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Morphometric and AFLP Re-evaluation of Tetraploid *Cardamine amara* (Brassicaceae) in the Mediterranean

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ABSTRACT. *Cardamine amara*, an Eurasian species comprising several subspecies, was examined through morphometric and amplified fragment length polymorphism (AFLP) analyses to re-evaluate subspecific status of tetraploid *C. amara* subsp. *olotensis* from Catalonia (NE Spain) and to resolve the taxonomic position of Central Italian populations usually placed within *C. amara* and/or *C. raphanifolia* s. l. A presumptive close relative from the Caucasus, diploid *C. wiedemanniana*, was also analyzed for AFLPs. Morphometric results show Catalonian and Italian populations to be similar to each other, but distinct from other *C. amara*. With both principal coordinate analysis and the neighbour-joining tree based on AFLP data, three main groups are delimited: (1) tetraploid Catalonian *C. amara* subsp. *olotensis* and Italian populations, (2) all other diploid and tetraploid subspecies of *C. amara*, and (3) diploid *C. wiedemanniana*. As a result of this study, Italian and Catalonian populations are herein treated as a single species, *C. amporitana*, distinct from *C. amara*. Catalonian populations appear genetically depauperate, perhaps having experienced bottlenecks during Pleistocene glaciation, or originating via long-distance dispersal from the Apennine Peninsula.

Recent taxonomic studies on *Cardamine amara* L. have shown considerable morphological variation throughout its distribution in Europe. Multivariate morphometric methods have provided new taxonomic insights, which have led to recognition of several subspecies both at the diploid and tetraploid levels (Marhold 1992, 1998, 1999; Marhold et al. 1996; Lihová et al. 2000). Whereas the typical diploid subspecies ($2n = 2x = 16$), *C. amara* subsp. *amara*, occurs in a major part of the distribution of the species (Jalas and Suominen 1994), other subspecies are restricted to different European mountain ranges. Other diploids are represented by *C. amara* subsp. *opicii* (J. Presl et C. Presl) Čelak. occurring in the Carpathians and Sudetes Mts. (Marhold 1992), subsp. *balcanica* Marhold, Ančev et Kit Tan in the mountains of Bulgaria and Greece (Marhold et al. 1996), and subsp. *pyrenaea* Sennen in the Eastern Pyrenees (Lihová et al. 2000). Tetraploids ($2n = 4x = 32$) are represented by *C. amara* subsp. *austriaca* Marhold from the Alps, most probably a late interglacial or postglacial autopolyploid derivative of *C. amara* subsp. *amara* (Marhold 1999; Lihová et al. 2000; Marhold et al. 2002a), and by *C. amara* subsp. *olotensis* O. Bolòs (Lihová et al. 2000). All these subspecies, currently recognized in *C. amara*, differ mostly in quantitative morphological characters such as number of leaves, number of leaflets, degree of congestion of leaves under the inflorescence, and width of stem base, and can be adequately distinguished using combinations of these features (Marhold 1992, 1998, 1999; Marhold et al. 1996; Lihová et al. 2000). This taxonomic treatment has also been recently supported by molecular analyses (RAPDs and isozymes). Support for *C. amara* subsp. *austriaca*, however, was low, perhaps due to its relatively recent autotetraploid origin and insufficient time for molecular differentiation (Lihová et al. 2000; Marhold et al. 2002a).

Cardamine amara subsp. *olotensis* has been regarded as endemic to the lowlands of Catalonia (NE Spain; Bolòs and Vigo 1990; Jalas and Suominen 1994). This taxon was originally described as *C. amporitana* Sennen et Pau (Sennen 1911), but later it was recognized as a subspecies within *C. amara* (Bolòs 1952). Adoption of the subspecific rank has been widely followed in recent floras (e.g., Bolòs and Vigo 1990; Rico 1993). Molecular analyses (RAPD, AFLP, isozymes) with a limited number of *C. amara* populations (Lihová et al. 2000; Marhold et al. 2002a, b) indicated an isolated position of the Catalonian tetraploid populations from core *C. amara*.

In Central Italy, populations of *Cardamine* of uncertain taxonomic position have been reported. Recent Italian floras mention both *C. amara* and *C. raphanifolia*, but admit to some confusion regarding their delimitation and proper affinities (Zángheri 1976; Pignatti 1982). In *Flora Europaea* (Jones and Akeroyd 1993), Central Italian populations are considered intermediate between Balkan *C. raphanifolia* subsp. *acris* (Griseb.) O. E. Schulz and *C. raphanifolia* subsp. *barbaraeoides* (Halácsy) Strid, whereas in the *Atlas Florae Europaeae* (Jalas and Suominen 1994), both *C. raphanifolia* subsp. *acris* and *C. amara* are cited for the central part of the Apennine Peninsula. Records in 19th century floras report only *C. amara* in that area (Bertoloni 1847; Arcangeli 1882; Parlatore 1890), and except for typical *C. amara*, only *C. amara* var. *grandifolia* Bertol. was recognized in Cen-

tral Italy (Bertoloni 1847). Our initial field observations revealed high overall morphological similarity between the taxonomically problematic Central Italian populations and the Catalonian taxon, *C. amara* subsp. *olotensis*. No populations resembling *C. raphanifolia* s. l. have been found in Central Italy.

Another taxon similar to *C. amara* has been reported from the easternmost part of Europe and from Asia, namely *C. wiedemanniana* Boiss. (= *C. lazica* Boiss. et Balansa ex Buser) occurring in western Transcaucasia and northern Turkey (Khatri 1988).

Throughout the genus *Cardamine* L., polyploidization has been frequent (e.g., the *C. pratensis* group, *C. raphanifolia* and related taxa, *C. flexuosa*; Lövkvist 1956; Jones and Akeroyd 1993), with both auto- and allopolyploidy having been documented or suggested for some taxa (Urbanska et al. 1997; Marhold 1999; Marhold et al. 2002a). A very recent polyploidization event has also been reported (Urbanska et al. 1997). In many cases, however, the origin of polyploid taxa of *Cardamine* is unknown. Due to this karyological complexity, knowledge of chromosome numbers of taxa and populations is crucial for resolving the taxonomy and evolutionary history of *Cardamine*, and this is particularly the case with *C. amara* and close relatives.

Morphometric analyses have already been successfully applied in polyploid complexes in which reliable qualitative characters are often lacking, but in which quantitative characters are especially important for distinguishing particular taxa (e.g., Brysting and Elven 2000). This approach has already helped delimit taxa and clarify relationships in several studies of *Cardamine* taxa (Marhold 1992, 1996, 1998).

Amplified fragment length polymorphisms (AFLPs), a recently developed high-resolution molecular fingerprinting technique, screens nuclear DNA regions distributed throughout the genome (Vos et al. 1995). This method has proven its potential in several systematic studies, especially when investigating relationships among closely related taxa (e.g., Kardolus et al. 1998; Hedrén et al. 2001; Koopman et al. 2001; Zhang et al. 2001; Marhold et al. 2002b).

In this study we apply a combination of morphometric, cytological, and molecular (AFLPs) approaches to explore relationships among subspecies of *C. amara* and related taxa with focus on (1) resolving the taxonomic placement of Central Italian populations of *C. amara* and/or *C. raphanifolia* (herewith called ''Italian'' populations), (2) evaluating their affinities to populations of the Catalonian *C. amara* subsp. *olotensis* (called ''Catalonian'' populations), and (3) providing a revised taxonomic treatment of *C. amara* subsp. *olotensis*.

Materials and Methods

Chromosome Numbers. Two to five plants from each population included in this study were examined for chromosome numbers (Table 1). These were determined from mitotic metaphases of root tips taken from plants collected in the field and cultivated at the Institute of Botany, Slovak Academy of Sciences, Bratislava, Slovakia. Root tips were treated as described in Marhold et al. (2002b).

Morphometrics—Plant Material. For morphometric analyses, population samples of six Catalonian and nine Italian populations were collected in the field, spanning the whole distribution area known in both regions (Table 1; Fig. 1). Each sample consisted of 20–40 individuals; thus a total of 176 Catalonian and 316 Italian specimens were studied. Data on *C. amara* subsp. *amara* (10 populations/380 individuals), and subsp. *austriaca* (10/368) also used in the analyses were taken from previous studies by Marhold (1992, 1998), and these originated from populations sampled across major parts of the distributions of the subspecies. All plants used for morphometric analyses were drawn randomly from their respective parent populations.

Morphometrics—Analyses. The following morphological characters were measured or scored for each plant, collected at the optimum flowering time, and dried: width of stem at the base (mm), number of leaves, maximum number of leaflets of leaves in the upper 4/5 of stem, degree of congestion of leaves under the inflorescence (expressed by the number of leaves reaching the base of the uppermost stem leaf), number of flowers (including buds) in the main inflorescence, and branching of stem (branched/unbranched). Floral organs of one randomly chosen flower per plant were taken when fresh, attached to transparent adhesive tape, and the following characters were measured after drying: length of sepals (mm), length of petals (mm), width of petals (mm), and maximum length of longer filaments (mm). The characters examined were those used in our previous studies of *C. amara*, which had been determined to be taxonomically significant. Characters known to be largely influenced by environmental conditions, such as size and shape of leaves and height of plants (Lövkvist 1957), were not among the characters evaluated. Fruits, typically providing valuable taxonomic characters in Brassicaceae, are not informative for differentiation in *C. amara* and closely related taxa (Marhold 1994b, 1995; Lihová et al. 2000), and were not considered in the analyses. The only fruit character showing some variation is the width of the stigma in comparison with the width of style (Lihová et al. 2000), but due to minute differences and difficulties in scoring such variation, we did not include this character in the morphometric analyses.

Multivariate morphometric analyses were performed on two data sets (matrices) to ascertain the relationship of Italian populations to *C. amara* subsp. *olotensis* and other subspecies morphologically close to this taxon: Matrix (A), a set including *C. amara* subsp. *amara*, subsp. *austriaca*, Catalonian (subsp. *olotensis*) and Italian accessions (i.e., 1237 individuals × 10 characters); matrix (B), a set including Catalonian and Italian accessions only (i.e., 15 populations × 10 characters, and 492 individuals × 10 characters). As shown by Lihová et al. (2000) and Marhold (1998), other subspecies of *C. amara*, namely subsp. *pyrenaea*, subsp. *opicii*, and subsp. *balcanica*, are morphologically well differentiated from *C. amara* subsp. *amara*, subsp. *austriaca*, and subsp. *olotensis*, and therefore, they were not included in these morphometric analyses.

Matrix (A): Canonical discriminant analysis (CDA; Klecka 1980) was performed to reveal the differentiation among *C. amara* subsp. *amara*, subsp. *austriaca*, and Catalonian and Italian accessions. Italian and Catalonian populations were treated as two groups. In this analysis, individuals were used as OTUs, and 95% isodensity circles (Podani 2000, 2001) were calculated. Total canonical structure showing correlations of morphological characters with particular canonical axes was computed.

Matrix (B): Both principal component analysis (PCA; Sneath and Sokal 1973; Krzanowski 1990), based on a correlation matrix and individuals as OTUs, and UPGMA clustering (Everitt 1986) of populations characterized by mean values of characters were performed. For evaluation of meaningful principal components, the broken stick model was used (Legendre and Legendre 1998). Following this approach, it would be meaningless to interpret the principal components that explain a fraction of the variance as

TABLE 1. Localities and chromosome numbers of studied populations of *Cardamine amara* and close relatives. The population code/AFLP sample size is followed by the locality and voucher information. Catalonian and Italian populations employed in the morphometric analyses are marked by asterisks. All voucher specimens are deposited in SAV. Collectors: *JL* = J. Lihová, *KM* = K. Marhold, *MP* = M. Perný. Chromosome numbers (2*n*) in bold represent new populational records; those with superscripts refer to numbers published in [1]Marhold 1994a, [2]Marhold et al. 1996, [3]Lihová et al. 2000, [4]Marhold et al. 2002b, and [5]Lihová and Marhold 2003.

Cardamine amara L. subsp. *amara*

A-bda/3—Slovakia, Vtáčnik Mts., Gepniarova dolina valley, 500 m, *MP,* Jul 2001 2*n* = **16**
A-zs/3—Slovakia, Bratislava, Železná studnička, 200 m, *JL,* 16 Sept 2001 2*n* = 16[1]
A-roz/3—Czech Republic, Pardubice, Rozhrna fishpond, 220 m, *KM,* 8 Aug 2000 2*n* = **16**

Cardamine amara subsp. *austriaca* Marhold

AU-kru/2—Slovenia, Predalpsko območje, Krumperk, 320 m, *JL* and *T. Bačič,* 18 Apr 2001 2*n* = 32[5]
AU-lit/3—Slovenia, Predalpsko območje, Zgornij Log, Sáva river, 240 m, *JL* and *B. Frajman,* 20 Apr 2001 2*n* = 32[5]
AU-sj/3—Slovenia, Predalpsko območje, the Šmartinsko jezero lake, 260 m, *JL* and *B. Frajman,* 20 Apr 2001 2*n* = 32[5]

Cardamine amara subsp. *balcanica* Marhold, Ančev et Kit Tan

B-an/3—Bulgaria, Vitoša Mts., Aleko, 1800 m, *M. Ančev,* Jul 2001 2*n* = 16[2]
B-vn/1—Bulgaria, Stara Planina, Tetevenska planina, Vežen, 1850 m, *MP* and *E. Georgieva,* 21 Jun 2000
B-gr/1—Bulgaria, Zapadni Rodopi Mts., Pamporovo, Goljamata Reka, 1490 m, *MP,* 25 Jun 2000 2*n* = **16**
B-vh/1—Bulgaria, Vitoša Mts., north-eastern slopes, ca. 1600 m, *MP* et al., 24 Jun 2000 2*n* = **16**

Cardamine amara subsp. *opicii* (J. Presl et C. Presl) Čelak.

OP-msd/2—Slovakia, Vysoké Tatry Mts., Malá Studená dolina valley, 1595 m, *JL,* 3 Sep 2001 2*n* = 16[3]
OP-zp/3—Slovakia, Vysoké Tatry Mts., Zelený potok valley, 1440 m, *JL,* 2 Sep 2001 2*n* = ***16***
OP-ids/2—Slovakia, Slovenské rudohorie Mts., Idčianske sedlo, 900 m, *JL,* 9 Jun 2001 2*n* = **16**

Cardamine amara subsp. *pyrenaea* Sennen

P-rmp/2—Spain, prov. Gerona, E Pyrenees, Planell de les Eugues, 1975 m, *KM,* 7 Jul 1997; *JL,* 30 Jun 2001 2*n* = 16[3]
P-nu2/2—Spain, prov. Gerona, E Pyrenees, Núria, Coma de Noufonts, 2600 m, *JL,* 27 Jun 2001 2*n* = 16[3]
P-nu6/1—Spain, prov. Gerona, E Pyrenees, Núria, Torrent de Finestreles, 2000–2100 m, *JL,* 28 Jun 2001 2*n* = 16[3]
P-mrg/2—Spain, prov. Lérida, E Pyrenees, Meranges, 2080 m, *JL,* 1 Jul 2001 2*n* = 16[4]

Catalonian populations (Spain, prov. Gerona; traditionally classified as *Cardamine amara* subsp. *olotensis* O. Bolòs)

OL-aru/2—Arbúcies, Molí de les Pipes, 340 m, *JL* and *J. Font García,* 4 May 2001 2*n* = **32**
OL-ol/2—*Olot, Parc Nou, 400 m, *KM* et al., 10 May 2001 2*n* = 323
OL-cll/2—*Cantallops, al mas Bell-lloc Petit, 140 m, *JL* and *J. Font García,* 2 May 2001 2*n* = **32**
OL-va/2—*La Vajol, als prats de Perdigó, 540 m, *JL* and *J. Font García,* 2 May 2001 2*n* = **32**
OL-gi/1—*Girona, Cami del Torin, 60 m, *JL,* 3 May 2001 2*n* = **32**
OL-stc/2—Santa Coloma de Farners, Santa Coloma river, 150 m, *JL* and *L. Villars,* 3 May 2001 2*n* = **32**
—*Olot, Paratges de la Deu, 400 m, *KM* et al., 10 Jun 1996 2*n* = 32[3]
—*Parc natural del Montseny, Santa Fe del Montseny, 1130 m, *KM* and *X. Giráldez,* 11 May 1996 2*n* = 32[3]

Italian populations

It-rip/2—Abruzzo, Gran Sasso, Rigopiano, 1090 m, *JL* et al., 27 Apr 2001 2*n* = **32**
It-of/2—Abruzzo, Gran Sasso, Brittoli, 905 m, *JL* et al., 27 Apr 2001 2*n* = **32**
It-pt/2—*Abruzzo, Gran Sasso, Valle del Rio Arno, Pietracamela, 1400 m, *JL* et al., 27 May 2000 2*n* = **32**
It-t/1—*Abruzzo, Parco Nazionale d'Abruzzo, Templo, 1200 m, *JL* et al., 29 May 2000 2*n* = **32**
It-x/2—*Abruzzo, Parco Nazionale d'Abruzzo, Passo Godi, 1530 m, *JL* et al., 29 May 2000 2*n* = **32**
It-cz/2—*Abruzzo, Parco Nazionale d'Abruzzo, Fosso della Padura, 1250 m, *JL* et al., 29 May 2000 2*n* = **32**
It-pg/2—*Umbria, Monti Sibillini, Pian Grande, 1250 m, *JL* et al., 26 May 2000 2*n* = **32**
It-r/1—*Marche, Monti Sibillini, Rapegna, 820 m, *JL* et al., 26 May 2000 2*n* = **32**
It-ci/2—*Marche, Monti Sibillini, Ussita-Casali, 1100 m, *JL* et al., 28 May 2000 2*n* = **32**
—*Abruzzo, Gran Sasso, Sorgenti del Vomano, Passo delle Capannelle, 1257 m, *JL* et al., 27 May 2000 2*n* = **32**
—*Abruzzo, Lago di Campotosto, Le Serre, 1340 m, *JL* et al., 27 May 2000 2*n* = **32**

C. wiedemanniana Boiss.

L-gal/3—Russia, W Great Caucasus, Mzymta valley, Galitsino, 200 m, *MP* and *B. S. Tuniev,* 21 Jun 2001 2*n* = **16**
L-mo/3—Russia, W Great Caucasus, Mzymta valley, Monastyr, 450 m, *MP* and *B. S. Tuniev,* 21 Jun 2001 2*n* = **16**
L-bel/3—Russia, W Great Caucasus, Mzymta valley, Krasnaya Polyana, the Beshenka stream, 680–720 m, *MP,* 21 Jun 2001 2*n* = **16**
L-gzl/3—Russia, W Great Caucasus, Kavkazskii zapovednik, Guzeripl', 700 m, *MP,* 29 Jun 2001 2*n* = **16**

small as or smaller than predicted by the broken stick null model. For UPGMA, characters were standardized to zero means and unit standard deviation, and distances were calculated using the Euclidean coefficient. Subsequently, CDA of Catalonian and Italian accessions as groups and individuals as OTUs was performed. This aimed to reveal the extent of morphological differentiation between Catalonian and Italian individuals, and the characters that contribute to differentiation. Whereas PCA represents a hypothesis-generating method, in which characters are equally weighted, and the ordination diagram shows overall variation among the individuals reduced into two or three dimensions, CDA differentially weights all characters in order to achieve the

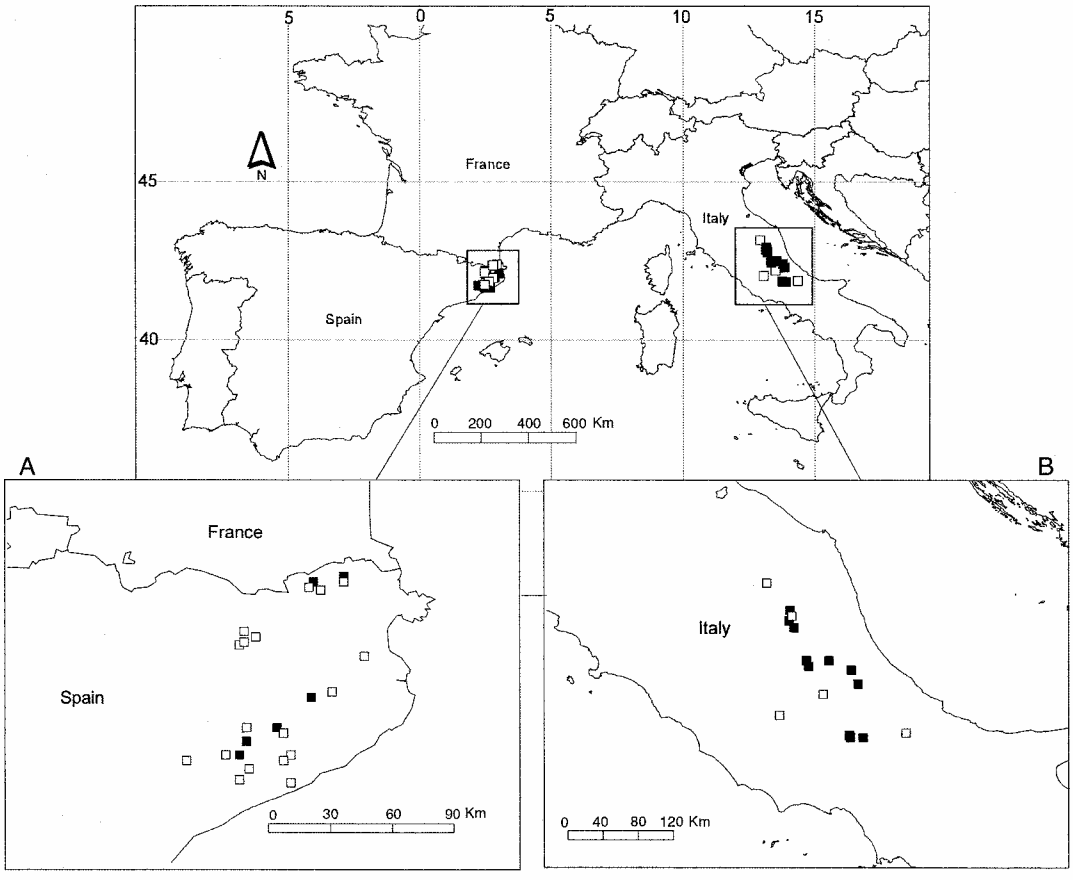

FIG. 1. Map showing distribution of *C. amporitana* in Catalonia (A) and Central Italy (B) based on authors' samples (solid squares, see Table 1), and herbarium material of other collectors (empty squares; see Lihová et al. 2000 and herbarium CAME)

best separation between predetermined groups. Standard descriptive statistics (mean, standard deviation and percentiles) of the examined characters were computed as well.

Standard descriptive statistics, PCA, and CDA (for matrix B) were performed using the SAS 8.2 package (SAS Institute 2000). Cluster analysis and CDA (for matrix A) were run using SYNTAX 2000 (Podani 2001).

AFLPs—Plant Material. Taxa investigated in the AFLP study included representatives of all subspecies of *C. amara*, Italian populations, and *C. wiedemanniana* (Table 1). One to three plants per population, 76 plants altogether, were analyzed from locations throughout the distributional area to represent a broad range of genetic variation. This sampling strategy of including as many populations as possible (to certain extent at the cost of number of individuals per population) was designed to address taxonomic, rather than population genetic questions.

AFLPs—Analyses. Genomic DNA was extracted from silica gel-dried leaves following the CTAB protocol (Doyle and Doyle 1987) with minor modifications and quantified photometrically (UV 160A Spectrophotometer, Shimadzu). The AFLP procedure followed the general protocol provided by Applied Biosystems (PE Applied Biosystems 1996) with a few modifications. Genomic DNA (ca. 0.5 µg per sample) was digested with two restriction endonucleases, *Eco*RI and *Mse*I (Promega, Madison, U.S.A.), and ligated to double-stranded *Eco*RI (5′-AATTGGTACGCAGTCTAC-3′, PE Applied Biosystems, Vienna) and *Mse*I adaptors (5′-TACTCAGGACTCAT-3′, PE Applied Biosystems, Vienna) in one step at 37°C for 2 hrs. Ligated DNA fragments were diluted 10-fold with $TE_{0.1}$ buffer. Preselective amplification using preselective primers (*Eco*RI: 5′-GACTGCGTACCAATTC-3′, *Mse*I: 5′-ATGAGTCCTGAGTA-3′, PE Applied Biosystems, Vienna) with 1-bp extensions at the 3′ end was performed in a thermal cycler (GeneAmp® PCR System 9700, PE Applied Biosystems). The PCR conditions were: 72°C for 2 min, 20 cycles of denaturation at 94°C for 1 sec, annealing at 56°C for 30 sec and extension at 72°C for 2 min, and finished by 30 min at 60°C and cooling to 4°C. After diluting 1:10 in $TE_{0.1}$ buffer, selective amplification was performed in 5 µl volume using the following three primer combinations with 3-bp extensions at the 3′ end: *Eco*RI -AAG-(HEX), *Mse*I -CTG; *Eco*RI -ATC-(6-FAM), *Mse*I -CAG; *Eco*RI -AGC-(NED), *Mse*I -CTG (VBC Genomics, Vienna & PE Applied Biosystems, Vienna). These were chosen from an initial primer test of 15 different primer combinations. The selective amplification was carried out in the following conditions: initial denaturation at 94°C for 2 min, followed by 32 cycles at 94°C for 1 sec, 30 sec annealing (initiated at 65°C, reduced by 1°C for the next 8 cycles and maintained at 56°C for the last 23 cycles) and 72°C for 2 min, finished by 2 min at 72°C and cooling to 4°C. The resultant AFLP products were combined and loaded on 4.5% polyacrylamide gels (with an internal size standard GeneScan®-500 ROX, PE Applied Biosystems). Electrophoresis was performed in an ABI Prism 377 sequencer.

Data Analysis. Fragment data generated by the sequencer were analyzed using GeneScan® software (PE Applied Biosystems). Presence or absence of fragments were scored for each sample in a range of 70–500 bp (ambiguous, faint fragments were excluded) and transferred into a binary matrix using GenoGrapher (version 1.6.0, Montana State University 1999; http://hordeum.msu.montana.edu/genographer/). Pairwise similarities were calculated using Jaccard's coefficient, and principal coordinate analysis (PCoA; Krzanowski 1990) was performed to recover

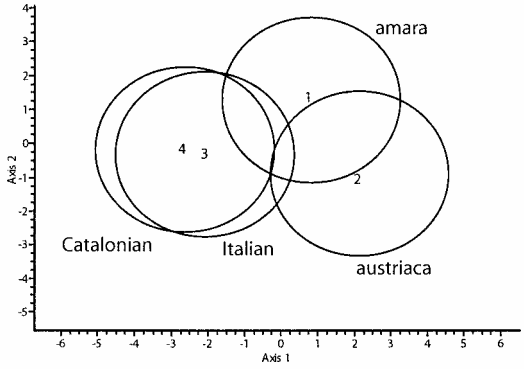

FIG. 2. Ordination graph of the canonical discriminant analysis of the individuals of *C. amara* subsp. *amara* (1), subsp. *austriaca* (2), Italian (3), and Catalonian (4) populations based on morphological characters. 95% isodensity circles calculated for the two canonical axes are drawn around the centroids. For total canonical structure see Table 2, columns CDA1. The first two axes explain 78.62% and 16.64% of variation among the groups.

nonhierarchical structure of AFLP data (SYN-TAX 2000; Podani 2001). Similarly as in PCA, a number of meaningful coordinates were evaluated using the broken stick model. Also, a neighbour-joining tree based on the Nei and Li (1979) genetic distance was constructed, and group support was assessed by repeated bootstrap analyses with 2000 replications (the TREECON program, version 1.3b; Van de Peer and De Wachter 1994).

Voucher specimens for all the analyses performed in this study are deposited in SAV.

RESULTS

Chromosome Numbers. Tetraploid chromosome numbers were determined for all Catalonian and Italian populations studied, being $2n = 32$. Data for Catalonian populations reported here confirm and extend previous records (Lihová et al. 2000). For Italian populations, the tetraploid level was already indicated by Landolt and Urbanska (unpubl.; localities Lago di Campotosto and Pietracamela), and we found it in 11 additional localities. Diploid chromosome numbers ($2n = 2x = 16$) determined for *C. wiedemanniana* in four localities represent the first published records for this species (Table 1).

Morphometric Analyses. Canonical discriminant analysis of *C. amara* subsp. *amara*, subsp. *austriaca*, Catalonian, and Italian accessions (matrix A) showed the latter two separated along the first canonical axis (Fig. 2). The characters differentiating them from subsp. *amara* and subsp. *austriaca* (number of leaflets and length of filaments) were those having the highest values of the total canonical structure coefficients (Table 2, column CDA1). In addition to these differences, there was a qualitative character, color of anthers, which was not included in the morphometric analyses, but clearly distinguished both Catalonian and Italian samples (yellow) from the rest of *C. amara* (violet). On the other hand, it was not possible to differentiate sufficiently between Catalonian and Italian populations, as their 95% isodensity circles almost coincided (Fig. 2).

PCA based on Catalonian and Italian individuals (matrix B) showed one group comprising plants from both Catalonian and Italian populations intermingled (Fig. 3). Only a minute shift along the first axis is seen. Neither the second nor the third axes showed further shifts or groupings. Indeed, as indicated by broken stick values, only the first axis is meaningful. Characters correlated most positively with the first axis were width of stem base and number of flowers, as seen from eigenvector values (Table 2, column PCA/PC1).

Similarly, UPGMA cluster analysis of the Catalonian and Italian populations resulted in a phenogram in which three Catalonian populations formed a separate cluster, and the other three populations grouped together with Italian populations (phenogram not shown).

Figure 4 presents the results of CDA based on in-

TABLE 2. CDA1: Canonical discriminant analysis of the individuals of *C. amara* subsp. *amara*, subsp. *austriaca*, and Catalonia and Italian populations (see Fig. 2). Total canonical structure expressing correlation of the characters with the canonical axes (CAN1, CAN2). PCA: Principal component analysis of the individuals of Catalonian and Italian populations (see Fig. 3). Eigenvectors showing correlation of the characters with the components (PC1, PC2). CDA2: Canonical discriminant analysis of the individuals of Catalonian and Italian populations (see Fig. 4). Total canonical structure (CAN1).

	CDA1		PCA		CDA2
Morphological character	CAN1	CAN2	PC1	PC2	CAN1
Width of stem base	−0.102	−0.122	0.447	−0.085	0.634
Number of leaves	−0.352	−0.426	0.213	−0.190	0.207
Max. number of leaflets of stem leaves	0.780	−0.197	0.334	−0.239	0.160
Congestion of leaves under inflorescence	−0.026	−0.079	0.275	−0.303	0.755
Number of flowers	−0.088	−0.121	0.430	−0.156	0.474
Branching of stem	−0.130	0.505	0.395	−0.164	0.430
Length of sepals	−0.028	−0.780	0.307	0.266	0.406
Length of petals	−0.314	−0.364	0.263	0.595	0.139
Width of petals	−0.409	0.032	0.231	0.405	0.373
Max. length of filaments	0.601	−0.265	0.102	0.410	−0.223

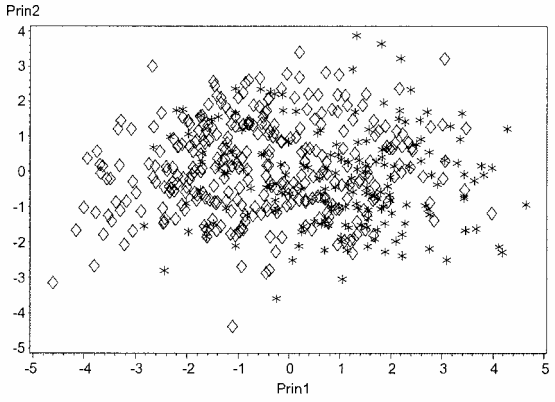

FIG. 3. Ordination graph of the principal component analysis of the individuals of Catalonian (asterisks) and Italian (diamonds) populations based on morphological characters. First two components explain 33.0% and 17.8% of variation. Corresponding broken stick values are 29.3% and 19.3%. For eigenvectors see Table 2, columns PCA.

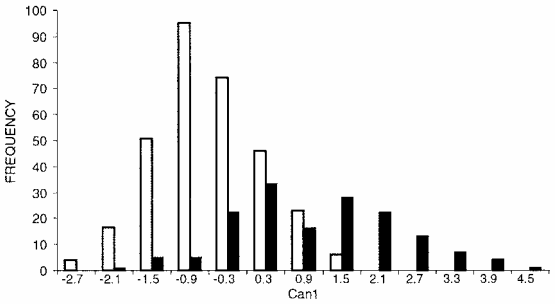

FIG. 4. Histogram of the canonical discriminant analysis of the individuals of Catalonian (black) and Italian (white) populations, based on morphological characters. Horizontal axis (Can1), discriminant function; vertical axis, frequency of plants. For total canonical structure see Table 2, column CDA2.

dividual plants as OTUs and Catalonian and Italian populations as two groups. Even in this analysis where characters are given different weights in order to achieve maximum separation, a rather large overlap was observed in the histogram. The characters congestion of leaves and width of stem base contributed most to the slight shift between the groups along the canonical axis (Table 2, column CDA2/CAN1).

To summarize, detailed analysis of individual characters revealed that there are no clear differences between Catalonian and Italian accessions, the 25–75 percentile ranges of all examined quantitative characters overlapping considerably (Fig. 5). There are only minute shifts in the characters number of flowers (Fig. 5E), congestion of leaves (Fig. 5H), and width of stem base (Fig. 5I), consistent with PCA and CDA results, in which these characters showed the highest correlations with the first axes. Catalonian populations tend to have a wider stem base; values as high as 9 mm in these populations have not been recorded among Italian individuals (Fig. 5I). Similarly, there is a tendency for greater congestion of leaves under the inflorescence in Catalonian populations in contrast to the Italian ones (Fig. 5H). Lastly, Catalonian populations have more flowers on average, but this character shows wide variation (Fig. 5E).

AFLP Analyses. In 76 *Cardamine* samples investigated here, a total of 184 scorable fragments were amplified with three primer combinations; 172 (93.48%) of them were polymorphic. 163 (88.59%) fragments were shared by two or more samples, and thus were potentially informative. Patterns of fragment distribution across studied taxa are presented in Table 3.

PCoA based on Jaccard's coefficient illustrates relationships of individual AFLP phenotypes (Fig. 6). Three clearly separated groups can be observed in the ordination graph: one dense group to the left along the first axis composed of populations of *C. wiedemanniana*, the second, somewhat looser group including both Catalonian and Italian samples to the right, and the third one including *C. amara* subsp. *amara*, subsp. *austriaca*, subsp. *opicii*, subsp. *pyrenaea* and subsp. *balcanica*. PCoA performed only on Italian and Catalonian populations showed much less genetic variation in the latter (Fig. 7). Broken stick values indicate that all three axes shown in both ordination diagrams are meaningful and worth interpreting.

Neighbour-joining based on genetic distance of Nei and Li (1979) produced a tree (Fig. 8) in which clustering corresponded to the three main groups of PCoA (Fig. 6). The tree was rooted with samples of *C. wiedemanniana*, as this taxon occupied the most isolated position in PCoA. Individuals of Catalonian and Italian samples formed one well-supported cluster (100% bootstrap) separated from *C. amara*. Within this cluster, several subclusters were resolved, mostly corresponding to populations from particular mountain ranges. One of these subclusters (99% bootstrap) was formed by genetically uniform Catalonian samples. Three diploid subspecies of *C. amara* each received high bootstrap support: subsp. *balcanica* (98%), subsp. *pyrenaea* (99%) and subsp. *opicii* (78%), and the latter two clustered together with moderate support (62%). *Cardamine amara* subsp. *amara* and subsp. *austriaca* formed an unresolved group, with the exception of some samples from the same populations forming well-supported small groups.

DISCUSSION

***Pattern of Diversity within* Cardamine amara *and Position of* C. wiedemanniana.** The AFLP study presented here is in concordance with a previous, more restricted RAPD analysis, in which only 29 individuals were included (Lihová et al. 2000). Both showed *C. amara*, excluding subsp. *olotensis*, as one cohesive but genetically variable group. This variability is also evident in our findings: as many as 72 unique AFLP fragments restricted to this species were found, but none

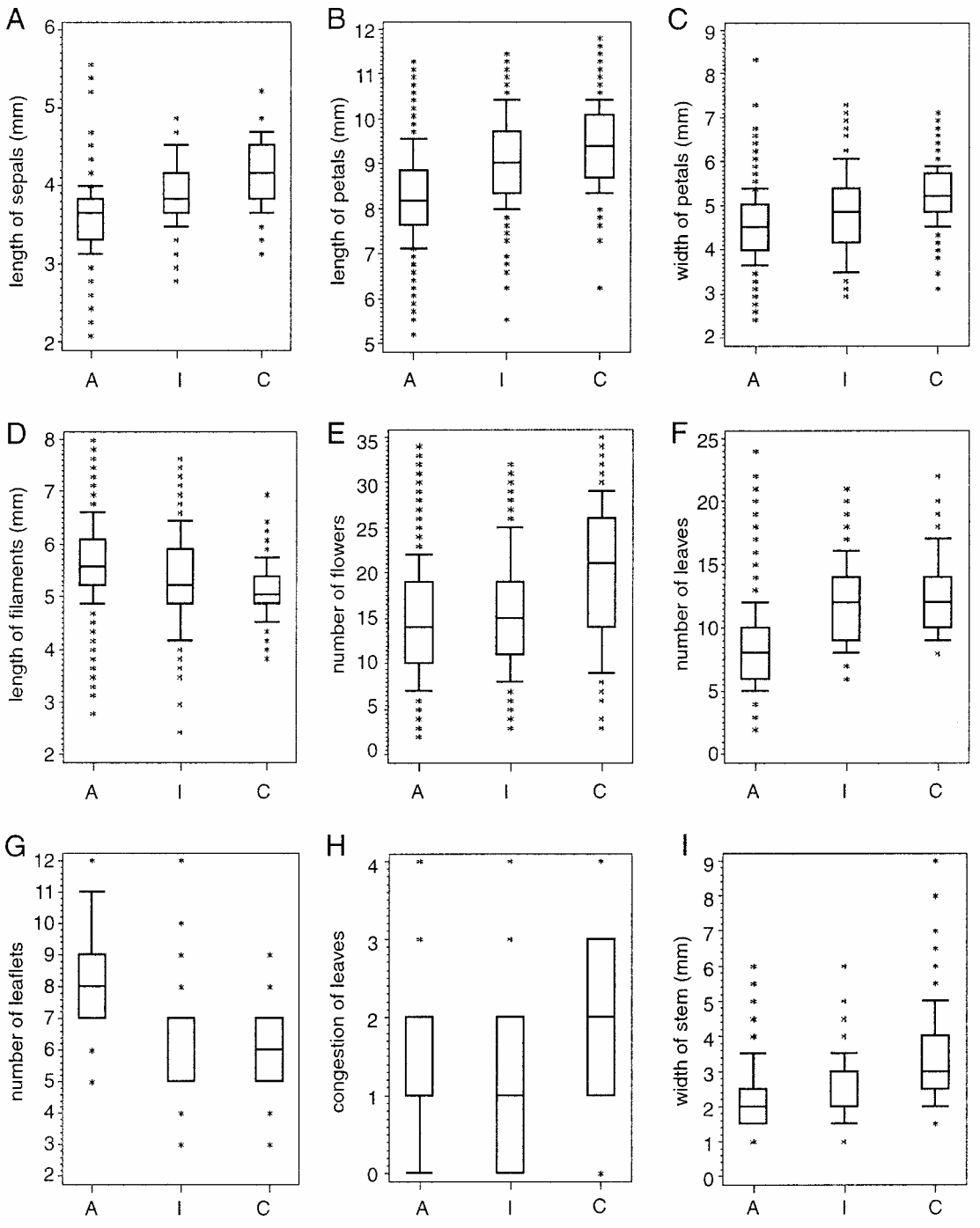

FIG. 5. Comparison of variation in morphological characters of Catalonian (C; 176 plants), Italian (I; 316 plants), and *C. amara* subsp. *amara* (A; 1457 plants) populations. Rectangles define 25 and 75 percentiles; horizontal lines show median; whiskers are from 10 to 90 percentiles; asterisks extreme values. A, length of sepals; B, length of petals; C, width of petals; D, length of longer filaments; E, number of flowers in the main inflorescence; F, number of leaves; G, maximum number of leaflets of leaves in the upper 4/5 of stem (median coincides with 25 percentile for Italian populations); H, degree of congestion of leaves under the inflorescence (median coincides with 25 percentile for subsp. *amara*); I, width of stem base (median coincides with 25 percentile for Italian populations).

TABLE 3. Distribution of AFLP fragments generated by three primer combinations across the investigated *Cardamine* samples. n = number of analyzed individuals; TF = total number of fragments; AF = average number of fragments per individual (± standard deviation); DF = number of fragments diagnostic for the taxon (i.e., fragments restricted to the taxon, present in all its samples); EF = number of fragments percent exclusively in the taxon (but not necessarily present in all samples of the given taxon). [1]Value in parentheses gives the number of fragments present in both Italian and Catalonian samples.

Taxon	n	TF	AF	DF	EF
C. amara (excluding Catalonian samples)	37	124	—	0	72
C. amara subsp. *amara* ($2n = 2x$)	9	67	47.2 (±1.20)	0	7
C. amara subsp. *austriaca* ($2n = 4x$)	8	79	57.6 (±2.50)	0	9
C. amara subsp. *balcanica* ($2n = 2x$)	6	65	51.5 (±1.38)	3	10
C. amara subsp. *opicii* ($2n = 2x$)	7	65	47.6 (±3.10)	0	12
C. amara subsp. *pyrenaea* ($2n = 2x$)	7	61	47.9 (±3.34)	2	7
C. wiedemanniana ($2n = 2x$)	12	48	43.1 (±0.51)	3	4
Catalonian populations ($2n = 4x$)	11	67	63.7 (±1.10)	0	1
Italian populations ($2n = 4x$)	16	93	65.2 (±4.53)	1	20
Catalonian and Italian populations ($2n = 4x$)	27	99 (62)[1]	64.6 (±3.59)	14	22

of them present across all individuals studied (Table 3). Consistent with RAPD (Lihová et al. 2000) and isozyme results (Marhold et al. 2002a), currently recognized subspecies of *C. amara* showed more (subsp. *balcanica*, subsp. *pyrenaea*) or less (subsp. *amara*, subsp. *austriaca*) strong differentiation from each other (Table 3, Fig. 8), providing additional support for their subspecific rank. *Cardamine wiedemanniana*, a diploid species also included in this study, is taxonomically placed within *Cardamine* sect. *Cardamine* subsect. *Amarae* Spasskaya, and considered close to *C. amara* (Khatri 1988). Despite nine unique AFLP fragments shared by *C. wiedemanniana* and *C. amara*, still they are genetically divergent from each other (see Figs. 6, 8). *Cardamine wiedemanniana* has four unique AFLP fragments, three of them present in all studied samples (Table 3). This species needs further study using both morphometric and molecular comparative analyses, especially with representatives of *C. amara* subsp. *amara* from neighbouring areas.

When comparing the average number of AFLP fragments amplified per individual, association with ploidy level is evident (Table 3). As already mentioned and documented by Kardolus et al. (1998), more fragments are expected in polyploids. In our case, tetraploid *C. amara* subsp. *austriaca*, and especially *C. amporitana* (see also below) produced more fragments than diploid taxa.

Relationships of Catalonian and Italian Populations. A distinct position of Catalonian populations traditionally classified as *C. amara* subsp. *olotensis* has been confirmed here with AFLP data, in agreement with previous studies (Lihová et al. 2000; Marhold et al. 2002a, b). Furthermore, both morphometric and molecular analyses showed strong relatedness between this Catalonian taxon and populations from Central Italy. The results of the multivariate morphometric analyses reveal that Catalonian and Italian populations are morphologically very close to each other

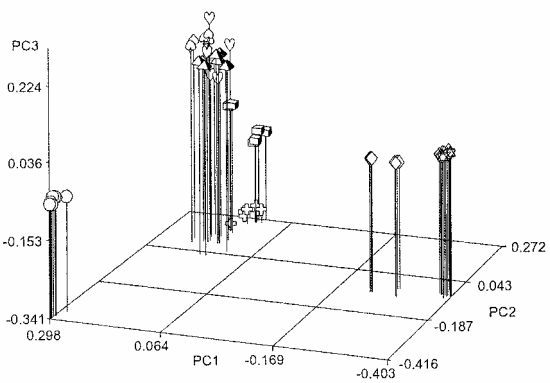

FIG. 6. Principal coordinate analysis of AFLP data including *C. amara* subsp. *amara* (pyramids), subsp. *austriaca* (hearts), subsp. *opicii* (cubes), subsp. *balcanica* (spades), subsp. *pyrenaea* (crosses), Catalonian populations (stars), Italian populations (diamonds) and *C. wiedemanniana* (circles). The first three principal coordinates explain 30.6%, 17.2%, and 7.5% of total variance; corresponding broken stick values are 6.5%, 5.1%, and 4.5%.

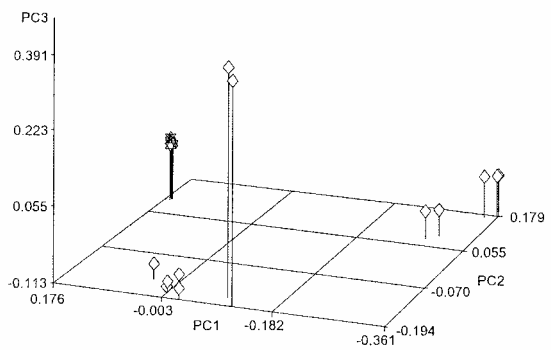

FIG. 7. Principal coordinate analysis of AFLP data of Catalonian (stars) and Italian (diamonds) individuals. The first three principal coordinates explain 37.3%, 23.2%, and 16.0% of total variance; corresponding broken stick values are 14.5%, 10.7%, and 8.8%, respectively.

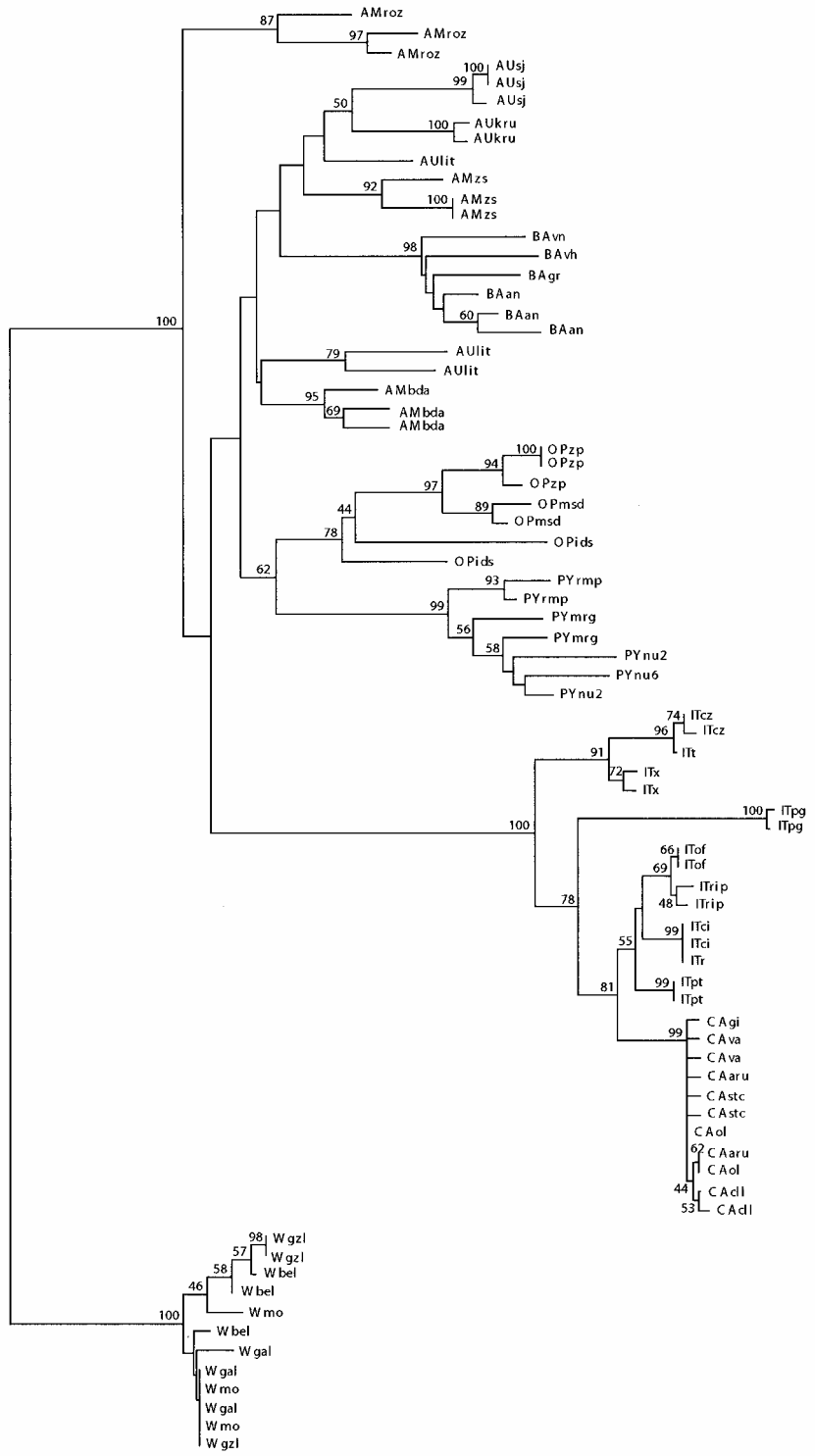

FIG. 8. Neighbour-joining tree of 76 *Cardamine* samples analyzed for AFLP variation. AU = *C. amara* subsp. *austriaca*, AM = subsp. *amara*, BA = subsp. *balcanica*, OP = subsp. *opicii*, PY = subsp. *pyrenaea*, W = *C. wiedemanniana*, IT = Italian populations, CA = Catalonian populations. For detailed information on samples see Table 1. Bootstrap values above 40% based on 2000 replicates are shown.

(Figs. 3, 4). Only minute differences are observed in the ranges of some characters (width of stem base, congestion of leaves under the inflorescence), but these do not allow sufficient discrimination between them (Fig. 5). Both Catalonian and Italian populations were compared also with *C. amara* subsp. *amara* and subsp. *austriaca*, being morphologically closer than other subspecies, but differences exist in number of stem leaves, leaflets of the stem leaves (Figs. 2, 5; Table 2, CDA1), and color of anthers (yellow anthers present in Catalonian and Italian populations vs. violet in the rest of *C. amara*). Bolòs (1952) and Bolòs and Vigo (1990) reported other diagnostic characters that should differentiate Catalonian populations, but these mostly refer either to traits showing wide variation and strong environmental plasticity (e.g., shape and size of leaves, number of flowers) or are useful for discrimination only from the other Iberian taxon, *C. amara* subsp. *pyrenaea*. AFLP data in both PCoA and neighbour-joining support morphology in clustering of Italian and Catalonian accessions together, both distant from the rest of *C. amara* (Figs. 6, 8). They both possess as many as 22 unique fragments, 14 of which are present in each individual examined (Table 3). Whereas Italian populations have considerable genetic variation (as seen from PCoA and high numbers of unique fragments; Table 3), Catalonian populations are genetically depauperate. This lack of genetic variation is responsible for strong support of the Catalonian branch in the neighbour-joining tree, rather than their genetic distinction from Italian populations (only one unique fragment was found in the Catalonian samples). Some authors have considered Italian populations close to or even identical with *C. raphanifolia* s. l. (Pignatti 1982; Jones and Akeroyd 1993; Jalas and Suominen 1994). Bolòs (1952) also mentioned a morphological similarity of Catalonian *C. amara* subsp. *olotensis* to *C. raphanifolia* s. str., but at the same time he listed a number of characters differentiating them (e.g., stolons, shape of stigma, color of petals). Based on our preliminary morphological and molecular studies (Lihová and Perný, unpubl.), however, Italian and Catalonian populations studied here are clearly distinct from *C. raphanifolia* s. l. The interpretation of Central Italian populations being close or equal to *C. raphanifolia* s. l., therefore, appears incorrect, and was probably due to morphological polymorphisms and taxonomic confusion centering around *C. raphanifolia* s. l., up to now poorly investigated.

Taxonomic Conclusions. Based on highly congruent morphological and molecular data, we herewith treat both Catalonian and Central Italian populations together as a separate species, distinct from *C. amara*. The validly published name *C. amporitana* Sennen et Pau (Sennen 1911) is already available for use (see Taxonomic Treatment).

In the region of Central Italy (Piceno, Monte di Bacucco), *C. amara* var. *grandifolia* was described by Bertoloni, differing from typical var. *amara* by larger leaves, more numerous leaflets, larger flowers and longer siliquae (Bertoloni 1847). He reported this taxon growing together with typical *C. amara*. Thus, his circumscription of *C. amara* var. *grandifolia* was narrower than that of our *C. amporitana*, because some of the populations that we classify as *C. amporitana* were considered by Bertoloni as *C. amara* var. *amara*. Typical *C. amara* does not occur in Central Italy (subsp. *amara* and subsp. *austriaca* grow only in the northern part of the country).

Evolutionary Hypotheses. *Cardamine amara* was reported to be partly autogamous, reproducing by outcrossing and selfing, and also, most efficiently, vegetatively by means of stolons (Lövkvist 1957). Later studies, however, have suggested self-incompatibility for this species (Urbanska-Worytkiewicz 1980). No information is available on the breeding or compatibility systems of *C. amporitana*, but formation of stolons is well developed in this taxon. In recent isozyme studies on *C. amara*, including all its subspecies and also two Catalonian populations of *C. amporitana*, several multilocus genotypes were detected in most of the population samples (consisting of 3 to 17 individuals; Marhold et al. 2002a), suggesting that outcrossing plays an important role in the reproduction of both *C. amara* and *C. amporitana*.

Distribution pattern and low morphological and molecular differentiation between populations of *C. amporitana* from two disjunct regions suggest that the contemporary populations may represent relicts of a previously more widespread ancestor. No hypotheses have been proposed for the origin of *C. amporitana*, whether auto- or allotetraploid. Higher numbers of AFLP fragments per individual in this taxon in comparison with presumptive autotetraploid *C. amara* subsp. *austriaca* (Table 3) might be supporting evidence for its allopolyploid origin. An ancient, preglacial polyploidization might also be suggested from the extent of genetic divergence. *Cardamine amporitana* occurs in regions that have been considered as potential refuges during the Quaternary (Taberlet et al. 1998). During Pleistocene climatic oscillations, many European taxa went through cycles of contraction and expansion of geographical ranges, which have had a strong impact on their present distribution and patterns of genetic diversity (Hewitt 1996; Comes and Kadereit 1998; Taberlet et al. 1998). Processes acting during glacial and interglacial periods could have caused changes in the distribution of *C. amporitana* and finally led to area fragmentation. The western-eastern orientation of the Pyrenees and Alps might have prevented northward recolonization, a hypothesis already suggested for several Mediterranean taxa (Hewitt 1996; Taberlet et al.

1998). The Mediterranean flora, in fact, is rich with endemics and taxa showing disjunct distributions, reflecting the effects of several processes and properties of this area on distributional patterns, genetic differentiation and speciation (Quézel 1985; Thompson 1999). Another taxon in Brassicaceae showing a similar central Italian-Iberian disjunction is *Jonopsidium savianum* (Caruel) Ball ex Arcang. It was described originally from Italy, but recent revision of Iberian representatives of the genus have shown that this rare species, being tetraploid, occurs also in Spain, in the province La Rioja (Morales Valverde 1992; Bencivenga et al. 1995; Jalas and Suominen 1996).

The data suggest that populations of *Cardamine amporitana* from Catalonia and Central Italy differ in their biogeographic history. Although they do not display marked genetic differentiation, Catalonian samples are genetically uniform and depauperate. It should be stressed that although within-population sampling was very low (only two individuals per population), it covered a major part of the entire distributional area, ranging from the southeastern foothills of the Pyrenees in the north to the Coloma river basin in the south (Fig. 1). It is unlikely, therefore, that the low genetic variation was caused by insufficient intrapopulational sampling. The loss of genetic variation might be associated with a more severe bottleneck experienced by the western lineage in contrast to the Italian one (which retained much more variation). In recent studies on *Saxifraga cernua* L., a similar strong contrast in genetic structure of populations from Scandinavia and the Alps was found (Bauert et al. 1998; Gabrielsen and Brochmann 1998). Whereas the arctic populations displayed considerable genetic variation, probably established by large-scale colonization from periglacial regions, the alpine ones were strongly bottlenecked. The latter could represent either fragmented glacial relicts, or alternatively selected immigrants to the Alps in postglacial time (Bauert et al. 1998).

An alternative explanation for the present disjunct distribution of *Cardamine amporitana* is that the Catalonian populations represent direct descendents of those from Italy. This view is favored by the fact that only a subset of genetic variation present in Italy is found in Catalonia, and moreover, that Catalonian samples possess only one unique fragment not found in Italian populations. Either migration through the Western Alps, Massif Central and Pyrenees, or long-distance dispersal from the Apennine to the Iberian Peninsula could have occurred in the past. With regard to the former hypothesis, however, one would expect a few remaining populations in connecting areas. These have not been found so far. Seeds of *C. amporitana* do not show any adaptation facilitating dispersal over large distances, but bird- or human-mediated transfer of seeds or stolon fragments may be possible. Colonization of new areas through long-distance dispersal may result in a lack of divergence and reduction of genetic diversity through founder effects. Introduced populations usually originate from a few founder individuals, which bear only part of the total genetic variation present in the original populations (Thompson 1999). In a recent AFLP study exploring the glacial and postglacial history of Alpine *Saponaria pumila* Janch. (Tribsch et al. 2002), besides regions with high genetic diversity, some peripheral populations with decreased genetic variation, but still possessing a high number of rare AFLP fragments, were identified, and these were suggested to be products of long-term isolation. Some populations, however, are genetically depauperate, lacking unique or rare (or with only a few) fragments, thus most probably originating via long-distance dispersal (Tribsch et al. 2002). In a comparative study of genetic variation in invasive *Rubus alceifolius* Poir. in its native range and areas of introduction, a cumulative decrease in genetic diversity was observed in the latter, attributed to founder events, and reinforced by largely vegetative reproduction (Amsellem et al. 2000).

Conservation Remarks. Based on our field observations and herbarium studies, *Cardamine amporitana* is a rather rare species, with only a few localities scattered in mountain ranges of Central Italy and lowland to mountainous region of Catalonia, NE Spain (Fig. 1). In some localities (e.g., Catalonia: Arbúcies, Santa Coloma de Farners; Italy: Templo; see Table 1) very small populations, consisting of only a few individuals (mostly no more than ten plants), were observed, but at other sites (e.g., Catalonia: Olot—Paratges de la Deu; Italy: Fosso della Padura), large populations appeared to persist well. Both small population size and low genetic diversity, especially pronounced among Catalonian populations, may reduce their ability to cope with changing environments for long-term survival. As the species requires wet, open to half-shady habitats, especially on stream banks, human activities such as forest plantations, road construction and landscape drainage might be threatening factors as well. We conclude, therefore, that this taxon deserves protection, and based on IUCN criteria (2001), we recommend treating the Catalonian populations as vulnerable (VU), and those from Central Italy as near threatened (NT).

Taxonomic Treatment

CARDAMINE AMPORITANA Sennen et Pau, Bull. Acad. Int. Géogr. Bot. 20 (259): 104–105. 1911. Ind. loc.: ''Espagne, Catalogne, ruisseau del Macho à Cabanas''.— *Cardamine amara* var. *parviflora* Cadevall, Fl. Catalunya 1: 136. 1915. Ind. loc.: ''al Empordá; reguerols de Cabanes (Sen!)''.—TYPE: Catalogne, Cabanas, ruisseau; Cabanas, bords du ruisseau Gordo del Macho, 4 Jun 1908, *Sennen* [Plantes

d'Espagne 530] (lectotype, chosen by Lihová et al. [2000]: MA 47582!).

Cardamine amara subsp. *olotensis* O. Bolòs, Collect. Bot. (Barcelona) 3: 187–188. 1952. Ind. loc.: ''Olot, Parc Nou''.— TYPE: Olot, in pratis humidis Parc Nou, 1 May 1950, *A. de Bolòs s.n.* (holotype: BC 112809!)

Cardamine amara var. *grandifolia* Bertol., Fl. Ital. vol. 7: 31–32. 1847. Ind. loc.: ''Piceno a Monteferino all' Acqua viva, monte di Bacucco''.—TYPE: unknown, original material was not retained (Mossetti, pers. comm.).

Cardamine amara var. *amara* auct. (p.p.) non L.: Bertol., Fl. Ital. vol. 7: 31–32. 1847.

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