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ORIGIN OF THE DISJUNCT TETRAPLOID *CARDAMINE AMPORITANA* (BRASSICACEAE) ASSESSED WITH NUCLEAR AND CHLOROPLAST DNA SEQUENCE DATA[1]

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Seventy-four nucleotide sequences from the ITS regions of nuclear ribosomal DNA and 76 from the *trnL-trnF* spacer of chloroplast DNA were used to address the origin of tetraploid *Cardamine amporitana*, the conspecifity of central Italian and northeastern Spanish populations, and the possible cause for such geographic disjunction. Because of the complex lineage relationships in *Cardamine*, the sampling included 22 taxa. In the results, both data sets are highly congruent in supporting a close relationship of *C. amporitana* to the widespread Eurasian *C. amara*. Low genetic variability in northeastern Spanish populations of *C. amporitana* suggests long-distance dispersal from central Italy. The interior position of the single northeastern Spanish haplotype in a statistical parsimony network of *trnL-trnF* haplotypes however does not support this scenario and invokes other plausible phylogeographic explanations. The disappearance of geographically intermediate populations and genetic impoverishment by migration and isolation, both probably associated with Quaternary climatic oscillations, appears as an alternative hypothesis to explain the phylogeographic pattern. A recent hybridization event is reported between *C. amporitana* and a diploid from the *C. pratensis* group in central Italy on the basis of additive polymorphisms in ITS for all the 22 distinguishing nucleotides.

Key words: *Cardamine amporitana*; concerted evolution; DNA sequences; geographic disjunction; internal transcribed spacer (ITS); polyploidy; *trnL-trnF* spacer.

The importance of polyploidy in plant evolution and diversification has been widely acknowledged. Application of molecular techniques in recent decades has expanded our knowledge of various aspects of this evolutionary phenomenon, including pathways and mechanisms of polyploid formation, polyploid stabilization and fitness, genome evolution during initial and advanced stages, and evolutionary significance of polyploids (Bayer, 1998; Ramsey and Schemske, 1998; Wendel, 2000). Recent polyploidization events may be easily recognized, and indeed, various molecular techniques have allowed the distinction between auto- and allopolyploidy, and the identification of parentage (Leitch and Bennett, 1997; see e.g., Barrier et al., 1999; Baumel et al., 2002a). Extensive studies on several model systems (*Brassica*, *Gossypium*, *Nicotiana*, *Tragopogon*; Song et al., 1995; Cook et al., 1998; Cronn et al., 1999; Volkov et al., 1999) and other taxa (*Microseris*, *Spartina*, *Saxifraga*, etc.; Roelofs et al., 1997; Brochmann et al., 1998; Baumel et al., 2002b), have revealed polyploids to be complex and dynamic. They usually undergo rapid changes in both genome structure and gene expression, coupled with interactions among duplicated genes and genomes (Soltis and Soltis, 1999; Wendel, 2000; Bowers et al., 2003). All these processes, together with polytopic and recurrent origin of many polyploids, account for the overall complexity observed in such taxa. Unraveling the origin and evolutionary history of a particular polyploid is therefore a challenging task.

Infrageneric relationships in *Cardamine* L. (Brassicaceae), a cosmopolitan genus of around 200 species, many of them polyploid, are poorly understood. Traditional sectional classification of the genus, based on a few morphological characters (Schulz, 1903), apparently does not reflect true phylogenetic relationships among the taxa (Sweeney and Price, 2000; Bleeker et al., 2002a). The few molecular studies published so far focus on selected taxa of the genus and have identified several groups of close relatives (Franzke et al., 1998; Franzke and Hurka, 2000; Sweeney and Price, 2000; Bleeker et al., 2002a; Marhold et al., 2004).

Cardamine amporitana Sennen & Pau is a tetraploid species with disjunct distribution in Catalonia (northeast Spain) and central Italy (Fig. 1). It is a perennial, rhizomatous herb morphologically resembling the widespread species *C. amara* L., which includes several diploid and tetraploid subspecies in Europe (Lihová et al., 2000, 2004a). The name *C. amporitana* was originally published for Catalonian populations (Sennen, 1911) although its subspecific treatment as *C. amara* subsp. *olotensis* O. Bolòs has been adopted by most authors (Bolòs, 1952; Bolòs and Vigo, 1990; Bolòs et al., 1993; Rico, 1993). Until our recent study (Lihová et al., 2004a), populations from central Italy have never been associated with the Catalonian taxon, and their taxonomic position was a matter of contro-

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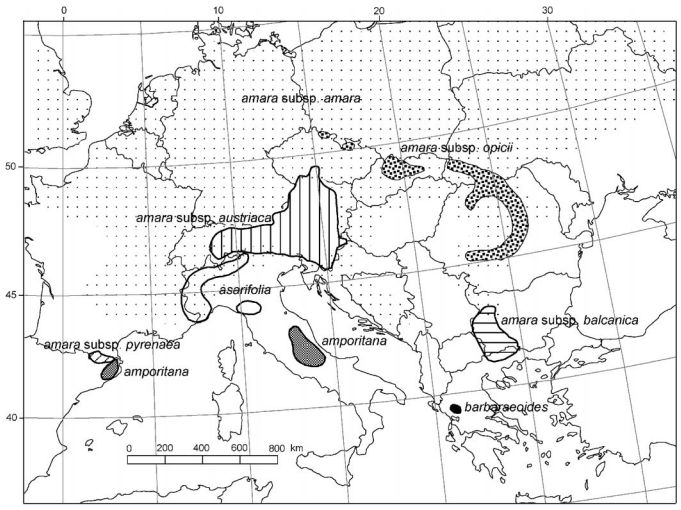

Fig. 1. Distribution areas of *Cardamine amporitana*, *C. amara* subsp. *amara*, subsp. *austriaca*, subsp. *opicii*, subsp. *pyrenaea*, subsp. *balcanica*, *C. barbaraeoides*, and *C. asarifolia*. Distribution area of *C. amara* subsp. *amara* covers a major part of Europe and extends to Asia.

versy. They were treated either as *C. amara*, without a subspecific affiliation, or considered close to Balkan or Iberian taxa of *C. raphanifolia* Pourr. sensu lato (Pignatti, 1982; Jones and Akeroyd, 1993; Jalas and Suominen, 1994). Recent morphometric and amplified fragment length polymorphism (AFLP) studies (Lihová et al., 2004a) strongly suggest that Catalonian and central Italian populations belong to a single species, distinct from *C. amara*.

The internal transcribed spacer (ITS) region of nrDNA is among the most widely used molecular markers for inferring phylogenetic relationships at low taxonomic levels (Baldwin et al., 1995; Soltis and Soltis, 1998). An important feature of the region to consider in phylogenetic studies is that it consists of multiple copies that are typically homogenized by concerted evolution (Graur and Li, 1999; Volkov et al., 1999). Even in recent hybrids and allopolyploids, different repeat units contributed by parental genomes can be rapidly homogenized towards one of the parental variant (e.g., Franzke and Mummenhoff, 1999; Fuertes Aguilar et al., 1999) or produce recombinant ITS sequences (Wendel et al., 1995; Mummenhoff et al., 1997; Nieto Feliner et al., 2004). However, this is not always the case because mechanisms of concerted evolution can also be strongly retarded, so that more than one copy is maintained in a genome (e.g., O'Kane et al., 1996; Widmer and Baltisberger, 1999; Hughes et al., 2002; Koch et al., 2003). Because the homogenization should proceed more efficiently within rather than among chromosomes, longer persistence of different ITS variants is expected in allopolyploids rather than in diploid taxa (O'Kane et al., 1996). The occurrence of single-site polymorphisms indicating intragenomic variation can, therefore, provide evidence for past hybridization events and allow the tracing of the taxa and genomes involved (Sang et al., 1995a, b). When concerted evolution fails to homogenize the repeat units, reticulate evolution may be easy to detect, but when homogenization proceeds intensively, the direct evidence for past hybridization is lost. In the latter case, ITS sequence data still can provide valuable information when compared with differently evolving and inherited markers. Some of the most frequently used for this purpose are the fast-evolving sequences of the noncoding *trnL-trnF* spacer region from the chloroplast DNA (e.g., in Brassicaceae, Franzke et al., 1998; Mummenhoff et al., 2001; Bleeker et al., 2002b).

The main aim of this study was to address the origin of tetraploid *Cardamine amporitana* within the complex relationships resulting from polyploidy that involved several lineages of the genus *Cardamine*. For this, we generated nrDNA ITS sequence data, which were lacking for polyploid representatives of the related *C. raphanifolia* and *C. amara* groups, and the *trnL-trnF* chloroplast spacer for which there were no available data from relevant taxa. Also, the conspecifity of Italian and Catalonian populations of *C. amporitana* suggested by AFLP data (Lihová et al., 2004a) is tested here and the origin of the geographic disjunction examined. In addition, our analyses of the two molecular data sets, containing a fair representation of the European species of *Cardamine*, were also used to shed light on the phylogenetic relationships within this genus.

MATERIALS AND METHODS

Taxon sampling—Sampling of *C. amporitana* was designed to cover a wide range of its geographic occurrence, including 11 Catalonian accessions from six localities and 14 Italian from eight localities. In addition, we sampled all five subspecies of *C. amara*, plus *C. wiedemanniana* Boiss., six taxa from the *C. pratensis* group, eight from the *C. raphanifolia* group, and also *C. asarifolia* L. (known to co-occur with *C. amara* and their presumed hybrid *C. ferrarii* Burnat; Bongini, 1916). Most of the taxa are represented by multiple accessions (Table 1, Fig. 1; see also the Appendix 1 in Supplemental Data accompanying the online version of this article). Chromosome numbers were counted in each population sampled (Lihová et al., 2000, 2003, 2004a, b, and unpublished data). Taxonomy and delimitation of groups mostly follows results from recent studies (Lihová et al., 2003, 2004a, b; Marhold et al., 2004). In this study, we use 44 ITS sequences mostly from polyploid taxa, plus a set of 30 recently obtained sequences of diploids (Marhold et al., 2004). With a few exceptions, accessions analyzed for the ITS region were the same as for the *trnL-trnF* spacer. The *trnL-trnF* region was sequenced here for 76 individuals (for the origin of samples see the Appendix 1 in Supplemental Data accompanying the online version of this article). Voucher specimens are deposited in the herbarium SAV. Sequences from the Australian *C. corymbosa* Hook. f. were used to root the trees, based on results by Franzke et al. (1998) and Bleeker et al. (2002a). These studies present a phylogeny of selected representatives of the genus *Cardamine*, where European lineages, including taxa studied here, were either weakly resolved or part of a polytomy. *Cardamine corymbosa* appeared in a clade external to all European and African species.

DNA isolation, amplification, and sequencing—Total genomic DNA was isolated from silica-gel-dried leaf samples using either cetyltrimethyl ammonium bromide (CTAB) extraction method (Doyle and Doyle, 1987) or a DNeasy Plant Mini kit (Qiagen, Valencia, California, USA) following the manufacturer's protocol. Polymerase chain reaction (PCR) amplifications were performed in 25-μL reaction volume with 0.5 μL of each primer (10 μmol/L) using Ready-To-Go PCR beads (Amersham Biosciences Europe GmbH, Cerdanyola, Barcelona, Spain) and run in a GeneAmp PCR system 9700 (PE Biosystems, Foster City, California, USA). For the ITS region (ITS1, 5.8S, ITS2), P1A and P4 primers (Francisco-Ortega et al., 1999) were used. The PCR cycle profile comprised an initial step of 94°C (5 min), 38 cycles with 94°C (30 s), 54°C (30 s), and 72°C (1 min), and a final extension step of 72°C for 10 min. Amplification of the *trnL-trnF* intergenic spacer was performed with the primers e and f (Taberlet et al., 1991), with the following PCR conditions: 94°C for 5 min, 35 cycles with 94°C (1 min), 52°C (1 min; in some samples the annealing temperature was modified to 54°C), and 72°C (45 s, every cycle decreased by 1 s), and final extension with 72°C for 10 min. Products were electrophoresed in 1.5% agarose gel in TAE (Tris-Acetate-

TABLE 1. List of *Cardamine* taxa sampled for ITS and *trnL-trnF* sequences. Localities, collection data, and GenBank accession numbers are in the Appendix 1 (see Supplemental Data accompanying the online version of this article).

Taxon	Distribution	Chromosome number
Cardamine amara group		
C. amara L. subsp. *amara*	Eurasia	$2n = 2x = 16$
C. amara subsp. *austriaca* Marhold	E Alps and adjacent areas	$2n = 4x = 32$
C. amara subsp. *balcanica* Marhold, Ančev & Kit Tan	SW Bulgaria, NE Greece	$2n = 2x = 16$
C. amara subsp. *opicii* (J. Presl & C. Presl) Čelak.	Carpathians, Sudety Mts.	$2n = 2x = 16$
C. amara subsp. *pyrenaea* Sennen	E Pyrenees	$2n = 2x = 16$
C. amporitana Sennen & Pau	Catalonia, central Italy	$2n = 4x = 32$
C. wiedemanniana Boiss.	W Transcaucasia, NE Turkey	$2n = 2x = 16$
Cardamine pratensis group		
C. apennina Lihová & Marhold	Central Italy	$2n = 2x = 16$
C. castellana Lihová & Marhold	Central Iberian Mts.	$2n = 2x = 16$
C. crassifolia Pourr.	E Pyrenees	$2n = 2x = 16$
C. granulosa All.	Piedmont (NW Italy)	$2n = 2x = 16$
C. matthioli Moretti	Central, SE Europe	$2n = 2x = 16$
C. pratensis L. s. str.	Eurasia, N Africa, N America	$2n = 2x-7x = 16–56$
Cardamine raphanifolia group		
C. acris Griseb.	Balkan Peninsula	$2n = 2x = 16$
C. barbaraeoides Halácsy	Pindos Mts. (NW Greece)	$2n = 4x = 32$
C. gallaecica (M. Laínz) Rivas-Mart. & Izco	NW Spain	$2n = 4x, 6x = 32, 48$
C. raphanifolia Pourr.	N Spain	$2n = 6x, 8x = 48, 64$
C. silana Marhold & Perný	Calabria (S Italy)	$2n = 6x = 48$
C. seidlitziana Albov	Caucasus Mts. (SW Russia, Georgia)	$2n = 2x = 16$
C. tenera J. G. Gmel. ex C. A. Mey.	Crimea (Ukraine), W Greater Caucasus (Russia), Talysh Mts. (Azerbaijan)	$2n = 2x = 16$
C. uliginosa M. Bieb.	Asia Minor, Caucasus Mts.	$2n = 2x = 16$
C. asarifolia L.	NW Italy, S Switzerland, SE France	$2n = 6x = 48$

EdtA) buffer, and stained with ethidium bromide. The PCR products were purifed using spin filter columns (PCR Clean-up kit, MoBio Laboratories, Solana Beach, California, USA), following the manufacturer's protocol. Cycle-sequencing reactions were performed with the same primers as in the amplifications, using fluorescently labelled dideoxynucleotide terminators (ABI Big-Dye Terminator kit, Applied Biosystems, Foster City, California, USA). Products were then separated on an ABI 377 DNA sequencer at the Centro de Investigaciones Biológicas, CSIC, Madrid. The *trnL-trnF* spacer was sequenced mostly in the forward direction only (after a pilot study that involved sequencing of both strands). For the ITS region both forward and reverse strands were sequenced with a 100% overlap. Electropherograms of ITS were carefully examined with EditView (Applied Biosystems), and consensus sequences between both strands were generated using the SeqApp program (Gilbert, 1992). Occurrence of 1-base pair (bp) substitutions between different ITS repeats in the same individual was detected by inspection of two overlapping peaks in the same position. Quantitative criteria to distinguish intraindividual ITS variation (i.e., intraindividual polymorphisms) from noise background follow Fuertes Aguilar and Nieto Feliner (2003). The IUPAC ambiguity codes were used for coding those polymorphic positions. When short (1- or 2-bp) indels appear, the co-amplification of both repeats produces a 1- or 2-bp shift in the double peaks pattern of the electropherogram. In these cases, co-occurring sequences were inferred by displacing one of the sequences 1- or 2-bp and double-checking the reverse primer electropherogram (Whittall et al., 2000; Wichman et al., 2002; Fuertes Aguilar and Nieto Feliner, 2003). All sequences were submitted to the GenBank (accession numbers are available in Appendix 1 in Supplemental Data accompanying the online version of this article). The sequences were aligned manually in the SeqApp program. The alignment of ITS sequences was straightforward, with only seven 1- or 2-bp indels introduced, but that of the *trnL-trnF* spacer resulted in 22 mostly overlapping gaps of 1 to 443 bp in length. For the *trnL-trnF* data matrix, gaps of 5 bp and longer (shorter gaps, i.e., those of 1 and 2 bp apparently caused by slipped-strand mispairing were excluded) were scored as 18 additional binary characters using the ''simple gap coding'' approach as suggested by Simmons and Ochoterena (2000; see data sets in the Appendix 1 and 2 in Supplemental Data accompanying the online version of this article).

Phylogenetic analyses—Maximum parsimony analyses were conducted with PAUP* version 4.0b10 (Swofford, 2001). Heuristic searches were made with the following settings: gaps treated as missing data, single-site polymorphisms as uncertainties, tree construction with stepwise addition, 100 replicates with random taxon addition (due to computer memory limitations only 10 replicates were used for ITS data, but 10 independent heuristic searches were run to make sure that no island of most parsimonious trees was missed), tree bisection-reconnection (TBR) branch swapping, no limit on the maximum number of trees saved (no MAXTREES limits), and saving all minimal trees found during branch swapping (MULTREES option in effect). For character-state optimization, the accelerated character transformation (ACCTRAN) option was used. Bootstrap analyses (Felsenstein, 1985) were performed using 10 000 resamplings with the fast-heuristic search as implemented in PAUP*. Previous studies (Mort et al., 2000; Álvarez Fernández et al., 2001) have shown that the fast bootstrapping produces very similar results to those using branch swapping and therefore can be considered reliable. This option was used due to the high number of most-parsimonious trees generated. The two data sets, ITS and *trnL-trnF,* were first analyzed separately. To check for congruence between them, a Templeton less parsimonious test (based on Wilcoxon signed-ranks test; Templeton, 1983) was performed in PAUP*. Because the number of most parsimonious trees obtained from the independent analysis of the two data sets was high, data sets were optimized on the strict consensus topology from the alternative data set instead of on fundamental trees. In addition, the index of Mickevich and Farris (I_{MF}) (Mickevich and Farris, 1981) was computed based on the parameters obtained from PAUP*, following Johnson and Soltis (1998). The maximum parsimony analysis of the two data sets combined was performed without accessions of *C. asarifolia* and *C. castellana* (see Results). Sequence divergence between taxa was calculated, using the uncorrected distance as implemented in PAUP*.

TABLE 2. Nucleotide site variation of the ITS region (ITS1-5.8S-ITS2) in tetraploid *Cardamine amporitana* compared with other taxa of two main clades A and B as appearing in the consensus tree (Fig. 2) and *C. asarifolia*. Nucleotide sites in *C. apennina* and *C. silana* (both from clade A) are given separately. Accession numbers are the same as in Figs. 2–5 and follow the Appendix 1 (see Supplemental Data accompanying the online version of this article). CA, accessions from Catalonia; IT, from central Italy.

Taxon/Position	24	28	47	55	56	57	63	76	123	138	140	146	208	210	211
C. amporitana															
1, 2—CA, Coloma	G	C	C	G	A	G	T	T	G	G	A	C	C	G	C
3, 4—CA, Girona	G	C	C	G	A	G	T	T	G	G	A	C	C	G	C
5, 6—CA, Vajol	G	C	C	G	A	G	T	T	G	G	A	C	C	G	C
7—CA, Cantallops	G	C	C	G	A	G	T	T	G	G	A	C	C	G	C
9, 10—CA, Olot	G	C	C	G	A	G	T	T	G/T	G	A	C	C	G	C
11—CA, Arbúcies	G	C	C	G	A	G	T	T	G/T	G	A	C	C	G	C
12, 13—IT, Rigopiano	G	C	C	G	A	G	T	T	G	G	A	C	C	G	C
14, 15—IT, Pietracamela	G	C	C	G	A	G	T	T	G	G	A	C	C	G	C
16—IT, Fosso d. Padura	G	C	T/C	G	A	G	T	T	G	G	A	C	C	G	C
18—IT, Brittoli	G	C	C	G	A	G	T	T	G	G	A	C	C	G	C
21—IT, Rapegna	G	C	C	G	A	G	T	T	G	G	A	C	C	G	C
22—IT, Rapegna	G	C	C	G	A	G	T	T	G	G	A	C	C	G	C
23, 24—IT, Ussita	G	C	C	G	A	G	T	T	G	G	A	C	C	G	C
C. amporitana × *apennina*															
IT, Pian Grande	A/G	C/T	C	A/G	A/G	A/G	C/T	A/T	C/G	G	A/G	C	C	G	C/T
Clade B	G[a]	C	C	G[b]	A	G	T	T	G	G	A	C	C	G	C
Clade A	A	C	C	A	G[c]	A	C	A	G	G	G	C	C	G	C
C. apennina	A	C	C	A	G	A	C	A	C	G	G	C	C	G	T
C. asarifolia	G	T	C	G	G	G	T	T	T	G	G	C	C	G	C
C. silana	A	C	C	A	G	A	C	A	C/G[d]	C/G[d]	G	C	C	G	C/T

[a] Except *C. raphanifolia* 1 with A.
[b] *C. amara* subsp. *amara* 4 with A/G.
[c] *C. tenera* 1, 2 with G/T.
[d] *C. silana* 2 with C/G, *C. silana* 1 with C.
[e] *C. crassifolia, C. castellana* with G.
[f] *C. raphanifolia* 1 with C/G.
[g] *C. tenera* 1, *C. uliginosa* 2 with C/T.
[h] *C. barbaraeoides* with G/T.
[i] *C. amara* subsp. *pyrenaea* 3, 4 with A/C.
[j] *C. amara* subsp. *opicii* 1 with C/T.
[k] *C. amara* subsp. *opicii* 1 with A.
[l] *C. tenera* 3, *C. uliginosa* 1 with T.
[m] *C. apennina* 2, 3 with C, *C. apennina* 1 with C/G.

Taking advantage of its uniparental inheritance and lack of recombination, the relationships between chloroplast sequences were also estimated under a phylogeographic frame (Avise, 2000). A haplotype unrooted cladogram was constructed using the program TCS 1.13 (Clement et al., 2000). This program applies statistical parsimony by implementing the algorithm described in Templeton et al. (1992). Under this method, unrooted cladograms that have a high probability (>0.95%) of being true based on a finite-site model of DNA evolution are identified. The program was run with gaps coded as missing, but with additional 18 binary characters reflecting indel structure.

RESULTS

The ITS and trnL-trnF sequence variation—The total length of the ITS region varied from 619 to 623 bp (268–270 bp ITS1, 164 bp 5.8S, 188–190 bp ITS2); 95 sites were variable, 72 of them parsimony informative, and 23 represented autapomorphies. Although PCR products were not cloned, intraindividual single-site polymorphisms could be detected from the occurrence of overlapping double peaks on both complementary strands, implying superimposition of different ITS repeats. Of 74 sequences analyzed, 47 contained single-site polymorphisms (polymorphic sites, PS) and 27 of them displayed an additive pattern (additive polymorphic sites, APS). Among the diploids, the highest numbers of PS (up to seven per sequence) were found in two Caucasian diploids, *C. uliginosa* and *C. tenera*, and also *C. matthioli* from Slovenia. In the polyploids (except *C. amporitana*, described separately later) the numbers of PS ranged from zero (in tetraploid *C. amara* subsp. *austriaca* and hexaploid *C. asarifolia*) to five (in hexaploid *C. silana*). Additive polymorphic sites were found only in two polyploid taxa, the tetraploid *C. barbaraeoides* and two accessions of hexaploid *C. silana* (Table 2). Distribution of PS and APS across 21 accessions of tetraploid *C. amporitana* is shown in Table 2. In one Italian individual of this taxon (Monti Sibillini, locality Pian Grande), co-occurring with diploid *C. apennina*, the number of PS (24) far exceeded those found in other samples, almost all of them additive (23). The pattern observed can be unambiguously interpreted as a result of recent hybridization between *C. amporitana* and *C. apennina*. Otherwise, PS were recorded in 10 samples of *C. amporitana*, but only five of them had additive patterns (Table 2). Two samples are from a single Italian locality (the Gran Sasso Mountains, locality Pietracamela, accession numbers 14, 15), while the other three are from two Catalonian localities (Arbúcies and Olot, accession numbers 9, 10, 11). Each of these samples has one APS either in position 123 (Catalonian samples) or in position 463 (Italian

TABLE 2. Extended.

215	216	219	227	246	249	463	476	523	539	541	547	558	588	589	604	605	606
C	C	C	C	A	A	C	C	A	C	C	G	C	T	T	-	-	G
C	C	C	C	A	A	C	C	A	C	C	G	C	T	T	-	-	G
C	C	C	C	A	A	C	C	A	C	C	G	C	T	T	-	-	G
C	C	C	C	A	A	C	C	A	C	C	G	C	T	T	-	-	G
C	C	C	C	A	A	C	C	A	C	C	G	C	T	T	-	-	G
C	C	C	C	A	A	C	C	A	C	C	G	C	T	T	-	-	G
C	C	C/T	C	A	A	C	C	A	C	C	G	C	T	T	-	-	G
C	C	C/T	C	A	A	C/T	C	A	C	C	G	C	T	T	-	-	G
C	C	C	C	A	A	C	C	A	C	C	G	C	T	T	-	-	G
C	C	C/T	C	A	A	C	C	A	C	C	G	C	T	T	-	-	G
C	C	C	C	A	A	C	C	A/C	C	C	G	C	T	T	-	-	G
C	C	C	C	A	A	C	C	A	C	C	G	C	T	T	-	-	G
C	C	C	C	A	A	C	C	A	C	C	G	C	T	T	-	-	G
C/T	A/C	C	C/T	A/G	A/G	C	C/T	A	C	C/T	A/G	C/T	C/T	A/T	T/-	T/-	C/G
C	C	C[f]	C	A	A[h]	C	C	A[i]	C	C[j]	G	C	T[k]	T	-	-	G
C	A[e]	C[g]	T	G	G	C	T	A	C	T	G	T	C[l]	A	T	T	G
T	A	C	T	G	G	C	T	A	C	T	G	T	C	A	T	T	C/G[m]
C	C	C	C	G	G	T	C	A	C	C	G	C	C	T	-	-	G
C/T	A	C	T	G	G	C	T	A	C	T	G	T	C	A	T	T	C/G

samples). The alternative base in the APS was found exclusively in *C. asarifolia* (Table 2).

Large variation was found in the length of the *trnL-trnF* spacer. It ranged between 394 bp in *C. castellana* and 747 bp in one accession of *C. tenera*. The aligned matrix had 889 nucleotide sites, plus the 18 additional presence/absence characters, representing indels. The matrix included 71 variable sites (substitutions), 42 of them were parsimony informative and 29 represented autapomorphies. Among the 18 indel characters, 13 were parsimony informative, and five were autapomorphic.

Four different haplotypes were found in the accessions of *C. amporitana*. All 11 Catalonian individuals shared a unique haplotype (H1), while three different haplotypes (H2–H4) were recorded among Italian samples, with one of them being common and widespread (H4). Another Italian haplotype (H2 shared by two accessions 16 and 17 from a single locality in the Parco Nazionale d'Abruzzo, southern range of the Italian distribution area) differed from the Catalonian by a single 6-bp indel. One Italian individual (accession number 21, locality Rapegna, Monti Sibillini, northern range of the Italian distribution area) had a private haplotype (H3), more distinct from the others, differing also by three substitutions. Another accession (number 22) from the same locality, however, presented the common Italian haplotype (H4). Haplotype H3 was also found in *C. amara* subsp. *opicii*, and the same indel distribution but with two nucleotide substitutions was observed in polyploid *C. raphanifolia* sensu stricto (s. str.) and *C. barbaraeoides*. The indel pattern of the Catalonian haplotype (H1) was also present in *C. amara* subsp. *amara*, subsp. *pyrenaea*, subsp. *austriaca*, *C. asarifolia*, but their sequences differed from those of *C. amporitana* by 2–5 substitutions. As most Italian samples, the putative hybrid individual detected by ITS sequence had haplotype H4.

Phylogenetic analyses—Maximum parsimony analyses of ITS data were performed both with and without the putative hybrid individual (*C. amporitana* × *C. apennina*). In the former, the heuristic search produced a high number of trees, and the search could not be completed due to computer memory limitations. The analysis excluding that single individual with 23 APS resulted in 175 702 most-parsimonious trees of 112 steps with a consistency index (CI) of 0.8977 and a retention index (RI) of 0.9823. The strict consensus tree shows three main clades: a clade of *C. asarifolia* (100% bootstrap) and two large clades, A (100% bootstrap) and B (69% bootstrap), both with extremely low within-clade resolution (Fig. 2). Pairwise sequence divergence ranged from 0 to 7.0%, with the highest value found between *C. asarifolia* and *C. granulosa*.

Clade B includes all accessions of *C. amporitana* plus all subspecies of *C. amara*, *C. wiedemanniana*, and three polyploids of the *C. raphanifolia* group: *C. barbaraeoides*, *C. raphanifolia* s. str., and *C. gallaecica*. Lack of resolution is caused by low nucleotide variation in the sequences. Pairwise sequence divergence within this clade ranged between 0 and 2.26%, with the highest value found between hexaploid *C. raphanifolia* and diploid *C. wiedemanniana*. Except for a few intraindividual polymorphisms (Table 2), all accessions of *C. amporitana*, *C. amara* subsp. *amara*, subsp. *austriaca*, subsp. *pyrenaea*, and also *C. barbaraeoides* had identical ITS sequences. The highest sequence divergences between *C. amporitana* and any other accessions of clade B were observed in the polyploid *C. raphanifolia* s. str. (1.46%, seven substitutions).

Clade A included another polyploid from the *C. raphanifolia* group, the Calabrian endemic *C. silana*, and three Caucasian diploids of this group (*C. tenera*, *C. uliginosa*, *C. seidlitziana*), together with taxa of the *C. pratensis* group. Sequence diversity within clade A was slightly higher than with-

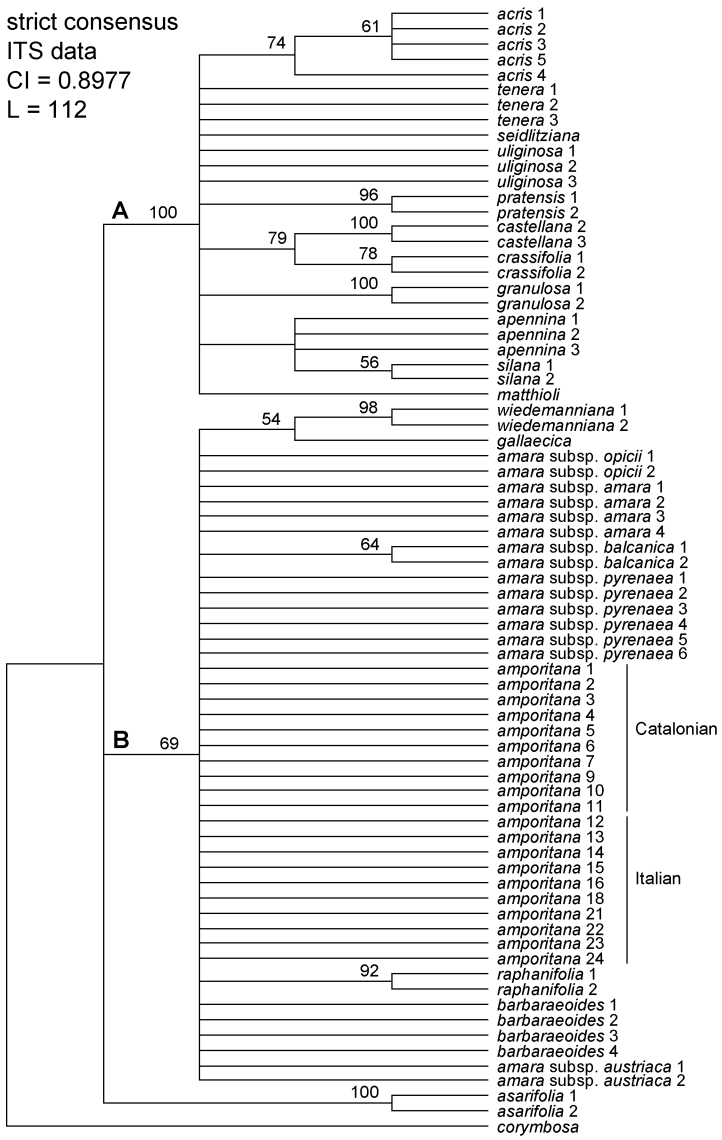

Fig. 2. Strict consensus of 175 702 most parsimonious trees based on nrDNA ITS sequence data. Bootstrap values above 50% are shown along the branches.

in clade B, although maximum pairwise sequence divergence only reached 2.42% (between *C. castellana* and *C. seidlitziana*). Relationships among the taxa remained largely unresolved, due to conflicting topologies of fundamental trees.

Parsimony analysis of *trnL-trnF* sequences yielded 68 201 trees of 141 steps with CI of 0.5377 and RI of 0.8721. In the strict consensus tree (Fig. 3) slightly more resolution was obtained in comparison with that based on ITS data, but several groupings emerging here did not receive bootstrap support higher than 50%. Two main clades, A and B, were obtained although they were supported only by two substitutions. These clades are the same as those in the ITS tree except for the placement of *C. castellana* and *C. asarifolia* in clade B, which were in clade A and in its own, respectively.

The Templeton less parsimonious test revealed significant incongruence between the ITS and *trnL-trnF* data sets (ITS data on *trnL-trnF* topology, $P < 0.0001$**; *trnL-trnF* data on ITS topology, $P = 0.0047$*). Because *C. castellana* and *C. asarifolia* were the main apparent discrepancies between the topologies of the ITS and *trnL-trnF* trees, we removed the sequences from both species and recomputed the Templeton test. This time, the number of extra steps was significant when checking the ITS data on the alternative topology but not the opposite (ITS data on *trnL-trnF* topology, $P = 0.0001$**; *trnL-trnF* data on ITS topology, $P = 0.066$). Therefore, homogeneity may be inferred (Johnson and Soltis, 1998). The I_{MF} computed without the two species indicated 17% incongruence, which is not negligible but, coupled with the results of the Templeton test, justifies combining the two data sets once the two most conflicting taxa were removed.

The combined data set consisted of 101 parsimony informative characters, and the parsimony analysis generated 16 628 most parsimonious trees of 260 steps (CI = 0.6337, RI = 0.9173). In the strict consensus tree (Fig. 4), two main clades came out as well-supported groups. Analyses with and without the putative hybrid individual produced almost equal number of most parsimonious trees, CI, and also the same topology of the strict consensus (the tree with the hybrid is

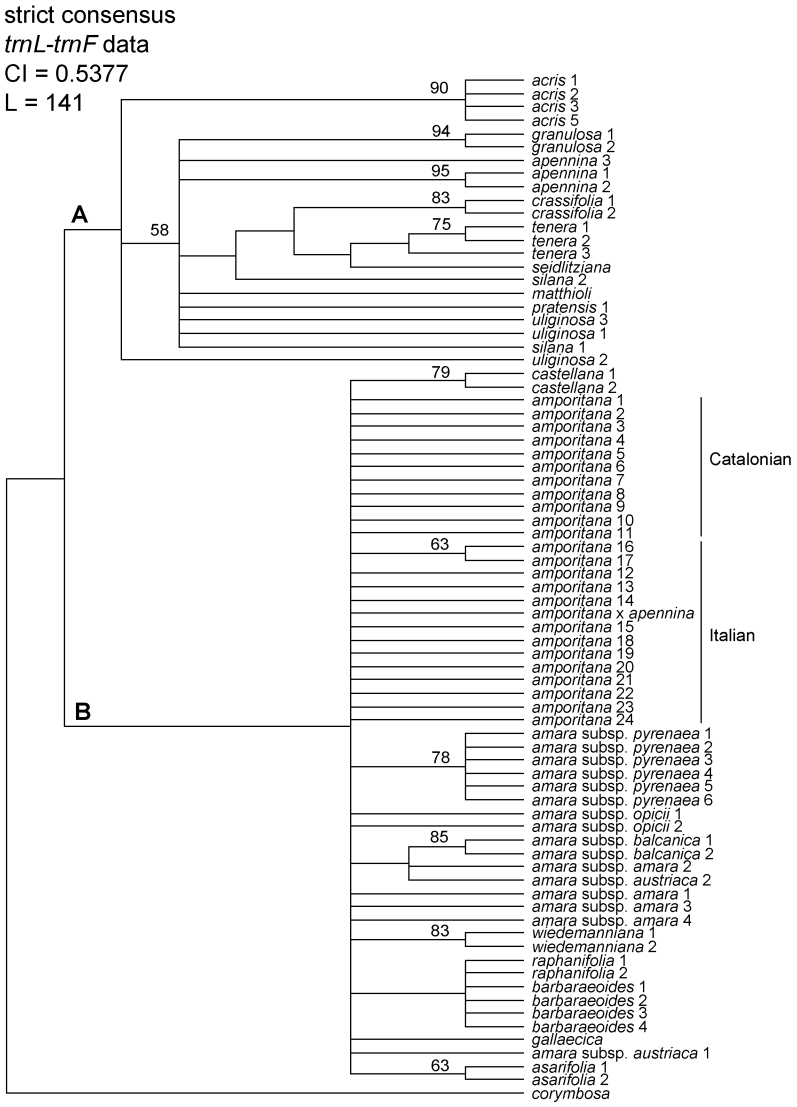

Fig. 3. Strict consensus of 68 201 most parsimonious trees derived from the analysis of the *trnL-trnF* cpDNA intergenic spacer. Bootstrap values above 50% are shown along the branches.

shown). But, in the analysis excluding the hybrid, bootstrap values increased for both main clades A and B. As compared to the topologies of the independent analyses of ITS and *trnL-trnF*, in the combined analysis, accessions of *C. amporitana* were placed in three different positions within clade B, although these were not well supported by bootstrap resampling. All Catalonian and one Italian (with haplotype H2) accessions were in a basal polytomy of the *amara* + *amporitana* subclade (B1); the rest of the Italian accessions (with haplotype H4) except one formed a clade with one *C. amara* subsp. *amara* accession, within subclade B1; a single Italian accession (with haplotype H3) was placed in a clade with *C. amara* subsp. *opicii* within another subclade (B2).

The statistical parsimony analysis of the *trnL-trnF* data yielded a single network including all the 37 haplotypes and containing 10 loops, which represent ambiguities (Fig. 5). The 10 haplotypes found in *C. amara* and *C. amporitana* plus that of *C. asarifolia* are connected by a single branch to the rest, except for the haplotype (H3) shared by the Italian sample of *C. amporitana* and *C. amara* subsp. *opicii*.

DISCUSSION

Origin of tetraploid Cardamine amporitana—In a recent study, we used AFLP fingerprinting to elucidate the taxonomic position and relationships among Eurasian taxa of the *Cardamine amara* group (Lihová et al., 2004a). The AFLP phenotypes recorded in the tetraploid *C. amporitana* were clearly distinct from those of all five subspecies of *C. amara*, as documented by a high number of diagnostic fragments (14 for *C. amporitana* vs. a maximum of three for the different subspecies of *C. amara*). Genetic differences between *C. amporitana* and four diploid subspecies of *C. amara* were larger than those between the latter and tetraploid *C. amara* subsp. *austriaca*, assumed to be of a recent (last interglacial or postglacial) autopolyploid origin (Marhold, 1999; Marhold et al., 2002). In contrast, neither of the sequence data sets presented here (ITS, *trnL-trnF*) provided synapomorphies for *C. amporitana*.

Except for a single individual (putative hybrid), PS in ITS sequences were very scarce in *C. amporitana*. Lack of polymorphisms suggests either a polyploid origin of *C. amporitana*

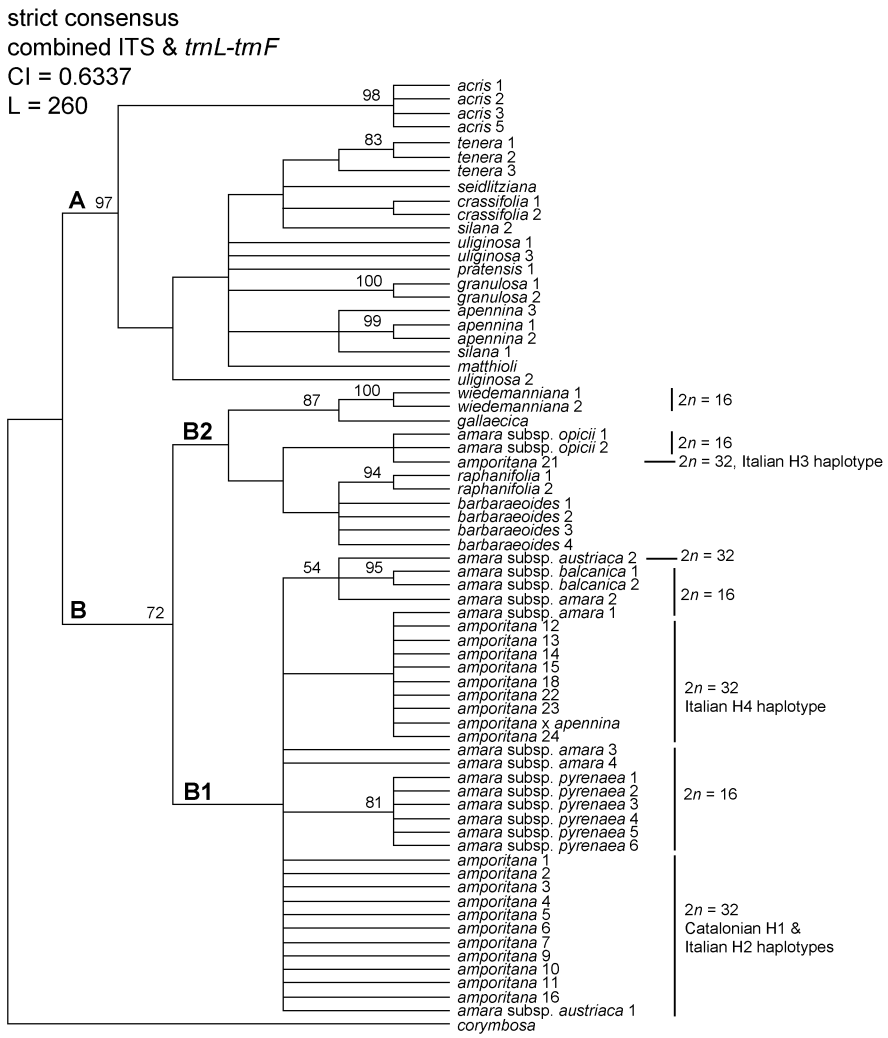

Fig. 4. Strict consensus of 16 628 most parsimonious trees resulting from the combined analysis of ITS and *trnL-trnF* data. Bootstrap values above 50% are shown along the branches. Chromosome numbers and *trnL-trnF* haplotypes for taxa of the *Cardamine amara* group are marked.

within *C. amara* or, alternatively, that concerted evolution has been active enough to erase traces of other possible parents. Data from the *trnL-trnF* chloroplast spacer were largely congruent with ITS sequences; cpDNA haplotypes identified in *C. amporitana* were closest to those of individual subspecies of *C. amara*, separated by a few mutational steps (Figs. 3, 5). Further, the fact that most haplotypes in *C. amara* and *C. amporitana* are monophyletic in the network suggests that they convey phylogenetic signal at the organismic level. The exception, i.e., haplotype H3 shared by a single Italian accession of *C. amporitana* and *C. amara* subsp. *opicii*, may be due either to lack of coalescence or to hybridization.

Although the sequence data obtained here do not allow specific inference of the origin of *C. amporitana* with confidence, based on the available independent evidence (ITS, *trnL-trnF* sequences, AFLPs, and morphology; Lihová et al., 2000, 2004a), the most likely hypothesis is that it originated within *C. amara*. A possible involvement of *C. barbaraeoides* or its diploid progenitor(s) also seems feasible based on morphological resemblances in various organs and on the AFLP data (M. Perný, unpublished data). At present, populations of *C. amporitana* do not overlap with those of *C. barbaraeoides* and *C. amara* (Table 1, Fig. 1). However, the old origin of *C. amporitana* (see next) suggests that current allopatry is not an obstacle.

The current distributional gap between the two areas of *C. amporitana* (Catalonia–central Italy) suggests a long-distance dispersal event. That only one *trnL-trnF* haplotype has been found in Catalonia (vs. three in Italy) and that AFLP variation in this region is a reduced subset of that found in Italy (Lihová et al., 2004a) support this hypothesis. This pattern could reflect a genetic bottleneck effect following a long-distance dispersal event, as discussed in detail by Lihová et al. (2004a). However, as none of the three Italian *trnL-trnF* haplotypes matches the Catalonian, a long-distance dispersal explanation appears to be unlikely, at least a recent one. This is supported by the statistical parsimony network; the haplotype in Catalonian populations (H1) is interior in the network, showing three connections to other haplotypes (Fig. 5). According to coalescence theory (Hudson, 1990; Fu and Li, 1999), this relative placement is consistent with an old haplotype; therefore Catalonian populations do not seem to be likely to have reached their current area by recent immigration from Italy. This raises also the question of the tip position of the Italian haplotype H2 in

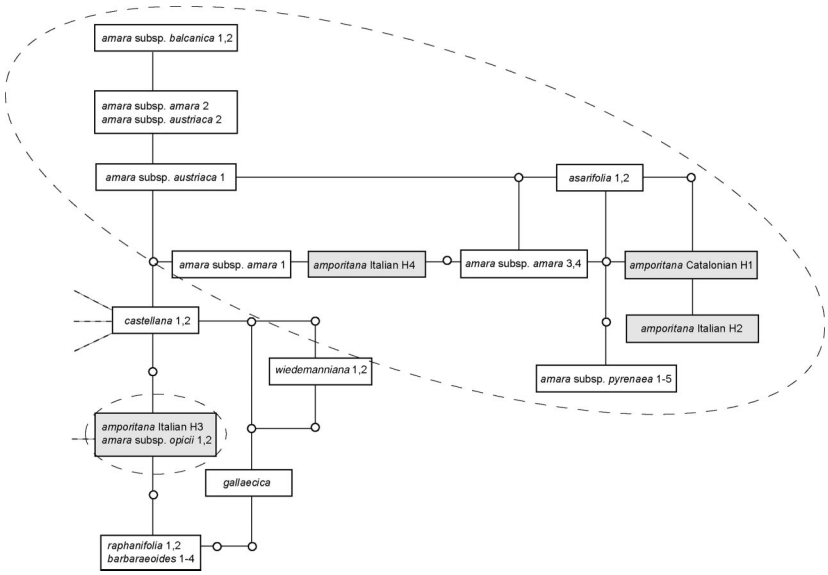

Fig. 5. Statistical parsimony network of haplotypes found in the *Cardamine amara* group (encircled), defined on the basis of chloroplast *trnL-trnF* sequences. Lines represent single mutational steps, small circles are haplotypes not found in any sample. The network presented is part of a larger one constructed with all the *trnL-trnF* sequences, connected to the rest as indicated with dashed lines.

this network. However, we cannot completely exclude the scenario that the Catalonian haplotype (H1) was also in Italy, gave origin to the Italian H2 haplotype, and later disappeared or still exists but has not been detected by our sampling. Therefore, in this context, the long-distance dispersal cannot be disregarded, but the pattern detected allows us to consider also other causes for the disjunction of *C. amporitana*.

Another scenario, the independent formation of the same tetraploid taxon in the two different areas, polytopism, cannot be rejected based on the differences in haplotypes. But morphological homogeneity, the AFLP data (Lihová et al., 2004a), and the lack of ITS differences found here (Table 2), indicate that Italian and Catalonian samples are very close. Therefore, a polytopic hypothesis would require independent selection (convergent evolution) of the same portions of the genomes contributed by the two progenitors, provided that it is an allotetraploid.

A plausible explanation to be discussed here is the disappearance of intermediate populations between the Pyrenees and central Italy as a result of Quaternary climatic oscillations. The geographical position of the Catalonian populations of *C. amporitana* is marginal to the area of the *C. amara* group, which spreads over central, northern and eastern Europe, and extends to Asia (Table 1, Fig. 1). Therefore, it is plausible that Catalonian populations arrived in Iberia from southeastern France, across the Pyrenees. According to this view, the low genetic variability may be a subset of the old range of this taxon, impoverished by isolation. The distributional gap seems to be due to disappearance of *C. amporitana* north of the Pyrenees, a region where Mediterranean vegetation was severely affected by glaciations (Frenzel et al., 1992; Jalut et al., 2002). Another possibility by which geographically intermediate populations, mostly French, might have disappeared is by being swamped by congeners. Currently, in those regions representing the *C. amporitana* gap, two taxa of the group can be found, *C. amara* subsp. *amara* and subsp. *austriaca*. But only the latter is tetraploid, and there is no sign of introgression of *C. amporitana* into *C. amara* subsp. *austriaca*. Still, a third explanation for the current absence of French populations of *C. amporitana* is ecological competition and replacement by diploid populations of *C. amara* subsp. *amara*. This subspecies is currently widespread in southern France in lowlands and has successfully inhabited northern and central European regions (Fig. 1).

Whatever the cause for the disappearance or absence of *C. amporitana* in France, the number of *trnL-trnF* haplotypes (four) as compared to the three subspecies of *C. amara* (subsp. *pyrenaea*, subsp. *austriaca*, and subsp. *balcanica*) supports the hypothesis of an old taxon. Not only the number of haplotypes but also their positions in the network (Fig. 5) are consistent with *C. amporitana* representing a significant sample of ancestral variation in the *C. amara* group. The haplotypes in *C. amporitana* are not isolated from the other members of the group, but connected through nodes that include haplotypes in *C. amara* subsp. *amara* and others (Fig. 5).

Although disjunct distribution patterns are frequent across southern European flora (Thompson, 1999), (southern and central) Italian–Iberian disjunction seems to be rather rare in contrast to, e.g., Italian–Balkan disjunctions (Thompson, 1999; see also Jalas and Suominen, 1994, 1996 for examples from Brassicaceae). Still, in Brassicaceae a similar central Italian–Iberian distribution pattern to the one in *C. amporitana* has been reported for *Jonopsidium savianum* (Caruel) Ball ex Arcang. (Morales Valverde, 1992; Bencivenga et al., 1995; Jalas and Suominen, 1996). Other cases of disjunct distribution between those two peninsulas, however, mostly refer to taxa with northern Italian–Iberian distribution, e.g., *Melampyrum catalaunicum* Freyn (Scrophulariaceae; northwest Italy and northeast Spain) (Pignatti, 1982; Real Jardín Botánico, CSIC, 2003).

Recent hybridization in Cardamine amporitana—The likely case of recent hybridization found in an individual from Monti Sibillini in the Umbria province (locality Pian Grande) is of interest as a counterpoint to the scarcity of PS in ITS from other taxa, particularly polyploids. The joint possession

of the common Italian *trnL-trnF* haplotype (H4) and additivity for numerous ITS nucleotide sites (Table 2) strongly support its hybrid nature from a cross between *C. amporitana* and *C. apennina*, the former acting as plastid donor (maternal plastid transmission occurs in Brassicaceae; Harris and Ingram, 1991). Hybridization involving taxa from the *C. amara* and *C. pratensis* groups, to which these taxa belong, has been considered rare, despite the fact that representatives of both groups are often sympatric (Lövkvist, 1956).

The other reported case of hybridization between representatives of those two groups with available ITS data provided a different pattern (Urbanska et al., 1997; Franzke and Mummenhoff, 1999). Despite being dated not earlier than in the 20th century, ITS sequences have been homogenized both in a triploid hybrid as well as in its autopolyploid derivative (Franzke and Mummenhoff, 1999). In comparison to this case, the individual from Monti Sibillini reported here might represent a very recent hybrid. The tetraploid chromosome number determined for that particular individual, as in all other individuals of *C. amporitana*, indicates that *C. apennina*, being a diploid, probably provided an unreduced male gamete. A wider sampling combined with a strategy of cloning ambiguous amplification products (as, e.g., by Koch et al., 2003) would be a more powerful approach in identifying multiple ITS copies and would help to clarify the origin of the polymorphic sites.

In contrast, the PS detected in two samples of *C. amporitana* showing an additive pattern with respect to *C. asarifolia* (sites 123 and 463 in three and two samples, respectively, Table 2) cannot be conclusively attributed to hybridization, at least a recent one. In each case, only one position was affected by APS, while the differing positions between *C. asarifolia* and *C. amporitana* are 19 (note that Table 2 shows only those sites that are variable within accessions of *C. amporitana*). Although we cannot reject the occurrence of an old hybridization event, whose traces have been partly lost through concerted evolution and allopolyploidy (also discussed earlier), an alternative possibility of independent mutations in those two positions still remains.

Implications for phylogenetic relationships in Cardamine—The gene trees allow some remarks on the phylogenetic relationships within *Cardamine*. Both ITS and *trnL-trnF* sequences provided moderate phylogenetic information, but no major disagreements were found between them, except for the different placement of *C. castellana* and *C. asarifolia*. *Cardamine asarifolia*, represented by two accessions from a single locality, appeared in the *trnL-trnF* haplotype network intermingled with *C. amara* and *C. amporitana* (Fig. 5). The marked difference between the topological placement of the ITS and *trnL-trnF* sequences of *C. asarifolia* has not a straightforward explanation (Figs. 2, 3). This is a hexaploid taxon that on morphological grounds has never been related to the *C. amara* group. A recent chloroplast capture is one possible explanation, supported by the fact that *C. asarifolia* and *C. amara* subsp. *amara* co-occur at several localities, have similar habitat requirements, and hybridization between these two taxa is assumed (Bongini, 1916). But allopolyploid evolution of *C. asarifolia* with a taxon from the *C. amara* group acting as a maternal parent and homogenization of ITS sequences towards strongly divergent variants of another parent might also be responsible for the pattern detected.

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