cruciferae) in the Carpathians and Pannonia. *Phyton (Horn, Austria)* 34: 19–34.
- Marhold K. (1994b) Taxonomy of the genus *Cardamine* L. (Cruciferae) in the Carpathians and Pannonia. I. *Cardamine pratensis* group. *Folia Geobot. Phytotax.* 29: 335–374.
- Marhold K. (1996) Multivariate morphometric study of the *Cardamine pratensis* group (Cruciferae) in the Carpathian and Pannonian area. *Plant Syst. Evol.* 200: 141–159.
- Marhold K. (1999) Taxonomic evaluation of the tetraploid populations of *Cardamine amara* (Brassicaceae) from the Eastern Alps and adjacent areas. *Bot. Helv.* 109: 67–84.
- Marhold K., Ančev M. E. (1999) *Cardamine penzesii*, a rediscovered taxon of the *C. pratensis* group (Cruciferae). *Ann. Bot. Fenn.* 36: 171–180.
- Marhold K., Ančev M. E., Kit Tan (1996) A new subspecies of *Cardamine amara* (Brassicaceae) from Bulgaria and Greece. *Ann. Bot. Fenn.* 33: 199–204.
- Mummenhoff K., Hurka H. (1995) Allopolyploid origin of *Arabidopsis suecica* (Fries) Norrlin: Evidence from chloroplast and nuclear genome markers. *Bot. Acta* 108: 449–456.
- Nei M. (1972) Genetic distance between populations. *Amer. Naturalist* 106: 283–292.
- Nei M. (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89: 583–590.
- Podani J. (2001) SYN-TAX 2000, Computer programs for data analysis in ecology and systematics. Scientia Publishing, Budapest.
- SAS Institute (1990a) SAS procedures guide, Version 6. Ed. 3. SAS Institute, Cary.
- SAS Institute (1990b) SAS/STAT[®] user's guide, Version 6. Ed. 4. SAS Institute, Cary.
- Swofford D. L., Selander R. B. (1989) BIOSYS-1 (Version 1.7). Illinois Natural History Survey, Champaign.
- Taberlet P., Fumagalli L., Wust-Saucy A.-G., Cosson J.-F. (1998) Comparative phylogeography and postglacial colonization routes in Europe. *Molec. Ecol.* 7: 453–464.
- Urbanska-Worytkiewicz K., Landolt E. (1974) Biosystematic investigations in *Cardamine pratensis* L. s.l. I. Diploid taxa from Central Europe and their fertility relationships. *Ber. Geobot. Inst. ETH Stiftung Rübel* 42: 42–139.

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