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Natural hybridization in *Cardamine* (Brassicaceae) in the Pyrenees: evidence from morphological and molecular data

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While researching *Cardamine* (Brassicaceae) in the Pyrenees, putative hybrid plants were found at two natural sites. Pollen grain viability, AFLP, and multivariate morphometric analyses were performed in order to assess the plants' presumed hybrid origin, establishing that natural hybridization between the diploids *C. crassifolia* and *C. amara* ssp. *pyrenaea* had occurred. A new diploid nothospecies, ***C.* ×*enriquei*** ($2n = 2x = 16$), is described. Examination of 18 morphological characters showed the intermediacy of the hybrid between the parental taxa in most characters. AFLP analyses of *C. amara* ssp. *amara*, ssp. *austriaca*, ssp. *olotensis* and *C. raphanifolia*, demonstrated the close position of the hybrid to *C. crassifolia,* and revealed that the highest number of markers were shared with the parents. Polymorphism found in the AFLP pattern of the hybrid suggested recurrent origin, segregation and/or backcrosses, although assessment of pollen viability indicated high male sterility. The hybridization event reported here represents the second documented case between the *C. pratensis* group and *C. amara*. An account of the nomenclature of *C. crassifolia* is also presented, including lectotypification of relevant names. *Botanical Journal of the Linnean Society*, 2002, **139**, 275–294.

ADDITIONAL KEYWORDS: chromosome numbers – lectotypification – multivariate morphometrics – new nothospecies – Spain.

INTRODUCTION

Interspecific hybridization is an important force in plant evolution and diversification; many studies and reviews illustrate its extent (e.g. Arnold, 1992; Rieseberg & Ellstrand, 1993; Ellstrand, Whitkus & Rieseberg, 1996; Rieseberg, 1998; Ellstrand & Schierenbeck, 2000).

From a morphological view, hybrids typically display a mosaic of parental and intermediate characters, although extreme and novel characters quite often appear in the hybrid phenotype as well. Morphological characters alone are of limited value when identifying natural hybrids (Rieseberg & Ellstrand, 1993) and molecular studies have greatly enhanced our knowledge of this field. An increasing number of introgression and hybrid speciation events have been documented, which prove that gene flow has obviously been underestimated when relying on morphology alone. Transgression seems to be the rule rather than the exception (Rieseberg, Archer & Wayne, 1999). Furthermore, marker systems such as isoelectric focusing (IEF) of the small and large subunits of rubisco (nuclear encoded SSU and chloroplast encoded LSU) and sequencing (e.g. *trn*L intron or *trn*L/F spacer region) provide information about maternal or paternal relationships of which there are several examples from Brassicaceae (Mummenhoff,

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Eschmann-Grupe & Zunk, 1993 for *Diplotaxis*; Mummenhoff & Hurka, 1995 for *Arabidopsis;* Urbanska *et al.,* 1997 for *Cardamine*; Bleeker, Huthmann & Hurka, 1999 for *Nasturtium*).

Fingerprinting methods such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and, more recently, amplified fragment length polymorphism (AFLP) have proven effective in studies on hybridization because they screen many loci scattered across the genome (Bachmann, 1994; Rieseberg, 1998). RAPD has been widely used in recent years (for Brassicaceae see Nolan, Skotnicki & Gibbs, 1996; Neuffer & Jahncke, 1997; Urbanska *et al*., 1997; Neuffer *et al*., 1999). However, the reproducibility of RAPD fingerprinting is difficult. AFLP was developed with the aim of combining the advantages of RFLP and arbitrary primer methods (Vos *et al*., 1995). Based on the selective amplification of genomic restriction fragments, it can be highly informative and reproducible, suitable for assessing genetic differences from the individual up to species level (Rieseberg, 1998; Mueller & Wolfenbarger, 1999; Sunnuncks, 2000) and also for the identification of the origin of hybrids (Han *et al*., 2000).

Cardamine sect. *Cardamine* involves several taxonomically difficult species complexes, e.g. *C. amara* L., *C. pratensis* L. and *C. raphanifolia* Pourr. Taxonomic studies on the *C. pratensis* group have revealed a rather complicated pattern of variation in Europe (Lövkvist, 1956; Urbanska-Worytkiewicz & Landolt, 1974; Marhold, 1994, 1996; Marhold & Ančev, 1999; Franzke & Hurka, 2000). Extensive polyploidization, hybridization and climatic changes in the Quaternary have presumably played a major role in its evolution (Lövkvist, 1956; Franzke & Hurka, 2000). *Cardamine crassifolia* Pourr., which occurs in the eastern Pyrenees (populations from the central part of the Iberian Peninsula which are also ascribed to this species require further study; Lihová *et al.,* work in progress), exhibits several morphological characters which are unique within the group: it has a creeping and stoloniferous rhizome, ascending stem, and lacks a rosette (Rico, 1993). Molecular data indicate that it represents an ancient lineage of the *C. pratensis* group (Franzke & Hurka, 2000).

Cardamine amara L. comprises several diploid and tetraploid subspecies that are, except for the widely distributed typical subspecies, restricted to European mountain ranges. Multivariate analyses were successfully employed for studies on morphological variation, as the subspecies differ almost exclusively in quantitative traits (Marhold, 1992, 1995, 1998, 1999; Marhold *et al.*, 1996; Lihová, Marhold & Neuffer, 2000). Molecular data (RAPD, isozymes) supported this taxonomic treatment, also suggesting the relic nature of diploid *C. amara* ssp. *balcanica* Marhold, Ančev & Kit Tan and *C. amara* ssp. *pyrenaea* Sennen from the Balkan Peninsula and eastern Pyrenees, respectively (Lihová *et al*., 2000; Marhold *et al.* submit.). Another Iberian taxon, *C. amara* ssp. *olotensis* O. Bolòs, seems to be more distinct morphologically and this is supported by molecular data. The species rank might be more appropriate for this taxon (Lihová *et al.,* work in progress). Representatives of the above mentioned groups are considered either outcrossing (*C. pratensis* group) or mixed selfing and outcrossing (*C. amara*) perennials with an effective vegetative propagation (Lövkvist, 1956, 1957). The hybrids between *C. amara* and the *C. pratensis* group are very rare and the only proven cases have been reported from Switzerland (Urbanska-Worytkiewicz & Landolt, 1972). Crossing experiments performed between diploid *C. amara* as one parent and representatives of the *C. pratensis* group at various ploidy levels as another revealed rather strong incompatibility between them (Lövkvist, 1956, 1957).

While researching *C. crassifolia* in the Pyrenees, we found unusual populations of uncertain taxonomic status at two sites. As the plants grew close to populations of *C. amara* ssp. *pyrenaea* and *C. crassifolia*, a hybrid origin was suggested. Plants morphologically similar to this putative hybrid have been found among the specimens of *C. crassifolia* in the Pourret herbarium at MAF, which may be important for the typification, and thus proper interpretation, of this name. To confirm or reject the assumed hybrid origin of the collected plants and the Pourret specimens we performed the following: (1) assessment of the fertility of pollen grains in both the assumed hybrid and the putative parents; (2) evaluation of genetic variation using AFLP and (3) multivariate analysis to reveal the patterns of variation in several morphological traits.

MATERIAL AND METHODS

Plant material

The origin of the plant material is listed in Table 1 and illustrated in Figure 1. Populations of putative parents were selected from the sites of the occurrence of the presumed hybrids (or their close vicinity) as well as from more distant places in order to include a wider sample of variation of these taxa. Chromosome numbers from all populations studied were either taken from our previous studies or represent new counts. For pollen fertility analyses a few plants (5–12) per locality were selected. Samples for AFLP analysis included not only the presumed hybrid populations and putative parents, but also plants of closely related *C. amara* ssp. *amara*, *C. amara* ssp. *austriaca*, *C. amara* ssp. *olotensis* and *C. raphanifolia*. For mor-

Table 1. List of populations and number of studied plants of *Cardamine crassifolia*, *C. amara* ssp. *pyrenaea*, *C. amara* ssp. *amara*, *C. amara* ssp. *austriaca*, *C. amara* ssp. *olotensis*, and *C. raphanifolia* and ***C.* ×*enriquei*** used for morphometric, pollen and AFLP analyses. The chromosome numbers (2*n*) without a number in superscript represent new data, those with the superscript are taken from previous papers:[1] Lihová *et al*. (2000),[2] Marhold (1999),[3] Ančev, unpubl.; '–' not used in the particular analysis

Population code, origin and collection data	2*n*	No. individuals		
		Morph	Pollen	AFLP
Cardamine crassifolia Pourr.				
C-MRG1 – Spain, Cerdanya, N of Meranges, at the lake Estany de Malmiu, 2260 m, 1.vii.2001, *Lihová*	16	39	5	–
C-MRG4 – Spain, Cerdanya, NW of Meranges, Riu Duran, near Refugi J. Folchi Girona, 2320 m, 2.vii.2001, *Lihová*	16	33	5	–
C-RMC – Spain, Ripollès, Planell de les Eugues, close to Refugi Manelic 1975 m, 30.vi.2001, *Lihová*	16	–	5	–
C-RA – Spain, Ripollès, between Llanars and Ribes de Freser, Rierra d′ Abella, 1580 m, 6.vi.2000, *Perný* & *Vicens*	–	26	5	–
C-NU1 – Spain, Ripollès, Núria, 2020–2250 m, 1996, *Franzke*, 8.vii.1997, *Marhold*; 27.vi.2001, *Lihová*	16	30	5	5
C-NU4 – Spain, Ripollès, Núria valley, Torrent de Fontalba, 2100 m, 28.vi.2001, *Lihová*	16	29	–	–
C-LB – France, Cerdagne, Lac de Bouillouses, 2100 m, 1996, *Franzke*	–	–	–	1
Cardamine amara ssp. *pyrenaea* Sennen				
P-MRG3 – Spain, Cerdanya, N of Meranges, below Refugi Malniu, 2080 m, 1.vii.2001, *Lihová*	16	18	5	–
P-RM – Spain, Ripollès, Planell de les Eugues, close to Refugi Manelic 1975 m, 7.vii.1997, *Marhold*	16[1]	29	5	–
P-SE – Spain, Ripollès, Pla dels Hospitalets, near the rivulet Clot de Coma Ermada 1925 m, 23.viii.1996, *Marhold* & *Vicens*	16[1]	35	5	1
P-FA – Spain, Ripollès, Núria, Torrent de Fontalba, 2080 m, 8.vii.1997, *Marhold*	16[1]	32	5	–
P-COE – Spain, Ripollès, Núria, Coma d′ Eina, 2065–2105 m, 8.vii.1997, *Marhold*	16[1]	27	5	1
P-NCR – Spain, Ripollès, Núria, Coma de Noucreus, 2130 m, 6.vii.1997, *Marhold*	16	–	–	2
P-NOC – Spain, Ripollès, Núria, Coma de Noucreus, 2290–2310 m, 6.vii.1997, *Marhold*	16[1]	–	–	1
P-HMO – Spain, Ripollès, Font de l'Home Mort 1850–1970 m, 5.vii.1997, *Marhold*	16[1]	–	–	1
P-VAM – Spain, Ripollès, Coma de Freser, near the bridge over the Torrent de Freser, 1560 m, 7.vii.1997, *Marhold*	16[1]	–	–	1
P-CRJ – Andorra, Grau Roig, close to Hostal Refugi de Cabana Roja, side tributary of Riu La Valira, 2095 m, 28.viii.1996, *Marhold & Vicens*	16[1]	–	–	1
Cardamine amara ssp. *amara*				
A-20-PUB – Slovenia, Loška dolina, river banks of Veliki Obrh, W of the village of Podub, 570 m, 21.v.1996, *Marhold* & *Jogan*	16[2]	–	–	1
A-61-WCH – Austria, Lower Austria, Wechselgebiet, along the rivulet Leidingbach S of the village Erlach, E of Leiding, 360 m, 11.v.1997, *Vitek*	16[2]	–	–	1
A-LEE – Germany, Leeden near Osnabrück, 80 m, *Kohrt*	–	–	–	1
A-07-GAS – Slovak Republic, Nízke Tatry Mts., close to the village Gašparovo, 540 m, 15.viii.1997, *Marhold*	16[1]	–	–	1
A-83-DRB – Slovak Republic, Slovenské rudohorie Mts., Drábsko, 980 m, 15.viii.1997, *Marhold*	16[1]	–	–	1

Table 1. *Continued*

Population code, origin and collection data	2*n*	No. individuals		
		Morph	Pollen	AFLP
Cardamine amara ssp. *austriaca* Marhold				
T-50-ANT – Slovenia, Štajerska, Pohorje, between Vuhred and Ribnica na Pohorju, 530 m, 22.v.1996, Marhold & Jogan	32[2]	–	–	1
T-60-HCH – Austria, Salzburg, Mt. Hochkönig nearby the village Dienten, track from Erichhütte to Stegmoosalm, 1590 m, 9.vii.1997, *Vitek*	32[2]	–	–	1
T-78-STS – Austria, North Tyrol, St. Anton am Arlberg, Steissbachtal 1800 m, 7.viii.1997, *Brandstätter*, *Chrtek & Mráz*	32[2]	–	–	1
Cardamine amara ssp. *olotensis* O. Bolòs				
L-45-SFE – Spain, Parc natural del Montseny, 1 km E of Santa Fe del Montseny, 1130 m, 11.v.1996, *Marhold & Giráldez*	32[1]	–	–	1
L-66-OL1 – Spain, Olot, Parc Nou, *c.* 400 m, 10.v.1996, *Marhold, Benedí & Vicens*	32[1]	–	–	1
L-66-OL2 – Spain, Olot, Paratges de la Deu, *c.* 400 m, 10.v.1996, *Marhold, Benedí & Vicens*	32[1]	–	–	1
Cardamine raphanifolia Pourr.				
RP-21-ERM – Spain, Ripollés, Setcases, Pla dels Hospitalets, nearby the rivulet Clot de Coma Ermada, 1925 m, 23.viii.1996, *Marhold*	48[3]	–	–	1
RP-31-SAL – Spain, Cerdanya, nearby the road from Ribes de Freser to Puigcerdá, *c.* 0.75 km E of Casilla de Saltèguet, near to the crossroad to Baga de Saltèguet, 1725 m, 28.viii.1996, *Marhold & Vicens*	48[3]	–	–	1
RP-62-CAL – Italy, Calabria, prov. di Cosenza, Sila Grande *c.* 13.5 km ENE of Camigliatello Silano, Macchialonga, 1510 m, 11.vi.1997, *Vitek*	48	–	–	1
Cardamine ×enriquei Marhold, Lihová & Perný				
MRG2 – Spain, Cerdanya, N of Meranges, nearby the path from Refugi Malniu to the lake Estany de Malniu, 2080 m, 1.vii.2001, *Lihová*	16	11	7	–
RMH – Spain, Ripollès, Planell de les Eugues, close to Refugi Manelic 1975 m, 7.vii.1997, *Marhold*; 30.vi.2001, *Lihová*	16	10 + 37	12	5

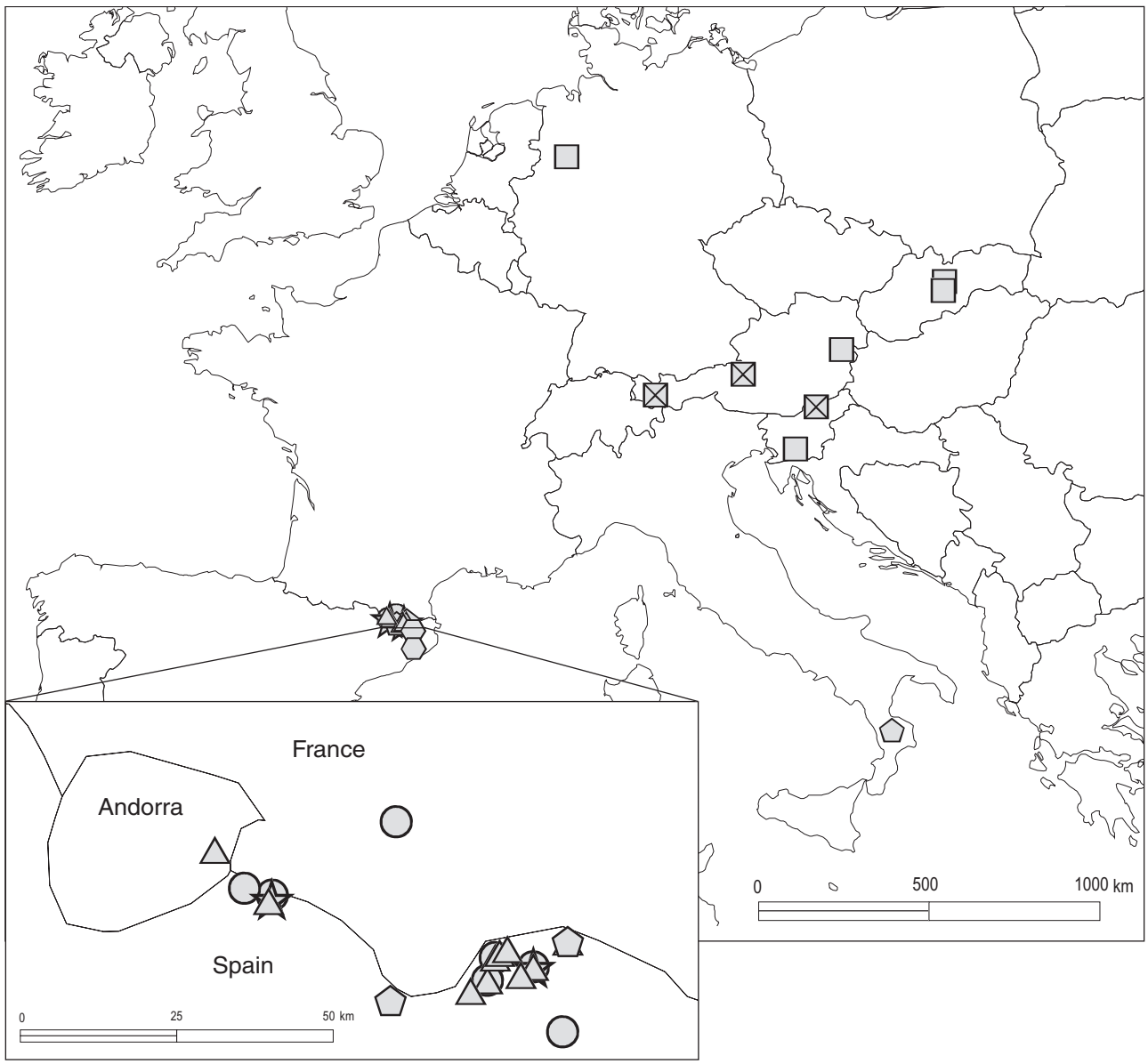

Figure 1. Map of distribution of sample sites of *Cardamine amara* ssp. *amara* (□), *C. amara* ssp. *austriaca* (⊠), *C. raphanifolia* (⬠), *C. amara* ssp. *olotensis* (⬡), *C. amara* ssp. *pyrenaea* (△), *C. crassifolia* (○), and ***C.* ×*enriquei*** (☆) (for sample site details see Table 1).

phological studies we selected population samples from both localities of presumed hybrids (58 plants) and five population samples from each putative parent, *C. amara* ssp. *pyrenaea* and *C. crassifolia* (141 and 157 plants, respectively).

CHROMOSOME NUMBERS

For most of the populations studied, chromosome numbers were assessed. New chromosome number counts were made using the methods described in Lihová *et al*. (2000) and Mártonfi *et al*. (1999). Root tips were obtained from the plants collected at the localities and cultivated at the Institute of Botany, Slovak Academy of Sciences, Bratislava. After pretreatment in 0.002 M aqueous solution of 8-hydroxyquinoline for 3 h, the root tips were fixed in a freshly prepared mixture of ethanol and acetic acid (3 : 1) for 1 h and stored in 75% ethanol. Subsequently, the root tips were hydrolysed in a mixture of concen-

trated hydrochloric acid and ethanol (1 : 1) for 3–5 min and rinsed in water. Squashes were made using a cellophane square and stained in 10% solution of Giemsa stock solution in Sörensen phosphate buffer for 1 h.

Pollen fertility

As a measure of male fertility, pollen viability was estimated using acetocarmine staining. Anthers were removed from a single flower bud and macerated in a drop of acetocarmine jelly on a microscope slide to release pollen grains; 100–160 grains were evaluated per individual and both viable (well-stained) and unviable (shrunken and unstained) grains were scored (Marks, 1954). Pollen quality was expressed as the percentage of viable grains detected.

AFLP fingerprinting

Genomic DNA was extracted from about 50 mg fresh leaf material (for DNA isolation protocol see Neuffer & Jahncke, 1997). After extraction, quantity and quality of DNA were determined photometrically. AFLP was performed as described by Vos *et al*. (1995), but with modifications. Genomic DNA (~0.8 µg per sample) was digested with the restriction enzymes *EcoRI* and *MseI*. Double stranded adaptors (Applied Biosystems, Weiterstadt, Germany) were ligated to the ends of DNA fragments. Restriction and ligation were done in a single reaction step (37°C, 2 h) in a thermocycler (Biometra, Göttingen, Germany).

Preselective amplification was carried out using primers with an additional base at the 3′end to reduce the number of fragments. The conditions for preselective amplification were 2 min at 72°C and 20 cycles of 1 s at 94°C, 30 s at 56°C, 2 min at 72°C; the final step was 30 min at 60°C.

Selective amplification was carried out in the following conditions: 2 min at 94°C, 9 cycles of 1 s at 94°C, 30 s at 65°C including a temperature reduction of 1°C per cycle, and 2 min at 72°C, 23 cycles of 1 s at 94°C, 30 s at 56°C, and 2 min at 72°C, and finally 30 min at 60°C. *EcoRI*-based dyes with the additional bases ACC, -AAC (yellow), -ACG, -AAG (green), -ACT and -ACA (blue) were used in combination with the *MseI*-based primer -CTA (Applied Biosystems).

AFLP fragments were detected using an ABI Prism 377 Sequencer; those ranging from 50 to 500 bases with a fluorescent intensity above 50 units were scored. AFLP reactions were analysed using GeneScan and Genotyper software (Applied Biosystems).

The data were analysed using the following techniques: (1) neighbour-joining distance analysis (Saitou & Nei, 1987), including the Nei & Li (1979) coefficient plus bootstrap option (1000 replications) (TREECON; van de Peer & de Wachter, 1994); (2) principal coordinate analysis (PCoA) using Jaccard's coefficient (SYNTAX 2000; Podani, 2001).

Morphometric analyses

For measurements of vegetative morphological characters, herbarium specimens collected from natural populations were used. Floral parts were attached by adhesive tape to paper and dried and the characters of each plant measured (see list in Table 2). Characters included those traditionally used in the taxonomic evaluation of both *C. amara* and the *C. pratensis* group, and others used to compare both groups and their presumed hybrid. Twelve vegetative and six floral characters were measured, and six ratios derived. Pearson and Spearman correlation coefficients were computed in order to eliminate highly correlated characters from further analyses and reveal groups of correlated characters differentiating studied taxa. Six vegetative characters were used only for computing ratios to eliminate size differences that might result from the influence of environmental factors. The colour of anthers and petals was scored as well. In order to depict morphological variation of the presumed hybrid and its putative parents, exploratory data analysis, principal component analysis (PCA) and cluster analysis were used as hypothesis generating methods and canonical discriminant analysis (CDA) used as a hypothesis testing method. In an R type of PCA (Sneath & Sokal, 1973; Krzanowski, 1990), based on a correlation matrix, individual plants were used as OTUs. Among clustering methods UPGMA (average clustering), complete linkage and centroid clustering methods (Everitt, 1986) were applied using population samples characterized by mean values of characters as OTUs. Euclidean distance coefficient was used and all characters were standardized so that they had zero means and unit standard deviations. In CDA (Klecka, 1980) the two parental taxa and hybrids were used as groups and individual plants as OTUs. This technique generally requires multivariate normality of characters and equality of the within-group covariance matrices, but as noted by Sneath & Sokal (1973: 127) "considerable robustness to violations of these assumptions has been demonstrated" (see also Thorpe, 1976). Therefore, although most of the characters used more or less deviated from the normal distribution, these deviations should not seriously disturb presented results of CDA (see Klecka, 1980 for detailed discussion). Total canonical structure presented in Results shows correlations of individual characters and particular canonical axes and, unlike standardized coefficients of canonical functions, they are not influenced by the correlations among characters. Cluster analyses were computed by SYNTAX 2000 (Podani, 2001). For other analyses SAS (SAS Institute, 1990a,b) was used.

Table 2. List of characters measured for morphometric analyses

Vegetative characters	
WIS	width of stem (mm)
LSI*	length of stem from the base to the lowest peduncle of flower (fruit) (cm)
LSL	length of stem from the base to the base of the uppermost stem leaf (cm)
NL	number of stem leaves
NLR	degree of congestion of leaves beneath the inflorescence, expressed by the number of leaves reaching the base of the uppermost stem leaf
SSL*	number of leaves in the lower half of LSL
LC2	length of middle stem leaf (cm)
NFS	number of pairs of lateral leaflets of the middle stem leaf[1]
LTS* and WTS*	length and width of terminal leaflet of the middle stem leaf (mm)
LLS* and WLS*	length and width of first lateral leaflet of the middle stem leaf (mm)
Floral characters	
LS and WS	length and width of sepals (mm)
LP and WP	length and width of petals (mm)
LFL	length of longer filaments (mm)
LFS	length of shorter filaments (mm)
Ratio characters	
LSI/LSL, SSL/(NL – SSL), NL/LSL, WTS/LTS, WLS/LLS, LFS/LFL	

*characters used only for computing ratios; [1] the leaf closest to the LSL/2 point

RESULTS

Chromosome numbers

Both putative parental taxa, *C. crassifolia* and *C. amara* ssp. *pyrenaea*, and the presumed hybrid are diploid ($2n = 16$; Table 1). New chromosome number data for both *C. crassifolia* and *C. amara* ssp. *pyrenaea* are in accordance with previously published records (Rico and Marhold in Franzke & Hurka, 2000; Lihová *et al*., 2000).

Pollen fertility

As revealed by its stainability, pollen of *C. crassifolia* appeared to be of good quality. In most individuals (e.g. populations C-MRG4, C-MRG1, C-RA) stainability exceeded 90%, although in the other two localities broader variation was found. Reduced pollen viability (as low as 21%) was detected in the population C-RMC, close to the site of putative hybrids. Investigation of *C. amara* ssp. *pyrenaea,* revealed almost invariably high male fertility, with stainability of 95–98% in most individuals studied (Table 3A).

The pollen viability of putative hybrid individuals, by contrast, differed strongly in comparison with that of the presumed parents. Of 12 individuals examined from the population RMH, 11 were completely sterile; in one individual only were viable grains ($N = 4$) detected. Low stainability was also recorded from MRG2; of seven analysed individuals four were found to be sterile, while the other three contained only ten viable grains between them (Table 3B).

AFLP fingerprinting

AFLP revealed 206 informative characters (Table 4). The presumed hybrid had many more characters than any other taxon, with four characters not found in any other taxon, including the presumed parents, although this may be due to sampling error. Of the other taxa studied, 14 specific characters of *C. amara* ssp. *olotensis* are worth mentioning. They have been observed in all plants of this subspecies and are unique to it. This could be an argument for the raising the rank of the taxon to species, (see Lihová *et al*., 2000 and work in progress; Marhold *et al*. submit.). Of the taxa studied, *C. crassifolia* and *C. amara* ssp. *pyrenaea* had the highest number of characters shared with the hybrid (67 and 57 characters, respectively). *C. crassifolia* shared 19 characters exclusively with the hybrid, followed by *C. amara* ssp. *pyrenaea* (three shared characters) and *C. raphanifolia* (two shared characters) (Table 4A). By comparing the number of bands shared by different pairs of the investigated taxa, *C. raphanifolia* and *C. crassifolia* were found to have the highest number (10) of fragments in common. These were absent in the other nonhybrid taxa, while six were found in the hybrid. *C. crassifolia* and *C. amara* ssp. *pyrenaea* shared five fragments, four of which were also present in the hybrid. The high

Table 3. A, Pollen fertility of putative parental taxa, *C. crassifolia* and *C. amara* ssp. *pyrenaea*. Minimum, maximum values and median, expressed in percentages (5–12 individuals per population and 100–160 pollen grains per individual out of one flower). B, Pollen fertility of ***C.* ×*enriquei*** expressed in number of investigated individuals and number of stained pollen grains found on particular slides. For population codes see Table 1

A.

crassifolia	C-NU 1	C-RA	C-MRG4	C-MRG1	C-RMC
min–max	33.33–92.80	93.14–99.10	90.52–93.88	71.43–98.37	20.66–86.99
median	75.63	98.23	91.53	92.17	35.93
pyrenaea	P-FA	P-COE	P-SE	P-MRG3	P-RM
min–max	86.09–99.34	98.13–99.34	58.59–98.48	96.77–98.68	94.34–99.34
median	97.74	98.72	95.59	98.04	96.77

B.

×***enriquei***	RMH	MRG2
number of investigated individuals	12	7
number of completely sterile individuals	11	4
number of individuals with at least one stained pollen grain found on a slide (number of stained pollen grains for particular individuals in brackets)	1 (4)	3 (1, 1, 8)

Table 4. Number of AFLP bands scored for the analysed *Cardamine* taxa. (A) C (characters) – bands present in a given taxon; UC (unique characters) – bands present in a given taxon, but not in other taxa; CHO (characters shared with hybrid only) – bands shared with the hybrid but not with other taxa; CH (characters shared with hybrid) – bands present in a given taxon and hybrid, regardless of their presence or absence in other taxa; (*n*) – number of analysed individuals. (B) Number of bands shared by the pair of taxa but not with any other nonhybrid taxon (i.e. including also bands present in that pair and the hybrid) (first row), and number of bands shared by the pair of taxa and the hybrid but not with any other taxon (second row); *au* – *C. amara* ssp. *austriaca*, *am* – *C. amara* ssp. *amara*, *ol* – *C. amara* ssp. *olotensis*, *pyr* – *C. amara* ssp. *pyrenaea*, *rap* – *C. raphanifolia*, *cra* – *C. crassifolia*

A.

Taxon (*n*)	C	UC	CHO	CH
austriaca (3)	82	0	0	45
amara (5)	84	2	0	45
olotensis (3)	85	14	1	45
raphanifolia (3)	86	8	2	47
crassifolia (6)	87	4	19	67
pyrenaea (8)	94	8	3	57
×***enriquei*** (5)	106	4	–	–
Total	206	40		

B.

Pair of taxa	*au*	*au*	*au*	*au*	*au*	*am*	*am*	*am*	*am*	*ol*	*ol*	*ol*	*rap*	*rap*	*cra*
Shared char.	*am*	*ol*	*rap*	*cra*	*pyr*	*ol*	*rap*	*cra*	*pyr*	*rap*	*cra*	*pyr*	*cra*	*pyr*	*pyr*
Each other	9	1	2	0	4	1	1	1	4	3	4	2	10	3	5
With hybrid	0	0	0	0	0	0	0	1	3	0	2	0	6	0	4

number of bands (9) shared exclusively by *C. amara* ssp. *amara* and ssp. *austriaca* can be attributed to the presumed autotetraploid origin of the latter (Table 4B). A neighbour-joining tree (Fig. 2) showed high bootstrap support for *C. crassifolia*, *C. amara* ssp. *pyrenaea* and *C. amara* ssp. *olotensis*. The hybrid also revealed high bootstrap support (71%) and was in a close position to *C. crassifolia* and *C. raphanifolia* (Table 4B). However, this may more readily be explained by the two species' relatedness as the latter

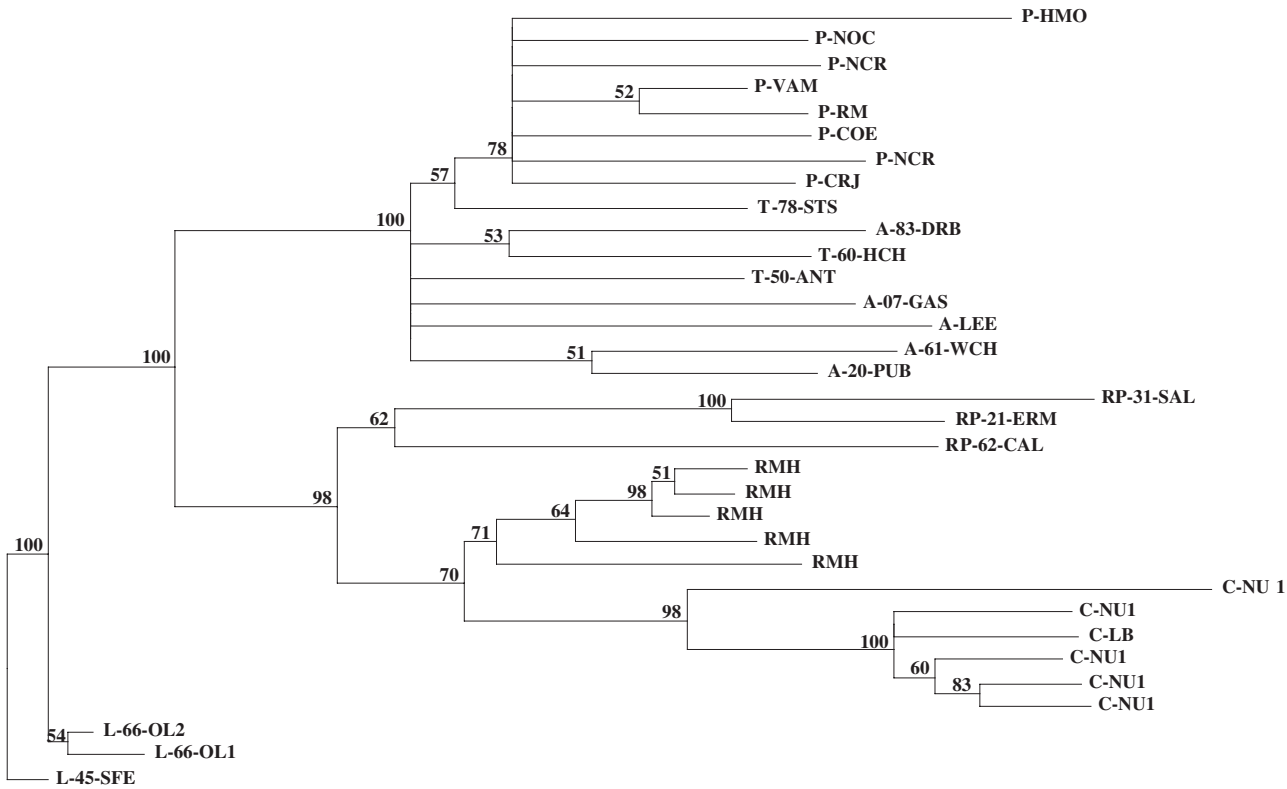

Figure 2. Neighbour-joining analysis of AFLP data of *Cardamine amara* ssp. *amara* (A), ssp. *austriaca* (T), ssp. *olotensis* (L), ssp. *pyrenaea* (P), *C. raphanifolia* (RP), *C. crassifolia* (C) and ***C. ×enriquei*** (RMH). Branches supported by less than 50% bootstrap values are drawn as unresolved. For population codes see Table 1.

species is hexaploid, excluding it as a potential parent of the hybrid. Populations of all included subspecies of *C. amara* (except ssp. *olotensis*) had very high support (100%). Low support for the separation of *C. amara* ssp. *amara* and ssp. *austriaca* furnished additional evidence for the recent autotetraploid origin of the latter subspecies (see also Marhold *et al.*, submit.). *Cardamine raphanifolia* appeared to be closer to *C. crassifolia* than any taxon of *C. amara*. PCoA (presenting nonhierarchical representation of data, which is more appropriate in the case of hybrids) of Iberian taxa (except *C. raphanifolia*) placed the hybrid plants in an intermediate position between the putative parents along the first coordinate, albeit closer to *C. crassifolia* (Fig. 3). *Cardamine amara* ssp. *olotensis* was clearly distant from all the other Iberian taxa.

Morphometric analyses

The ordination diagram of PCA (Fig. 4) showed two distinct groups of *C. crassifolia* and *C. amara* ssp. *pyrenaea* with the hybrid plants in clearly intermediate positions along the first axis. The first three component axes accounted for 53.07%, 17.34% and 5.38% of the variation among OTUs, respectively. Almost all characters (except length of sepals and petals, width of petals and length of longer filaments) contributed equally to division among the groups along the first axis. The hybrid plants were slightly separated from the putative parents along the second axis, which was more strongly correlated with the floral characters: length of sepals and petals, width of petals and length of longer filaments (Table 5).

Cluster analysis using several clustering algorithms (UPGMA, complete linkage, centroid method, dendrograms not shown) resulted in three main clusters representing groups of populations of the hybrids and the putative parents. Hybrid populations were at a higher level clustered together with *C. crassifolia*, indicating further close morphological connections.

CDA with individual plants as OTUs and the parental taxa and putative hybrids as groups showed three distinct groups with only one plant of *C. crassifolia* (population C-RA) revealed as being closer to the hybrid plants, and one hybrid plant (from population RMH) as being closer to *C. crassifolia* (Fig. 5). As shown from canonical correlation coefficients (Table 6), analysis confirmed that all of the vegetative and two

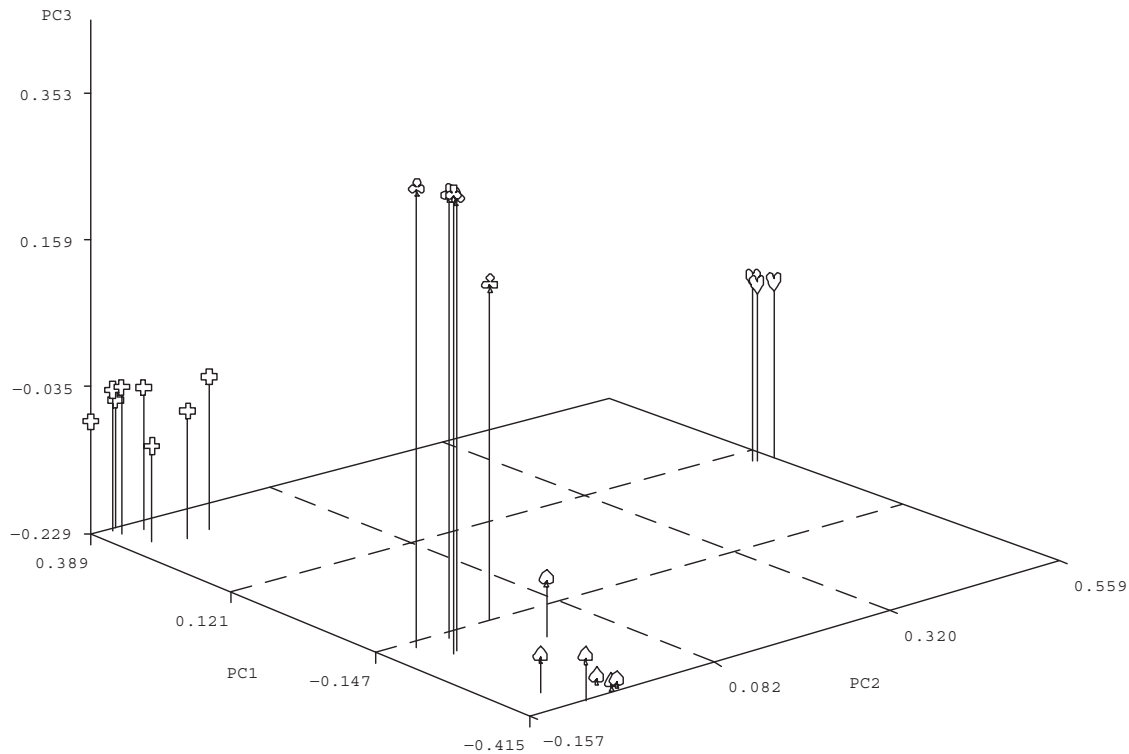

Figure 3. Principal coordinate analysis of AFLP data of *C. crassifolia* (♤), *C. amara* ssp. *pyrenaea* (✠), *C. amara* ssp. *olotensis* (♡) and ***C.* ×*enriquei*** (♧). The first three coordinates explaining 28.9%, 15.5% and 11.5% of variation, respectively.

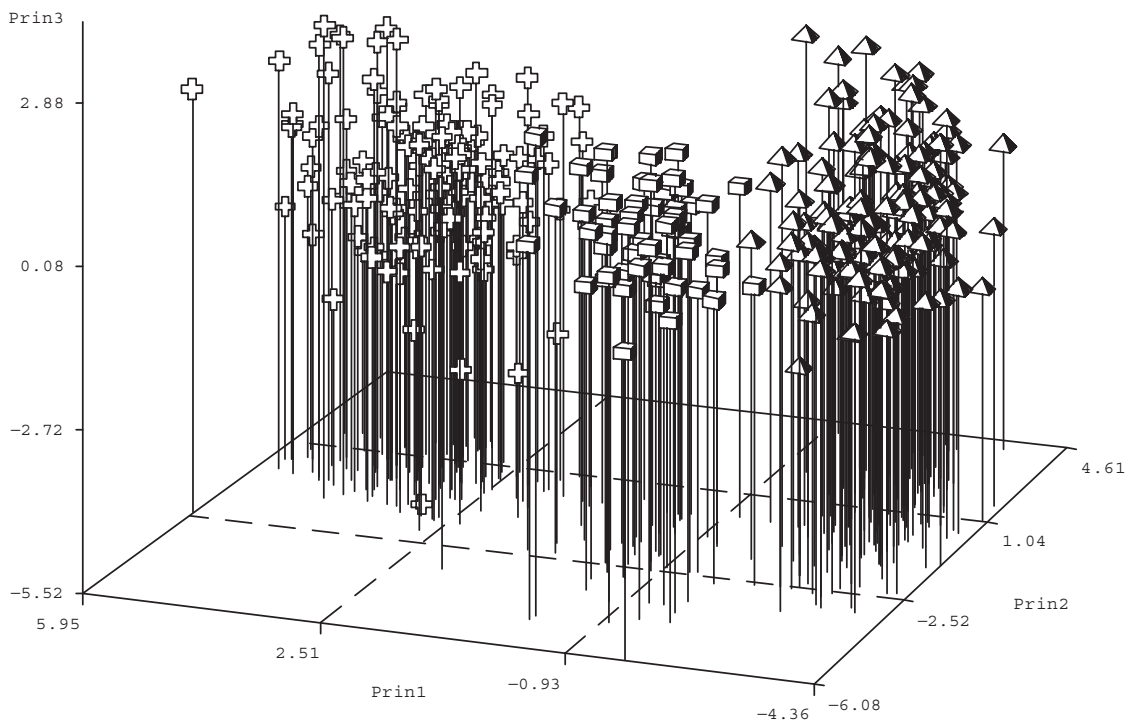

Figure 4. Principal component analysis based on 18 morphological characters of individuals of *C. crassifolia* (✠, $N = 157$), *C. amara* ssp. *pyrenaea* (◭, $N = 141$), and their hybrid ***C.* ×*enriquei*** (◰, $N = 58$).

Table 5. Principal component analysis of *C. crassifolia*, *C. amara* ssp. *pyrenaea* and their hybrid ***C. ×enriquei*** (see Fig. 4). Component loadings showing contributions of the characters to the principal components (PC1, PC2, PC3). For character abbreviations see Table 2

Character	PC1	PC2	PC3
WIS	0.289902	–0.023058	–0.092279
LSL	0.219660	–0.136737	0.625183
NL	0.304393	–0.021191	–0.120967
NLR	0.288213	0.010531	–0.102166
LC2	0.273272	–0.065453	0.384935
NFS	0.266502	–0.065062	0.101306
LS	0.108023	0.421889	0.084726
WS	0.212205	0.151803	–0.119547
LP	–0.017839	0.531802	0.139772
WP	–0.057090	0.448560	0.147323
LFL	0.138561	0.434310	–0.049494
LFS	0.274545	0.221055	–0.002001
SSL/(NL-SSL)	0.207924	–0.127190	0.118817
NL/LSL	0.257774	0.009066	–0.540384
WTS/LTS	0.239512	–0.091916	–0.114121
WLS/LLS	0.263322	–0.007919	–0.147930
LSI/LSL	0.272983	–0.155876	0.093002
LFS/LFL	0.283776	0.009027	0.047641

Table 6. Canonical discriminant analysis of *C. crassifolia*, *C. amara* ssp. *pyrenaea* and their hybrid ***C. ×enriquei*** (see Fig. 5). Total canonical structure (correlation coefficients of morphological characters and canonical axes (CAN1, CAN2)). Those exceeding the value 0.6 are marked in bold. For character abbreviations see Table 2

Character	CAN1	CAN2
WIS	**0.854186**	–0.122574
LSL	**0.638758**	–0.113500
NL	**0.918677**	0.020719
NLR	**0.857858**	0.058742
LC2	**0.798446**	–0.045136
NFS	**0.796192**	–0.096542
LS	0.300640	0.245189
WS	0.574081	0.068466
LP	–0.116547	**0.602395**
WP	–0.295837	0.364051
LFL	0.389278	**0.772899**
LFS	**0.840643**	0.414323
SSL/(NL-SSL)	**0.675483**	–0.230915
NL/LSL	**0.815833**	0.002692
WTS/LTS	**0.748677**	0.012468
WLS/LLS	**0.814452**	0.152119
LSI/LSL	**0.863780**	–0.335362
LFS/LFL	**0.888283**	0.006099

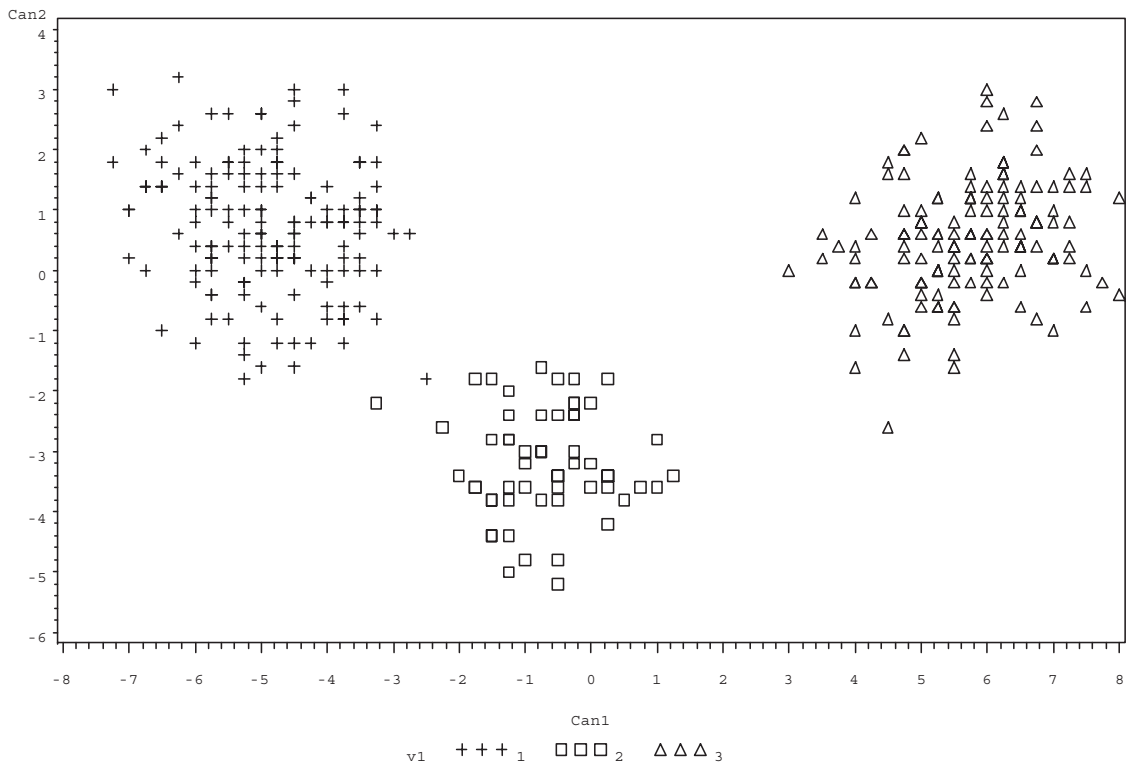

Figure 5. Canonical discriminant analysis based on 18 morphological characters of individuals of *C. crassifolia* (+, $N = 157$), *C. amara* ssp. *pyrenaea* (△, $N = 141$), and their hybrid ***C. ×enriquei*** (□, $N = 58$).

of the floral characters (length of shorter filaments, ratio of length of shorter and longer filaments) were important for separation of the groups along the first axis, while floral characters (length of longer filaments, length of petals) separated the hybrid plants from the putative parents along the second axis.

Spearman (rank) correlation coefficients (used because the character values were not normally distributed) exceeding an arbitrary value of 0.6 are presented in Table 7 (the Pearson coefficients, not shown here, differed only slightly). In a pooled matrix of all plants, vegetative characters prevail among those with higher correlations, which implies that they can be used for taxon identification (Table 7A). On the other hand, the within-group correlations, which characterize variation within corresponding taxa, are generally higher for floral characters (Table 7B).

Further detailed analysis of individual characters revealed that *C. crassifolia* and *C. amara* ssp. *pyrenaea* strongly differed in a number of morphological characters (Table 8). The 25–75 percentile ranges of most vegetative characters for the two taxa did not

Table 7. Spearman correlation coefficients (SCC) exceeding the arbitrary level of 0.6, based on pooled data matrix (A), and on matrices of particular taxa (B)

A

Characters	SCC	Characters	SCC
WIS–LSL	0.63351	LC2–WTS/LTS	0.63915
WIS–NL	0.89329	LC2–WLS/LLS	0.64839
WIS–NLR	0.87159	LC2–LFS	0.60889
WIS–LC2	0.79600	LC2–LFS/LFL	0.72981
WIS–NFS	0.79896	NFS–NL/LSL	0.69836
WIS–LSI/LSL	0.82040	NFS–WTS/LTS	0.62267
WIS–SSL/(NL-SSL)	0.63708	NFS–WLS/LLS	0.60180
WIS–NL/LSL	0.82292	NFS–LFS	0.62527
WIS–WTS/LTS	0.61744	NFS–LFS/LFL	0.67067
WIS–WLS/LLS	0.66941	LSI/LSL–NL	0.88561
WIS–LFS	0.65179	LSI/LSL–NLR	0.84101
WIS–LFS/LFL	0.75691	LSI/LSL–LC2	0.77565
LSL–NL	0.67148	LSI/LSL–NFS	0.77665
LSL–NLR	0.60946	LSI/LSL–SSL/(NL-SSL)	0.63944
LSL–LC2	0.79152	LSI/LSL–NL/LSL	0.73782
LSL–NFS	0.61699	LSI/LSL–WTS/LTS	0.66599
LSL–LSI/LSL	0.75674	LSI/LSL–WLS/LLS	0.69015
NL–NLR	0.89514	LSI/LSL–LFS	0.63323
NL–LC2	0.76970	LSI/LSL–LFS/LFL	0.73004
NL–NFS	0.80021	SSL/(NL–SSL)–NL/LSL	0.66212
NL–SSL/(NL–SSL)	0.68365	SSL/(NL–SSL)–FS/LFL	0.66066
NL–NL/LSL	0.89515	NL/LSL–WTS/LTS	0.62682
NL–WTS/LTS	0.68538	NL/LSL–WLS/LLS	0.71617
NL–WLS/LLS	0.73368	NL/LSL–LFS	0.66480
NL–LFS	0.68469	NL/LSL–LFS/LFL	0.72349
NL–LFS/LFL	0.77673	WTS/LTS–WLS/LLS	0.79785
NLR–LC2	0.80614	WTS/LTS–LFS/LFL	0.63062
NLR–NFS	0.79668	WLS/LLS–LFS	0.61980
NLR–SSL/(NL-SSL)	0.62079	WLS/LLS–LFS/LFL	0.66834
NLR–NL/LSL	0.84542	LS–LP	0.62950
NLR–WTS/LTS	0.67533	LS–LFL	0.60484
NLR–WLS/LLS	0.73690	LP–WP	0.65955
NLR–LFS	0.69859	LP–LFL	0.67534
NLR–LFS/LFL	0.77144	LFL–LFS	0.78238
LC2–NFS	0.76005	LFS–LFS/LFL	0.83349
LC2–SSL/(NL-SSL)	0.62285		

B

Taxon/Characters	SCC
C. crassifolia	
LSL–LC2	0.63815
LSL–NL/LSL	–0.82099
LS–LP	0.78325
LS–WP	0.65333
LS–LFL	0.62527
LS–LFS	0.59527
LP–WP	0.74492
LP–LFL	0.81450
LP–LFS	0.70768
LFL–LFS	0.71532
LFS–LFS/LFL	0.67532
C. ×enriquei	
LS–LP	0.67287
LS–WP	0.65330
LS–LFL	0.64419
LP–WP	0.90056
LP–LFL	0.89230
LP–LFS	0.82501
WP–LFL	0.86850
WP–LFS	0.81549
LFL–LFS	0.84382
LFS–LFS/LFL	0.74170
WTS/LTS–WLS/LLS	0.60169
C. amara ssp. *pyrenaea*	
WIS–NL	0.64455
LFL–LFS	0.72952

Table 8. Results of the exploratory data analysis of *C. crassifolia*, *C. amara* ssp. *pyrenaea* and ***C. ×enriquei***. Values in bold represent mean and standard deviation, values in brackets 5 and 95 percentiles, respectively

Character	*C. crassifolia* (*N* = 157)	***C. ×enriquei*** (*N* = 58)	*C. amara* ssp. *pyrenaea* (*N* = 141)
WIS	(0.50–) **0.75 ± 0.26** (–1.26)	(1.50–) **2.00 ± 0.58** (–3.00)	(2.00–) **3.00 ± 1.10** (–5.00)
LSL	(4.96–) **9.25 ± 4.21** (–18.10)	(10.00–) **18.00 ± 3.69** (–20.23)	(10.00–) **22.25 ± 6.06** (–30.50)
NL	(2.00–) **2.50 ± 0.69** (–4.00)	(6.00–) **10.00 ± 2.73** (–14.15)	(13.00–) **22.00 ± 7.28** (–36.00)
NLR	(0.00–) **0.00 ± 0.38** (–1.00)	(1.00–) **1.00 ± 0.82** (–3.15)	(2.00–) **2.50 ± 1.81** (–7.00)
LC2	(1.10–) **2.15 ± 1.00** (–4.24)	(2.27–) **3.80 ± 1.39** (–6.30)	(3.80–) **5.95 ± 1.82** (–9.50)
NFS	(2.00–) **2.00 ± 0.79** (–4.00)	(3.00–) **3.50 ± 0.38** (–4.00)	(4.00–) **4.50 ± 0.62** (–6.00)
LS	(2.78–) **3.47 ± 0.54** (–4.51)	(3.10–) **3.38 ± 0.32** (–4.02)	(3.47–) **3.99 ± 0.30** (–4.16)
WS	(1.39–) **1.65 ± 0.27** (–2.12)	(1.39–) **1.65 ± 0.31** (–2.13)	(1.74–) **2.26 ± 0.36** (–2.78)
LP	(5.90–) **7.98 ± 1.47** (–10.41)	(5.15–) **6.25 ± 0.85** (–7.63)	(6.59–) **8.15 ± 0.67** (–9.02)
WP	(3.47–) **5.12 ± 0.72** (–5.90)	(2.78–) **4.08 ± 0.73** (–5.21)	(3.47–) **4.68 ± 0.54** (–5.21)
LFL	(3.82–) **4.86 ± 0.75** (–6.25)	(2.60–) **3.56 ± 0.68** (–4.68)	(5.03–) **5.55 ± 0.51** (–6.77)
LFS	(2.08–) **2.95 ± 0.58** (–3.82)	(1.39–) **2.43 ± 0.66** (–3.47)	(4.16–) **5.03 ± 0.56** (–5.90)
LSI/LSL	(0.58–) **0.67 ± 0.09** (–0.86)	(0.84–) **0.96 ± 0.04** (–0.97)	(0.93–) **0.97 ± 0.02** (–1.00)
SSL/(NL-SSL)	(0.00–) **0.75 ± 0.33** (–1.00)	(0.66–) **0.69 ± 0.39** (–1.52)	(0.69–) **1.52 ± 0.52** (–2.29)
NL/LSL	(0.15–) **0.27 ± 0.13** (–0.57)	(0.38–) **0.55 ± 0.21** (–1.05)	(0.65–) **0.99 ± 0.55** (–2.13)
WTS/LTS	(0.16–) **0.37 ± 0.16** (–0.65)	(0.35–) **0.59 ± 0.10** (–0.63)	(0.50–) **0.69 ± 0.11** (–0.83)
WLS/LLS	(0.18–) **0.27 ± 0.11** (–0.50)	(0.25–) **0.49 ± 0.09** (–0.51)	(0.47–) **0.72 ± 0.13** (–0.86)
LFS/LFL	(0.44–) **0.61 ± 0.08** (–0.69)	(0.53–) **0.68 ± 0.10** (–0.84)	(0.73–) **0.91 ± 0.07** (–0.97)

overlap (Fig. 6). Only LSL (see Table 2 for abbreviations) and the ratio WTS/LTS were differentiated less strongly. By contrast, the parental taxa are less differentiated by most flower characters. LFS and the ratio LFS/LFL did not overlap. However, apart from the quantitative characters mentioned above, two qualitative floral characters distinguish putative parents: *C. crassifolia* has pink petals and yellow anthers while *C. amara* ssp. *pyrenaea* has white petals and violet anthers (cf. Lihová *et al*., 2000).

The hybrid plants showed a high degree of intermediacy in vegetative characters (Fig. 6A–C). With respect to floral characters, hybrids are either more similar to *C. crassifolia* (in the length of sepals and length of shorter filaments), to *C. amara* ssp. *pyrenaea* (in the width of petals), or showed intermediate values (width of sepals and ratio of length of shorter and longer filaments, Fig. 6D). Ranges of length and width of petals, and length of longer filaments are shifted in respect of both parents. The values of these characters are generally smaller in the hybrid plants (Fig. 6E, F). Another quantitative character used for identification of *C. amara* and the *C. pratensis* group is the width of the stigma in comparison with the style. In *C. amara* the stigma is of the same width or narrower than the style, while in the *C. pratensis* group the stigma is much wider than the style. The hybrid plants were intermediate with respect to this character. Qualitative floral characters showed intermediate states in the hybrid plants: petals were pale reddish-violet and anthers were of mixed yellow-violet colour. An interesting case is the population of *C. amara* ssp. *pyrenaea,* collected from the neighbourhood of 18 hybrid plants in locality P-MRG3; the anthers of 13 plants were yellow, two were yellow-violet and three were violet. This may be an indication of further introgression.

As already mentioned, apart from two hybrid populations discovered in the field, putative hybrids were also found among the plants collected by Pourret, most probably in Núria, and labelled *C. crassifolia* (herbarium sheet MAF 4721). Two plants on the sheet proved to have completely sterile pollen and fell morphologically within the putative hybrid populations MRG2 and RMH, e.g. in respect of the characters WIS (values for two specimens: 2 mm, 1.5 mm), NL (10, 8), NLR (2, 3), LC2 (4.5 cm, 4.3 cm) and NFS (4, 4) (Fig. 7).

DISCUSSION

In the literature several examples of recent hybridization are supported by morphological markers and some of these examples are already well studied (e.g. *Spartina*: Gray, Marshall & Raybould, 1991; Daehler & Strong, 1997; Ferris, King & Gray, 1997; Ayres *et al*., 1999). For a better understanding of biological strategies and evolutionary processes further case studies are urgently required (Hurka, Bleeker & Neuffer, 2002). Within the Brassicaceae many examples of hybridization, introgression and hybrid speciation are known and this is in part explained by the rather young age of this family in geological terms

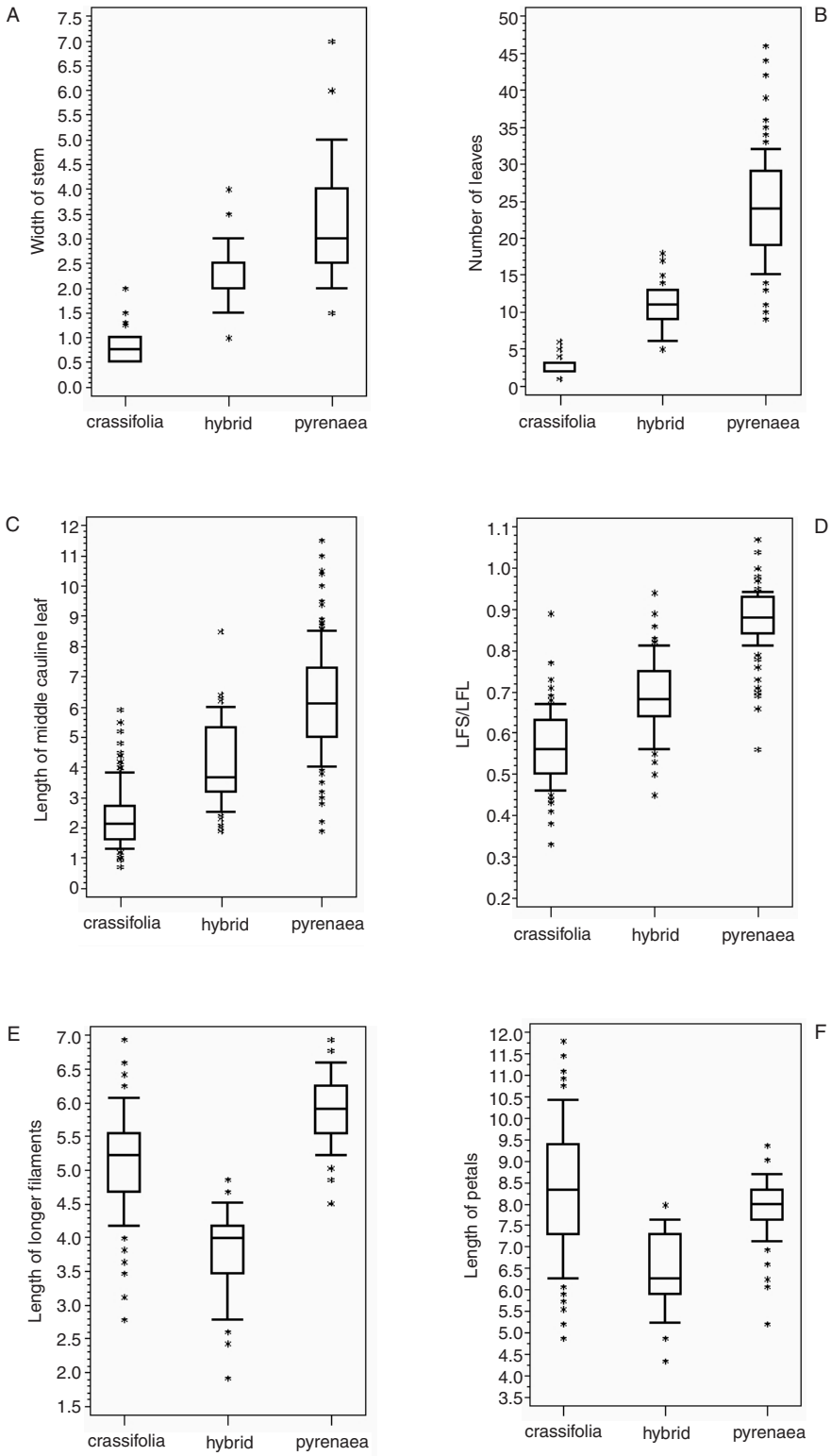

Figure 6. Variation in selected morphological characters of *C. crassifolia* ($N = 157$), *C. amara* ssp. *pyrenaea* ($N = 141$) and their hybrid ***C.* ×*enriquei*** ($N = 58$). Box is defined by 25 and 75 percentiles and whiskers are from 10 to 90 percentile. A, width of stem; B, number of leaves; C, length of the middle cauline leaf; D, ratio length of shorter filaments/length of longer filaments; E, length of longer filaments; F, length of petals.

Figure 7. Pourret's specimen representing mixed sample of *C. crassifolia* and ***C. ×enriquei*** deposited at MAF (MAF 4721); A, the whole sheet; B, detail of the hybrid plant at the upper left corner of the sheet. The plants marked with arrows are hybrid individuals discussed in the text.

(Hurka *et al*., in press). *Cardamine* would appear to be a prime example of ongoing evolution, as evidenced by various ploidy levels within the *C. pratensis* complex or polyploid species like *C. flexuosa* With. of hitherto unknown origin.

The *C. pratensis* group and *C. amara* represent two well separated assemblages within *Cardamine* sect. *Cardamine* with respect to their morphological and molecular characters. Although representatives of both groups are often sympatric, hybrids are extremely rare. The events reported here represent the second documented case of natural hybridization between the groups, the first being *C. ×insueta* Urbanska discovered in Switzerland (Urbanska-Worytkiewicz & Landolt, 1972; see below). In our studies, pollen, AFLP and morphological data strongly support the presumed hybrid status and parentage of the studied populations from two sites in the eastern Pyrenees. Hybrid individuals were also found among Pourret's herbarium material of *C. crassifolia*. We

suspect that other localities of hybrid populations will eventually be found following detailed examination of plants in the field, as the parental taxa have the same ploidy level, possess similar ecological requirements, and often grow close to each other. We describe these hybrids as a new nothospecies in the Appendix.

The diploid hybrid *C.* ×*enriquei* occupied an intermediate position for most of the morphological characters compared with the parental taxa (*C. crassifolia* and *C. amara* ssp. *pyrenaea*). Smaller floral parts of hybrid plants than those of the parental taxa might be associated with the male sterility detected for this hybrid. The occurrence of such 'extreme' characters in hybrids, especially in later generations, has also been reported by Rieseberg & Ellstrand (1993). From morphological and AFLP analyses, it is apparent that the hybrids are closer to *C. crassifolia* than to *C. amara* ssp. *pyrenaea*. The hybrid populations exhibited considerable morphological and molecular variability (e.g. RMH) despite their rather small numbers, suggesting a recurrent origin, segregation and/or backcrossing with parents. Yellow anthers in some individuals of *C. amara* ssp. *pyrenaea* observed in P-MRG3 and considerably decreased pollen fertility of *C. crassifolia* in C-RMC could indicate gene flow from the hybrids to both parents, although nearly full male sterility was found in hybrid individuals. Similarly, Urbanska *et al*. (1997) detected broader variation in pollen quality and the occurrence of almost pollen sterile plants in the population of *C. pratensis* (*C. rivularis* auct. non Schur), the female parent of *C.* ×*insueta*, and attributed this to backcrossing. In that case, the hybrid, although triploid, possessed some viable pollen grains, and formed functional gametes. However, vegetative propagation has made the major contribution to the establishment and persistence of *C.* ×*insueta*, and this likely to be the case of the stoloniferous *C.* ×*enriquei*.

Hybrids between *C. pratensis* and *C. amara* have often been reported both in the literature (Kuntze, 1867; Oudemans & Suringar, 1883; Brügger, 1886; Schulz, 1903; Zapałowicz, 1912) and in herbaria, and even described under various names – *C.* ×*ambigua* O. E. Schulz, *C.* ×*zapalowiczii* Domin (*C. dubia* Zapał., nom. illegit.), *C.* ×*killiasii* Brügger. However, as Schulz (1903) and Lövkvist (1956) pointed out, these refer either to the pink-flowered plants of *C. amara* (also described as *C. amara* var. *erubescens* Peterm.) or polyploid *C. dentata* Schult. from the *C. pratensis* group. The critical evaluation by Schulz (1903) of the specimens previously classified as hybrids resulted in the rejection of hybrid status for all but one specimen collected in the Ukraine, in the vicinity of Kharkov (1819, Tschernajew, LE, four plants in all), which represents the holotype of the name *C.* ×*ambigua*. However, our investigation of pollen quality of two plants from this specimen showed that they have fully fertile pollen grains (94 and 99%). While the taxonomic classification of these plants is not precise (they are poorly preserved and might represent either *C. amara* or *C. tenera* J. G. Gmel. ex C. A. Mey.), we strongly doubt their hybrid status. We have not been able to trace the specimen with allegedly hybrid plants reported by Brügger (1886) from Switzerland (U. Engadin, Uinna-da-dora, leg. E. Killias, 29. vi. 1883) in any of the herbaria where Brügger's specimens are deposited (Lanjouw & Stafleu, 1954). However, according to our present knowledge of variation in *C. amara,* Brügger's description does not provide any character that would indicate hybrid origin of his plants (an opinion expressed by Schulz, 1903; who had not seen the specimen either). Brügger's plants most probably belong either to *C. amara* ssp. *amara* or to ssp. *austriaca* as both these taxa occur in the given region of Switzerland (Marhold, 1999). The name *C.* ×*zapalowiczii* refers to *C. amara* ssp. *opicii*, according to the holotype deposited in KRAM (Marhold, 1995). Specimens collected by Harz in 1909 and 1910 in Schesslitz (Bavaria, Germany), classified by him as hybrids of *C. amara* and *C. pratensis* and sent to several European herbaria (e.g. PRC and Z) also represent fully fertile pink-flowered *C. amara*; the size of the pollen grains indicates that they belong to diploid ssp. *amara*.

The only known and proven spontaneous hybrids between the representatives of the groups were found in 1970 and 1971 at two localities in Switzerland (Urbanska-Worytkiewicz & Landolt, 1972). At both localities triploid hybrids were reported as the result of the cross between *C. amara* and diploid *C. rivularis* Schur. At Oberengadin (Canton Graubünden), the *C. amara* parent was represented by the tetraploid cytotype, at Urnerboden (Canton Uri) by the diploid. The hybrids from Urnerboden were later described as *C.* ×*insueta* by Urbanska-Worytkiewicz (1977). Swiss populations classified by Urbanska-Worytkiewicz & Landolt (1972) as *C. rivularis* were later shown to be significantly different from this species (confined to the Balkan Peninsula; Marhold & Rayner, 1994), and should be classified at present as *C. pratensis s.s*. The tetraploid cytotype of *C. amara* from Oberengadin belongs to the recently described *C. amara* ssp. *austriaca* Marhold (Marhold, 1999), while the diploid one from Urnerboden remains in *C. amara* ssp. *amara*. From the nomenclatural point of view, the name *C.* ×*insueta* should be applied to all hybrids of *C. pratensis* and *C. amara* (including all its subspecies). Hybrids from Oberengadin and Urnerboden could, however, be treated as different nothosubspecies. In the hybrid from Urnerboden, *C. amara* was detected as a male parent, whereas *C. pratensis*

(= *C. rivularis* auct.) provided an unreduced female gamete (Urbanska *et al*., 1997). Since its discovery, extensive biosystematic and molecular investigations have been performed demonstrating its parentage, morphological and molecular variation, reproductive behaviour and ecological preferences (Urbanska-Worytkiewicz, 1980; Neuffer & Jahncke, 1997; Urbanska *et al*., 1997). Unidirectional introgression between the partially fertile *C.* ×*insueta* and *C. pratensis* was observed, and autopolyploidization of the hybrid led to the formation of the fertile hexaploid *C. schulzii* Urbanska (Urbanska-Worytkiewicz, 1977). Both *C.* ×*insueta* and *C. schulzii* have successfully established themselves at Urnerboden and serve as a model case for the study of man-influenced hybrid speciation and evolution (Urbanska *et al*., 1997; Urbanska & Landolt, 1999).

With *C.* ×*enriquei* we detected another model system between *C. amara* and one member of the *C. pratensis* complex. Hybridization on the diploid level needs more detailed research, including advanced cytological and comparative isozyme analysis.

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APPENDIX

TAXONOMY AND NOMENCLATURE OF *C. CRASSIFOLIA* AND *C.* ×*ENRIQUEI*

(For a taxonomic and nomenclatural account of *C. amara* ssp. *pyrenaea* see Lihová *et al*., 2000).

Cardamine crassifolia Pourr., Mém. Acad. Sci. Toulouse 3: 310, 1788

Ind. loc. "Ibid. [Dans les Pyrénées, à Salvanaire]"

Lectotype (designated here): *Cardamine crassifolia* P./foliis pinnatis carnosis, foliolis integris ovatis, floribus umbellatis, *s.a.* [*Pourret s.n.*] (P).

≡ C*ardamine pratensis* ssp. *crassifolia* (Pourr.) P. Fourn., Quatre Fl. France: 413, 1936.

Note: There are three specimens, labelled by Pourret as *C. crassifolia*, deposited in MAF, G and P, which might be considered for lectotypification. The specimen MAF 4721 (Fig. 7), labelled "*Cardamine crassifolia* Pourr./*Cardamine pratensis* parvoflore purpureo . . ./in pratis circa . . . vs. m. da [N]uriá [?] julio floret" represents a mixed sample, as discussed above, with *C. crassifolia* mixed with plants apparently of *C.* ×*enriquei*. As the locality on the label (only partially legible) does not correspond to the protologue and even morphologically typical plants of *C. crassifolia* might be influenced by introgression, we excluded this specimen from further consideration as the lectotype. The specimen in G bearing a single plant is labelled "*Cardamine crassifolia* Pourr./Pyren." in Pourret's hand with a later note indicating that the specimen was sent by Pourret to Étienne Pierre Ventenat. The specimen from P bearing three plants is labelled in Pourret's hand "*Cardamine crassifolia* P./foliis pinnatis carnosis, foliolis integris ovatis, floribus umbellatis" [almost literally corresponding to the description in the protologue]. Another, printed, label reads: "Collection de l'Abbé Pourret extraite de l'Herbier légué par M. le D' Barbier 1847". Both specimens (from G and P) correspond closely to the present concept of *C. crassifolia*, although there is no collection date on either of them. The specimen with the citation of the description from the protologue (a slight difference in formulation seems to indicate that the label was written prior to the publication of the name) was selected as the lectotype.

= *Cardamine nuriae* Sennen, Treb. Institute Catalana Hist. Nat. 3: 70, 1917.

Ind. loc."Hab.-Catalogne. Pyrénées, tourbières et bords des torrents à Nuria entre 2000 et 2300 m."

Lectotype (designated here): Catalogne: Pyrénées à Núria, ruisseaux et tourbières, 2000 à 2200 m, vii. 1914, *Sennen, Sennen – Plantes d'Espagne 1906* (BC-Sennen 805517); isolectotypes: BM, M.

≡ *Cardamine pratensis* ssp. *nuriae* (Sennen) Sennen, Monde Pl., Ser. 3, 30 (63–178): 7, 1929.

= *Cardamine mariae* Sennen, Bol. Soc. Ibér. Ci. Nat. 25: 65, 1926.

Ind. loc. "Hab.-Cerdagne: Les Escaldes, dans les praires, vers 1350 m (F. Jude Marie); Vallée d'Angoustrine, vers 1550 m; Vallée d' Eyne, aux bords du torrent, 1900 à 2150 m".

Lectotype (designated here): Cerdagne: Vallée d'Angoustrine, praires humides vers. 1550 m, 17.vi. 1919, *F. Jude Marie*, *Sennen – Plantes d'Espagne 3656* (BC-Sennen 839298)

≡ *Cardamine pratensis* ssp. *mariae* (Sennen) Sennen, Monde Pl., Ser. 3, 30 (63–178): 7, 1929.

CARDAMINE ×*ENRIQUEI* MARHOLD, LIHOVÁ & PERNÝ **NOTHOSP. NOV.**

Diagnosis: Hybrida e *Cardamini crassifolia* et *C. amara* ssp. *pyrenaea* genita, inter parentes media; differt ab *Cardamini crassifolia* Pourr. petalis dilute rubelloviolaceis (non rubelloviolaceis), antheris violaceis luteo-vittatis (non luteis), caule basi latiore [(1.0–) 1.5–3.0 (–3.7) mm non (0.5–) 0.5–1.3 (–1.5) mm], foliorum numero [(6–) 6–14 (–17) non (2–) 2–4 (–5)]; insuper differt ab *C. amara* ssp. *pyrenaea* petalis dilute rubelloviolaceis (non albis), antheris violaceis luteo-vittatis (non violaceis), caule basi angusto [(1.0–) 1.5–3.0 (–3.7) mm non (1.5–) 2–5 (–6.6) mm], foliis paucioribus [(6–) 6–14 (–17) non (9–) 13–36 (–43)], filamentis brevioribus [(2.2–) 2.6–4.7 (–4.9); (1.2–) 1.4–3.5 (–3.9) mm non (4.7–) 5.0–6.8 (–6.9); (3.8–) 4.2–5.9 (–6.3) mm].

Type: Spain, Ripollès, Planell de les Eugues, close to Refugi Manelic, 1975 m a.s.l., 30.vi.2001, *Lihová s.n.* (holotype: SAV; isotypes: BC, MA, SALA).

Description: Perennial herb (11.8–) 14.3–28.6 (–33.1) cm tall. Rhizome long, prostrate to ascending. Stolons present. Stem ascending, simple or rarely branched above, glabrous (1.0–) 1.5–3.0 (–3.7) mm wide at the base. Leaves not forming a basal rosette, cauline leaves (6–) 6–14 (–17), pinnate to pinnatisect, equally spread on the stem, not congested under the inflorescence, glabrous or with scattered to dense hairs on the margin; middle stem leaves (2.0–) 2.3–6.3 (–7.3) cm long, with (3–) 3–4 (–4) pairs of lateral leaflets or segments, their terminal leaflet or segment (8.6–) 10.0–24.0 (–28.0) mm long (3.0–) 4.0–12.7 (–15.6) mm wide, lateral leaflet or segment (5.6–) 7.0–16.7 (–19.4) mm long (1.3–) 2.0–8.0 (–9.4) mm wide; terminal leaflets or segments elliptic to obovate, shallowly lobed or with several crenae, lateral leaflets or segments obovate, elliptic, entire or seldom with few crenae, both terminal and lateral leaflets in the upper part of stem elliptic to narrowly elliptic. Inflorescence racemose (corymbose at anthesis), simple or compound, with (5–) 7–20 (–23) flowers in the main inflorescence; peduncles glabrous. Sepals ovate-lanceolate with membranous margins (2.8–) 3.1–4.0 (–4.2) mm

long and (1.1–) 1.4–2.1 (–2.6) mm wide. Petals pale reddish-violet, obovate (4.6–) 5.2–7.6 (–7.8) mm long and (2.6–) 2.8–5.2 (–5.2) mm wide with short claw, apex truncate to emarginate, glabrous. Stamens 6, tetradynamous, shorter filaments (1.2–) 1.4–3.5 (–3.9) mm long, longer filaments (2.2–) 2.6–4.7 (–4.9) mm long; anthers reddish- to blackish-violet with yellow strips, with overwhelmingly sterile pollen grains. Stigma approximately as wide as style.

Etymology: The new nothospecies is dedicated to Enrique Rico, author of the account of the genus *Cardamine* in *Flora iberica*, to whom we are indebted for long discussions and help in the field.