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WORLDWIDE PHYLOGENY AND BIOGEOGRAPHY OF *CARDAMINE FLEXUOSA* (BRASSICACEAE) AND ITS RELATIVES[1]

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Phylogenetic relationships, biogeography, and taxonomy of a group of taxa putatively related to the tetraploid *Cardamine flexuosa* were explored using sequences of the internal transcribed spacer region of nrDNA (ITS) and the *trnL-trnF* region of cpDNA. Taxon sampling focused on eastern Asia, North America, and Europe, and included 19 taxa represented by 177 and 182 accessions for each data set, respectively. Our analyses provided unequivocal evidence that Asian weedy populations traditionally assigned to *C. flexuosa* form an independent evolutionary lineage and represent a distinct taxon from European *C. flexuosa*. The allopolyploid origin of this common weed in paddy fields, its origin, and/or spread associated with the establishment of suitable man-made habitats are suggested. It is also found as an introduced weed in Australia and North America. Phylogenetic relationships and the associated taxonomic implications are presented and discussed for the group as a whole. Contrasting patterns of genetic variation (particularly in cpDNA) among different species were revealed. While very little haplotype diversity was found in widespread *C. hirsuta* and *C. flexuosa*, greater variation, showing phylogeographic structure, was observed in the tetraploid *C. scutata* within a relatively small area of Japan.

Key words: Brassicaceae; cpDNA; Cruciferae; DNA sequencing; nrDNA ITS; phylogeography; polyploidy.

Species number is not evenly distributed across genera within plant families, and some genera contain several hundred species often reflecting complex evolutionary processes. *Cardamine* L. is one of the largest genera of the Brassicaceae, comprising at least 200 species distributed worldwide. This number of species is far beyond the average number of species per genus within this family that consists of more than 3700 species in 338 currently recognized genera (Al-Shehbaz and Beilstein, 2006). The genus shows great morphological and karyological diversity and complex evolutionary history strongly affected by both historical and more recent reticulation events (reviewed by Lihová and Marhold, 2006), providing an opportunity to study mechanisms of plant diversification. Major centers of diversity, assessed by species richness and endemism, occur in the Far East and the Himalayas, with about 70 *Cardamine* taxa reported (Al-Shehbaz, 1988). Several species have been reported to have spread as weeds beyond their natural ranges following introduction to distant areas and even different continents. Apart from the European species, most of which have been thoroughly investigated taxonomically (Lihová and Marhold, 2006), the taxonomy of species from other continents, e.g., South America (Sjöstedt, 1975), Australia, and New Zealand (Hewson, 1982; Webb et al., 1988) or eastern Asia (Ohwi, 1984; Zhou et al., 2001), is very complex and remains in many cases controversial and unresolved.

Several molecular phylogenetic studies of *Cardamine* species have been published in recent years (Franzke et al., 1998; Sweeney and Price, 2000; Bleeker et al., 2002a; Lihová et al., 2004). The traditional sectional classification proposed by Schulz (1903) apparently does not reflect phylogenetic relationships within the genus. Most sections analyzed so far in molecular studies have been shown to be polyphyletic. The evolutionary history of *Cardamine* is complicated by widespread reticulation and polyploidization, making the reconstruction of an overall phylogeny challenging. Disentangling reticulate from divergent relationships requires use of multiple independent markers with different modes of inheritance (Doyle, 1992; Linder and Rieseberg, 2004; Vriesendorp and Bakker, 2005). Investigation of the additivity of molecular markers, their (in)congruence, and additional data such as morphology, chromosome numbers, and geographic distribution are often crucial (Vriesendorp and Bakker, 2005). Exclusion of putative hybrids or allopolyploids from phylogenetic analyses to construct an initial diploid phylogeny can provide a useful starting point for subsequent analysis of polyploid origins (Bachmann, 2000; Marhold et al., 2004), but

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TABLE 1. List of the analyzed *Cardamine* taxa, including most common synonyms, ploidy levels, and distribution ranges (those with asterisks refer to the non-native occurrence). Ploidy levels without superscript were previously published, and the references are available in the database of chromosome numbers of the genus *Cardamine* (Kučera et al., 2005). The name for the Asian populations traditionally assigned to *C. flexuosa* needs to be designated; these populations are informally named as 'Asian *C. flexuosa*'.

Taxon name, synonyms	Ploidy level	Distribution range
C. flexuosa With.	$2n = 4x$	Europe, Australia*, New Zealand*, Africa*, America*, southern Africa*
'Asian *C. flexuosa*'	$2n = 8x$[a]	eastern Asia, Australia*, North America*
C. hirsuta L.	$2n = 2x$	Europe, western Asia, eastern Asia*, Australia*, New Zealand*, America*, Africa*
C. oligosperma Nutt. = *C. oligosperma* var. *oligosperma* = *C. hirsuta* subsp. *oligosperma* (Nutt.) O. E. Schulz	$2n = 2x$	western North America
C. umbellata Greene = *C. oligosperma* var. *kamtschatica* (Regel) Detling = *C. oligosperma* subsp. *kamtschatica* (Regel) Cody = *C. hirsuta* subsp. *kamtschatica* (Regel) O. E. Schulz	$2n = 4x$, $6x$	amphi-Beringian (northwestern North America, Alaska, Aleutian islands, Kamtschatka, Kuriles)
C. pensylvanica Willd. = *C. flexuosa* subsp. *pensylvanica* (Willd.) O. E. Schulz	$2n = 4x$ (?$8x$)	North America
C. fallax (O. E. Schulz) Nakai = *C. flexuosa* subsp. *fallax* O. E. Schulz = *C. flexuosa* var. *fallax* (O. E. Schulz) Nakai = *C. scutata* subsp. *fallax* (O. E. Schulz) H. Hara	$2n = 6x$[b]	eastern Asia (Japan, China, Korea)
C. scutata Thunb. = *C. regeliana* Miq. = *C. flexuosa* subsp. *regeliana* (Miq.) O. E. Schulz	$2n = 4x$	eastern Asia (Japan, Korea, China, eastern Russia)
C. niigatensis H. Hara	$2n = 4x$[b]	Japan (Honshu)
C. dentipetala Matsum.	$2n = ?$	Japan (Honshu)
C. longifructus Ohwi = *C. dentipetala* var. *longifructus* (Ohwi) Hiyama	$2n = ?$	Japan (Honshu, Shikoku, ?Kyushu)
C. parviflora L. var. *parviflora*	$2n = 2x$	Eurasia
C. parviflora var. *arenicola* (Britton) O. E. Schulz = *C. arenicola* Britton	$2n = 2x$	North America
C. impatiens L.	$2n = 2x$	Eurasia, North America*, southern Africa*
C. pectinata Pall. ex DC. = *C. impatiens* subsp. *pectinata* (Pall. ex DC.) Stoj. & Stef.	$2n = 2x$	Balkan, Turkey, Caucasus, Iran
C. corymbosa Hook. f.	$2n = 6x$	Australia, Great Britain*, North America*
C. paucijuga Turcz.	$2n = ?$	Australia
C. debilis DC.	$2n = ?$	New Zealand
C. amara L. subsp. *amara*	$2n = 2x$	Eurasia

[a] Unpublished records by our research group, by K. Watanabe (Kobe University, Japan), and B. Lövkvist (unpublished data deposited in the herbarium of the University of Uppsala, Sweden).

[b] J. Lihová and K. Marhold, preliminary unpublished data.

in genera with a large number of polyploids this approach has limited application (Vriesendorp and Bakker, 2005).

This study focuses on a cosmopolitan group of taxa that are putatively related to two Eurasian taxa, tetraploid *C. flexuosa* With. and diploid *C. hirsuta* L. (see Table 1). Much controversy surrounds the taxonomy of these species, and little is known about their phylogenetic relationships. The group is characterized by overall morphological similarity (e.g., pinnate or pinnatisect leaves; small, white flowers) and includes annual or perennial herbs, most of which are predominantly autogamous, but outcrossing occurs as well. Diploids to high polyploids are represented (Table 1).

Cardamine flexuosa was originally described from Great Britain (Withering, 1796), and there are no doubts about its circumscription within Europe, except for occasional misidentifications as *C. hirsuta*. *Cardamine flexuosa* is a tetraploid species and is widely distributed in Europe west of Belorussia and Ukraine, with a few localities in the western part of Russia (Jalas and Suominen, 1994). It has also been reported as native to northern Africa (Ouyahya, 1999) and southeastern Asia (Ohwi, 1984; Zhou et al., 2001; Pak, 2005) as well as introduced to other continents (Hewson, 1982; Webb et al., 1988; Rollins, 1993), although doubts remain about the identity and relationships of populations outside Europe (Marhold, 1995; Kudoh et al., 2006). The polyploid origin of *C. flexuosa* has been a challenging problem for several decades. Initially, an autotetraploid origin from diploid *C. hirsuta* was suggested (Banach, 1950), but later an allopolyploid origin with *C. hirsuta* and *C. impatiens* L. as parents has been favored, based essentially on morphology and geography (Ellis and Jones, 1969).

Cardamine scutata Thunb. was described from Japan (Thunberg, 1794), without a precise locality. This, together with difficulties in the interpretation of the type specimen (deposited at UPS), probably led some Japanese authors to adopt the later name *C. regeliana* Miq. (Hara, 1952; Kitagawa, 1982) for this taxon. This latter name has also been widely used in Russia (e.g., Busch, 1915; Berkutenko, 1988). The illustration referred to in the protologue of *C. regeliana* (Regel, 1862: Table 5, Figs. 1, 2) leaves no doubt that notwithstanding the problems of nomenclature, *C. regeliana* represents the same taxon as *C. scutata*. Several other taxa, apparently related to *C. scutata*, were described from Japan and still are considered to be endemic to that country, namely *C. niigatensis* H. Hara, *C. dentipetala* Matsum., and *C. longifructus* Ohwi (Hara, 1983; Ohwi, 1984; Fukuoka and Kurosaki, 1992).

Cardamine hirsuta is a nearly cosmopolitan diploid species. However, the precise native range remains a mystery because it occurs as a weed throughout the world including Europe (its most likely area of origin). *Cardamine parviflora* L. occurs in Eurasia (Jalas and Suominen, 1994; Zhou et al., 2001) and northern Africa (Almeida, 1973). It is diploid and grows in moist open habitats of various kinds. Similar populations from sandy, open moist soils along the Atlantic coast of North America were described as a separate species, *C. arenicola* Britton (Britton, 1892). Rollins (1993) recently treated all North American populations as *C. parviflora* var. *arenicola* (Britton) O. E. Schulz as different than var. *parviflora* in other parts of the distribution area of this species. *Cardamine impatiens*, one of the putative parental species of *C. flexuosa*, is a widespread Eurasian diploid (Khatri, 1989; Jalas and Suominen, 1994). A morphologically similar species with a more restricted distribution, *C. pectinata* Pall. ex DC., has been sometimes treated as conspecific with the former, but our investigations confirmed that it is distinct (Kučera et al., 2006). Finally, several highly variable and taxonomically confusing taxa, namely *C. debilis* DC., *C. corymbosa* Hook. f., *C. paucijuga* Turcz., which seem to be related to the *C. flexuosa* group, have been reported from Australia and New Zealand (Hewson, 1982; Webb et al., 1988). Previous molecular phylogenetic analyses of southern hemisphere *Cardamine* species have shed some light on the relationships of these species as closely related to South American *C. glacialis* (G. Forst.) DC. (Bleeker et al., 2002a).

Here we aim to resolve phylogenetic relationships and address both biogeographic and taxonomic questions within the *C. flexuosa* group using sequence data from the 5.8S subunit and flanking internal transcribed spacers ITS1 and ITS2 of nrDNA, and the *trnL-trnF* region of cpDNA. Specifically we focus on the morphologically variable populations assigned to *C. flexuosa* from different areas and continents, which often behave as serious and invasive weeds, as well as assess genetic diversity patterns among the studied taxa across geographic areas.

MATERIALS AND METHODS

Taxon sampling—Altogether 19 *Cardamine* taxa were sampled (Table 1), with a focus on *C. flexuosa, C. hirsuta*, and other taxa, which have been associated with either of these two species, or considered to be closely related based on morphology or previous DNA sequence data (Franzke et al., 1998; Bleeker et al., 2002a). Our preliminary screening of phylogenetic relationships in the genus (J. Lihová, K. Marhold, and K. K. Shimizu [University of Zürich, Switzerland], unpublished data) suggested that the Eurasian diploid *C. amara* is also closely related to *C. flexuosa* justifying its inclusion despite its distinct morphology. Most taxa were represented by multiple accessions with 177 ITS sequences and 182 *trnL-trnF* sequences in total, representing accessions from all continents, but with the main focus on Europe, North America, and eastern Asia. Material was either field-collected or obtained from herbarium specimens at MO, UBC, PERTH, and HEID (acronyms follow Holmgren et al., 1990). Voucher specimens and GenBank accession numbers are listed in the Appendix. In addition to the new sequences, a few previously published sequences of selected southern hemisphere species, *C. corymbosa, C. debilis, C. paucijuga*, and *C. glacialis* (Bleeker et al., 2002a), were included in the analyses (Appendix). Representatives of most closely related genera *Barbarea* R. Br. and *Rorippa* Scop. (Franzke et al., 1998) were used as outgroups.

DNA extraction, PCR amplification, and sequencing—Total genomic DNA was isolated from silica-gel-dried or herbarium material using a modified CTAB extraction method with either isopropanol or ethanol with sodium acetate used for nucleic acids precipitation (Doyle and Doyle, 1987). PCR amplification of the target regions, ITS of nrDNA, chloroplast *trnL* intron and *trnL-trnF* intergenic spacer, was performed using the universal primers P1A, P4 by Francisco-Ortega et al. (1999) and the primers e, f, c, d by Taberlet et al. (1991). The PCR conditions were as described in Lihová et al. (2004). For problematic herbarium material, a "touch-down" PCR approach was applied with annealing temperature starting at 65°C and each cycle decreasing by 0.5°C. Amplification products were purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Freiburg, Germany) following the manufacturer's protocols. Sequencing was performed using the original PCR primers at Heidelberg Institute of Plant Science, Germany and the Max-Planck Institute for Chemical Ecology in Jena, Germany. At the former, sequencing reactions and post-reaction cleanups were performed using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences), and the sequences were resolved on a MegaBACE 500 DNA Analysis System (Amersham Biosciences). In the latter, cycle sequencing was performed using the TaqDyeDeoxy Terminator Cycle Sequencing Kit (ABI Applied Biosystems, Darmstadt, Germany), and products were analyzed on an ABI 3730XL DNA Analyser automated sequencer.

ITS electropherograms were carefully inspected for the occurrence of double or overlapping peaks in the same position that might suggest presence of different ITS copy types within individuals (see e.g., Rauscher et al., 2002; Nieto Feliner et al., 2004).

Phylogenetic analyses—Sequences were aligned manually using BioEdit (version 5.0.9; Hall, 1999). The *trnL* intron and *trnL-trnF* spacer sequences were first aligned separately and then combined for phylogenetic analyses. In both these alignments, indels of 4 bp or longer were scored as additional gap characters (three indels in the *trnL* alignment and six in the *trnL-trnF*). One complex indel of variable length across accessions was coded as a four-state character. Otherwise gaps were coded as binary characters (see e.g., Simmons and Ochoterena, 2000). Due to multiple *trnF* gene duplications and subsequent pseudogene formation (Koch et al., 2005), the *trnL-trnF* sequences varied considerably in length. Multiple overlapping gaps at the 3′ end of the spacer were identified after alignment. These ambiguous regions were eliminated from the alignment, and only 335 aligned positions from the 5′ end downstream were used in the phylogenetic analysis.

Both data sets (ITS, *trnL+trnL-trnF*; Appendices S1, S2, see Supplemental Data accompanying online version of this article) were subjected to a heuristic maximum likelihood phylogenetic analysis (ML), as well as a Bayesian inference based on a Markov chain Monte Carlo algorithm (MCMC; Huelsenbeck and Ronquist, 2001). Prior to the ML analyses, accessions with identical sequences were detected and merged using MacClade 4.0 PPC (Maddison and Maddison, 2000) to reduce computation time. Models of nucleotide substitutions and the gamma distribution shape parameter were selected using MODELTEST 3.6 (Posada and Crandall, 1998) in conjuction with PAUP*, version 4.0b10 (Swofford, 2001). The Akaike information criterion (AIC) was used to determine the model that best fits the data. The ML analyses were run in PAUP* using the resulting model settings and a neighbor-joining tree based on the logdet distance as a starting tree. Heuristic searches were performed with 10 random addition replicates, TBR (tree-bisection-reconnection) branch swapping, and a reduced scope of tree rearrangements using the ReconLimit option set to 10.

In the Bayesian analysis (MrBayes, version 3.0beta4) four chains were run for four to ten millions generations, adjusting the temperature difference between the cold and heated chains to achieve efficient swaps between the chains. Six substitution rates (nst = 6) and a gamma distribution (rates = gamma) with (for cpDNA data) or without (for ITS data) invariable sites were assumed. The trees were sampled every 100 generations, and finally, a majority-rule consensus tree was computed excluding the trees found in the burn-in phase (i.e., those generated before the likelihood values reached a plateau and fluctuated within a stable range, this was assessed individually for each run). The node's posterior probability indicated on the consensus tree is estimated by examining the proportion of trees containing each node. At least three independent Bayesian analyses were run for each data set, and the topologies of the consensus trees were compared. Except for minor differences in posterior probability values for some nodes, the topology was stabilized, indicating that the results are robust. Posterior probabilities shown here are values averaged across independent analyses.

For the cpDNA sequences also, a haplotype network was constructed using the parsimony method of Templeton et al. (1992), implemented in TCS 1.13 program (Clement et al., 2000). The 95% probability limit of parsimonious connections was applied to produce the network. The analysis was run with gaps coded as missing data, but with an additional nine characters reflecting indel structure.

RESULTS

***ITS region**—Sequence characteristics*—Sequencing of the ITS region was in most taxa, including European tetraploid *C. flexuosa*, straightforward, resulting in completely homogenized sequences or with only a few intra-individual single-nucleotide polymorphisms. Conversely, in numerous accessions of Asian *C. flexuosa*, *C. dentipetala*, and *C. longifructus* high numbers (up to 16 per accession) of polymorphic sites were observed, suggesting presence of divergent ITS copies. Comparison of the individual ITS sequences retrieved from Asian *C. flexuosa* revealed a complex pattern (Table 2). Altogether 25 positions where at least one accession displayed an intra-individual polymorphism were identified. In such positions, either both nucleotides were observed simultaneously (i.e., intra-individual polymorphism) or only one of them, depending on the accession. This may imply differential partial homogenization of originally divergent ITS copies within this taxon, although bias caused by preferential amplification or quantitative masking of minority ITS variant(s) cannot be excluded (Rauscher et al., 2002). Similar sequence variation has been found in introduced plants in Australia and North America. Most of the intra-individual polymorphisms in Asian *C. flexuosa* displayed an additive pattern, with alternative nucleotides found in Asian taxa *C. fallax* and *C. scutata* (Table 2). This suggests an allopolyploid origin of Asian *C. flexuosa*, although future studies are needed to address this issue in more details.

Given that the abundance of intra-individual polymorphic sites and especially those with additive patterns, has a negative impact on the tree resolution and prolongs computing time, we assembled two ITS alignments. One alignment included all 177 ingroup sequences (complete ITS data set). In the second alignment 22 accessions with numerous polymorphic sites were excluded (reduced ITS data set). Both alignments were 634 bp long, the complete ITS data set had 165 variable positions (128 informative and 37 autapomorphic), and the restricted one had 164 variable positions (127 informative and 37 autapomorphic).

Phylogenetic analyses—The optimal likelihood model for the ITS data set was SYM + G. To reduce computing load of the maximum likelihood analysis, this was run only on the reduced ITS data set, where, in addition, identical sequences were merged. The load was still too high to complete the search. We present here only the consensus tree produced by the Bayesian inference (Fig. 1).

Diploids were resolved in three well-supported clades comprising clade I, Eurasian *C. impatiens* and *C. pectinata*; clade II, Eurasian *C. parviflora* var. *parviflora* being sister to North American *C. parviflora* var. *arenicola*; and clade III, North American *C. oligosperma* and European *C. hirsuta*, again in sister positions (Fig. 1). All the polyploids (plus diploid *C. amara*) were placed in clade IV. Polyploids from New Zealand (*C. debilis, C. corymbosa*) and amphi-Beringian *C. umbellata* grouped together (IVa), being apparently very closely related to each other. *Cardamine amara* was placed in the basal position of the large subclade (IVb) comprising all the other polyploids from Europe, Asia, and North America. The relationships among these were poorly resolved, with some groupings suggested (most of them kept only in the tree based on the reduced ITS data set, see Fig. 1), which generally received only low posterior probability values. Accessions of the Asian *C. flexuosa* were placed either among those of *C. fallax* or *C. scutata*. This is consistent with ITS variation patterns resolved in these taxa as shown in Table 2.

***trnL-trnF* region**—The combined *trnL* intron and *trnL-trnF* intergenic spacer alignment of 182 accessions comprised 853 characters plus nine indel characters, of which 89 were variable (79 informative, 10 autapomorphic). Of 182 accessions, 87 different sequences were identified by MacClade. For the maximum likelihood analysis only the reduced data matrix (of 87 sequences) was used.

Based on the Akaike information criterion, the model K81uf + I + G was selected for this data set. The maximum likelihood analysis produced an ML tree with −ln likelihood of 1951.26 (not shown). Its topology fully corresponded to that of the Bayesian consensus tree (Fig. 2), with the only exception of the placement of two accessions of *C. pensylvanica* as indicated on the tree. Nevertheless, in the Bayesian tree they were placed in a clade that received only a low posterior probability value. Four main groups of haplotypes can be delimited on the tree, corresponding to four isolated (disconnected) haplotype networks produced by the parsimony method of Templeton et al. (1992) with the 95% probability connection limit (not shown). The groups comprised clade I, Eurasian diploids *C. impatiens* and *C. pectinata*; clade II, diploids *C. hirsuta* and *C. oligosperma*; clade III, European tetraploid *C. flexuosa* and diploid *C. amara*; and clade IV, remaining taxa, including the Asian *C. flexuosa*. Highly divergent levels of haplotype diversity were observed among the analyzed taxa. Very low diversity was found in the weedy cosmopolitan *C. hirsuta* with only two haplotypes identified among 42 different accessions; in *C. oligosperma* with two haplotypes differentiated from *C. hirsuta* by four or five mutations, respectively; and in 17 accessions of *C. flexuosa* with only three haplotypes found in Europe and two of them in populations apparently introduced to North America. Among the remaining taxa forming clade IV, a number of closely related haplotypes were resolved (Fig. 3). A few mutations differentiated a group of related haplotypes found in taxa from New Zealand; *C. corymbosa* and *C. debilis, C. glacialis* from South America; and amphi-Beringian *C. umbellata*. This is congruent with the ITS data, for which these taxa were also placed in a single clade. All 29 accessions of the Asian *C. flexuosa* (including accessions representing introduced weedy populations in Australia and North America), Asian *C. fallax*, and Australian *C. paucijuga* shared a single haplotype. In contrast, greater haplotype variability was observed in *C. parviflora,* North American *C. pensylvanica*, and Asian *C. scutata* and its related species. In the latter two, geographic pattern was also apparent. Four different haplotypes were identified in seven accessions of *C. pensylvanica*, two western haplotypes (haplotype a: British Columbia; b: Washington, Oregon), one eastern (d: New York), and one southern haplotype (c: Arkansas). In *C. scutata* and its related Japanese endemics *C. niigatensis, C. dentipetala,* and *C. longifructus*, altogether 12 haplotypes were observed, in which two groups of northern (A–E) and southern (F–J) haplotypes

Taxon/ accession	Alignment position																								
	24	36	42	66	76	89	91	99	117	118	123	233	248	250	265	461	462	535	566	584	587	602	605	606	610
Asian *flexuosa*																									
JP11	C	C	G	G	T	T	G	C	T	C	T	T	T	C	G	A	C	C	T	T	C	T	A	A	?
JP5, AUS5	Y	Y	K	G	T	C	G	Y	C	Y	C	Y	Y	C	G	T	Y	Y	W	Y	C	Y	A	A	Y
JP27	Y	Y	K	G	T	C	G	Y	C	C	C	Y	Y	C	G	T	Y	Y	W	Y	C	Y	A	A	T
JP13	C	C	K	G	T	Y	G	Y	Y	C	Y	T	Y	C	G	W	Y	Y	W	Y	C	Y	A	A	C
JP18	C	Y	K	G	T	Y	G	Y	Y	C	Y	Y	Y	Y	G	W	Y	Y	W	Y	C	Y	A	A	Y
JP1, JP28, CHI2, VIE	C	T	T	G	T	C	G	T	C	C	C	T	C	C	G	T	T	T	A	C	C	C	A	A	T
CHI11	C	Y	K	G	T	C	G	Y	C	C	C	Y	Y	C	R	T	Y	Y	W	Y	C	Y	W	A	Y
CHI4	C	Y	K	G	T	C	?	Y	C	C	C	Y	Y	C	R	T	Y	Y	W	Y	C	Y	A	A	Y
CHI10	C	Y	K	R	T	C	K	Y	C	C	C	Y	Y	C	R	T	T	Y	W	Y	C	Y	W	W	Y
CHI6	C	Y	K	R	T	C	K	Y	C	C	C	Y	Y	C	A	T	Y	Y	W	C	C	C	A	A	Y
CHI1	C	Y	K	R	T	C	K	Y	C	C	C	Y	Y	C	R	T	Y	Y	W	Y	Y	Y	W	W	Y
CHI13	C	Y	K	G	T	Y	G	Y	Y	Y	Y	T	Y	C	G	W	Y	Y	W	Y	C	C	A	A	Y
THA2	Y	Y	K	G	T	C	G	Y	C	Y	C	Y	Y	C	G	T	Y	Y	W	Y	C	Y	A	A	Y
THA1	C	Y	K	G	T	C	K	Y	C	C	C	Y	Y	C	R	T	Y	Y	W	Y	C	Y	W	A	Y
TAI2	Y	Y	K	G	T	C	G	Y	C	Y	C	Y	Y	C	G	T	Y	Y	W	Y	C	Y	A	A	Y
TAI3, CAN3-CAN5	C	Y	K	G	T	Y	G	Y	Y	C	Y	Y	Y	C	G	W	Y	Y	W	Y	C	Y	A	A	Y
US33-US35	C	C	G	G	A	C	T	C	C	C	C	C	T	C	A	T	Y	C	T	T	C	T	T	A	C
US8	C	C	G	G	T	T	G	C	T	C	T	C	T	T	G	A	C	C	T	T	C	T	A	A	C
MEX	C	C	K	G	W	C	K	C	C	C	C	C	T	C	A	T	C	C	T	T	C	Y	T	A	C
AUS9	Y	Y	K	G	T	C	G	Y	C	Y	C	C	Y	C	G	T	Y	Y	W	Y	C	Y	A	A	C
flexuosa, Europe	C	C	G	G	T	C	G	C	C	C	C	C	T	C	G	T	C	C	T	C	C	T	A	A	C
fallax	C	T	T	G	T	C	G	T	C	C	C	T	C	C	G	T	T	T	A	C	C	C	A	A	T
scutata	T,C	C	G	G	T	T,C	G	C	T,C	C	T,C	C	T	C	G	A,T	T,C	C	T	T	C	T	A	A	C
niigatensis	C	C	G	G	T	T,C	G	C	C	C	C	C	T	C	G	T	C	C	T	T	C,T, Y	T	A	A	C
longifructus	T,Y	C	G	G	T	T,Y	G	C	C,Y	C	C,Y	C	T	C	G	W,T	C	C	A,W	A,W	C	C,Y	A	A	T,Y
dentipetala	T	C	G	G	T	T,Y	G	C	C	C	C	C	T	C	G	T	C	C	W	W	C	Y	A	A	T,Y
pensylvanica	C	C	G	G	T	C	G	C	C	C	C,T	C	T	C	G	T	C	C	T	C	C	T	A	A	C
parviflora	T	C	G	G	T	C	G	C	C	C	C	C	C	C	G	T	T	C	A	C	C	C	A	A	C
arenicola	T	C	G	G	T	C	G	C	C	C	C	C	C	C	G	T	T	C	A	C	C	C	A	A	C
hirsuta	C	C	G	G	T	C	G	C	C	C	T,C, Y	C	T	C	G	T	C	T	T	*A*	C	C	A	T	C
oligosperma	C	C	G	G	T	C	G	C	C	C	T	C	T	C	G	T	C	T	T	*A*	C	C	A	T	C
impatiens	T	C	G	G	T	C	*A*	T,C	C	C	C	C	T	C	A	T	T	C	T	C	C	C	A	A	C
pectinata	*A*	C	G	G	T	C	*A*	C	C	C	C	C	T	T	*T*	T	T	C	T	C	C	C	A	*C*	C
umbellata	C	C	G	G	T	C	G	T	C	C	C	C	T	C	G	T	T	T	A	C	C	C	A	A	T
paucijuga	C	T	T	G	T	C	G	C	C	C	C	C	T	C	G	T	T	C	T	C	C	C	A	A	C
debilis, *corymbosa*	C	C	G	G	T	C	G	C	C	C	C	C	T	C	G	T	T	C	T	C	C	C	A	A	C
amara	C	C	G	G	T	C	G	C	C	C	C	C	T	C	G	T	T	T	T	C	C	C	A	A	T

can be delimited, respectively, with the overlap in central Honshu where the main haplotype diversity is found (Fig. 3). One widespread haplotype (K), present across the whole area of Japan, was identified.

DISCUSSION

Phylogeny reconstruction in the studied Cardamine taxa—Our phylogenetic study comprising several putatively closely related diploid and polyploid *Cardamine* species incorporated both maternally inherited cpDNA markers being essential for teasing apart maternal vs paternal contributions to polyploid species, as well as biparentally inherited nuclear DNA data. The nrDNA region has been widely employed in similar studies, although its multicopy constitution and molecular evolutionary processes that may impact ITS sequence variation should be considered (Baldwin et al., 1995; Álvarez and Wendel, 2003). In the present study, different patterns of nrDNA sequence evolution in polyploids have been suggested. While *C. scutata, C. niigatensis, C. fallax, C. pensylvanica*, and European *C. flexuosa* displayed largely uniform ITS sequences, significant ITS sequence heterogeneity was found in Asian *C. flexuosa, C. longifructus*, and *C. dentipetala* (Table 2). This may indicate different modes of polyploid evolution (auto- or allopolyploidization) or different ages of polyploid origins, but molecular genetic processes that impact on nrDNA evolution often proceed in an unpredictable way, and, in addition, this was based only on examination of direct ITS sequences. The ability to detect multiple ITS types is strongly dependent on their copy number in the genome, and thus rare repeat types can remain undetected (Rauscher et al., 2002).

Several approaches have been adopted to reconstruct patterns of reticulate evolution, which are apparently present in the studied genus, and they are currently thoroughly reviewed and discussed (see e.g., Linder and Rieseberg, 2004; Hegarty and Hiscock, 2005; Vriesendorp and Bakker, 2005). The importance of the use of multiple independent markers and additional data (e.g., morphology, geography, cytogenetics) for reconstructing relationships among potentially reticulating species has been stressed, as well as the need of comparative sequencing of single-copy nuclear genes. The present study provides a phylogenetic framework for taxa poorly explored so far, and as such it serves as a basis for future detailed studies, which should address proposed reticulations and focus on individual polyploids and their origins. Recently, the allopolyploid origin of European hexaploid *C. asarifolia* has been resolved, first based on and invoked from strong incongruence between cpDNA and ITS gene trees of European taxa (Lihová et al., 2004). Employment of a single-copy nuclear gene has proven useful to address this issue (Lihová et al., 2006) and should be followed in other polyploids of the genus as well.

Comparison of ITS and cpDNA phylogenies in the present study identified areas of both congruence and incongruence. Generally, diploid taxa were well resolved and supported, while low genetic differentiation was apparent among several polyploids (although in ITS this may be due to different patterns of ITS sequence evolution and homogenization). The most striking conflict between the two data sets is the placement of diploid *C. parviflora* (including var. *parviflora* and var. *arenicola*). Although in the ITS tree, it was placed among other diploids, its cpDNA haplotypes were close to Asian and North American polyploids (Figs. 1, 3). We sequenced several accessions of both the Eurasian and American varieties from different parts of their distributions, so a single hybridization event or an artefact due to sparse sampling can be excluded. Even when considering that the effective population size of maternally inherited cpDNA markers is smaller than that of nuclear markers, the *trnL-trnF* region of cpDNA has been reported to evolve more slowly than ITS and may retain traces of ancestral polymorphisms. To a certain extent, this may explain the ambiguities (loops) in the haplotype relationships, with several taxa bearing non-monophyletic haplotypes (*C. scutata*, *C. pensylvanica*). However, the incongruence might also be connected with the polyploid origins of Asian and North American species, which possess related haplotypes. *Cardamine parviflora* (including both varieties) is widely distributed, covering the whole of Eurasia and North America. Considering this, its ecology (weedy behavior, invading open habitats free of competition), and common reticulate evolution in the genus, this species might have contributed to the origin of several geographically disjunct polyploids. Such a scenario would explain the close relationship of *C. parviflora* cpDNA haplotypes to Asian and American polyploids. A somewhat less striking, but still an apparent discrepancy concerns European *C. flexuosa*, probably reflecting its allopolyploid evolution (see the next subsection).

Cardamine flexuosa*—Populations from Europe and Asia represent two distinct lineages of different polyploid origins*—*Cardamine flexuosa* in Europe is a morphologically well-defined tetraploid, growing usually in mesic natural to seminatural habitats (e.g., along forest roads). Its distribution area in Europe does not extend far into Asia (Jalas and Suominen, 1994). Populations assigned to this species have, however, also been reported from southeastern Asia (see introduction). There has been no consensus as to whether these Asian populations can be considered native or introduced, but their weedy behavior has been often mentioned alongside their great morphological variability and phenotypic plasticity (Ishiguri et al., 1994; Kudoh et al. 1995, 1996). Populations in Japan typically occur in cultivated areas, such as paddy fields, moist gardens, and orchards but not in natural habitats (Kimata, 1983; Kudoh et al., 1993; Yatsu et al., 2003). In fact, they represent a serious and dominant weed there. Maekawa (1944) pointed to the possibility of its historic (archaeophytic) introduction together with the rice cultivation.

Our analyses provided unequivocal evidence that Asian

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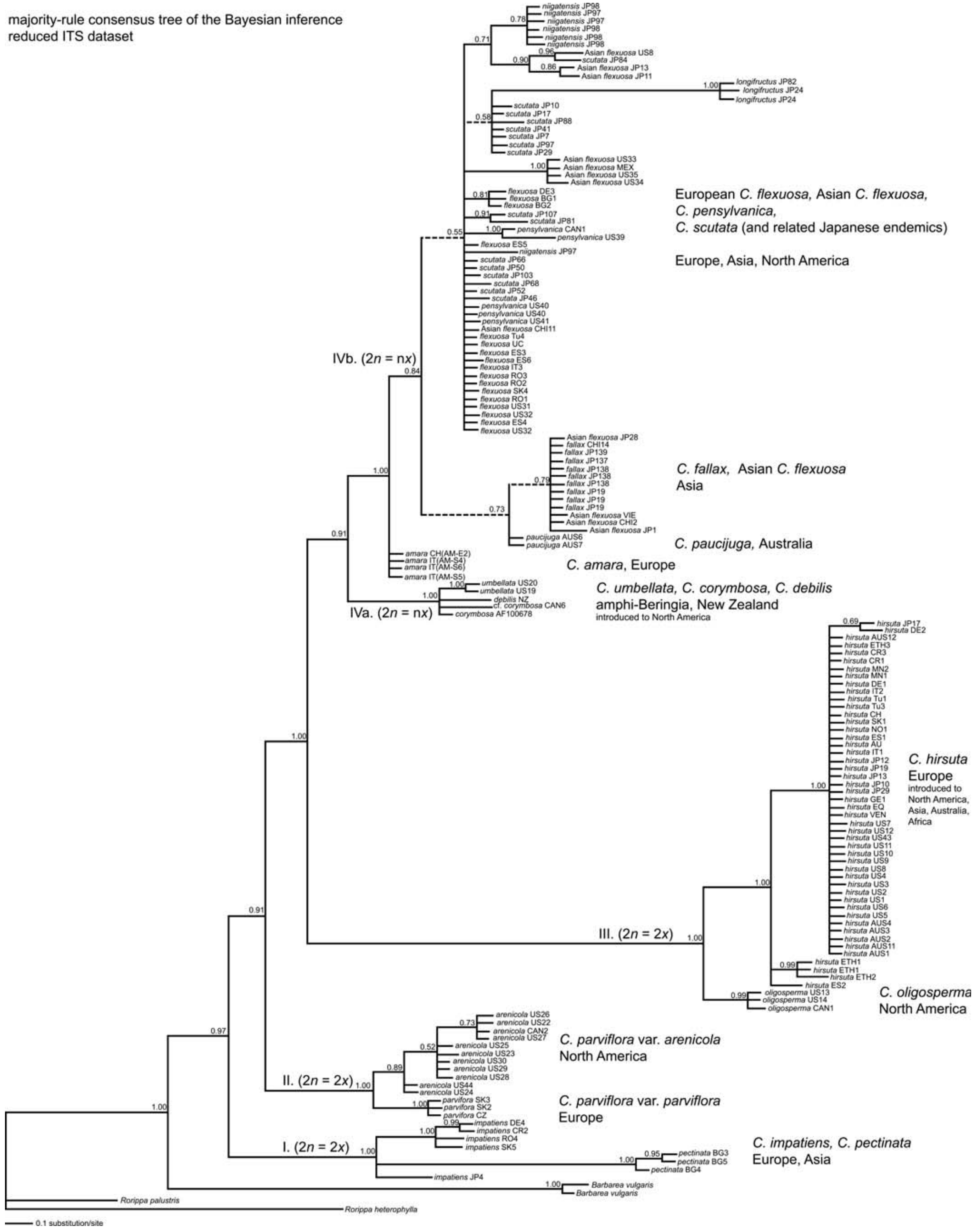
Table 2. Nucleotide site variation of the ITS region (ITS1-5.8S-ITS2) in the Asian *Cardamine flexuosa*, compared with other taxa. Denotation of accessions follows the Appendix and Figs. 1 and 2. (JP = Japan, CH = China, VIE = Vietnam, THA = Thailand, CAN = Canada, US = USA, MEX = Mexico, and AUS = Australia). Grey background highlights positions with both nucleotides (i.e., intra-individual variation with IUPAC ambiguity codes), alternative nucleotides are marked in the white and black backgrounds. Another (third) nucleotide, if present, is in italic. IUPAC ambiguity codes: Y = T/C, K = G/T, W = A/T, R = A/G.


majority-rule consensus tree of the Bayesian inference
reduced ITS dataset
niigatensis JP98
niigatensis JP97
niigatensis JP97
niigatensis JP98
niigatensis JP98
niigatensis JP98
0.78
0.71
0.96
Asian flexuosa US8
scutata JP84
0.90
0.86
Asian flexuosa JP13
Asian flexuosa JP11
1.00
longifructus JP82
longifructus JP24
longifructus JP24
scutata JP10
scutata JP17
0.58
scutata JP88
scutata JP41
scutata JP7
scutata JP97
scutata JP29
1.00
Asian flexuosa US33
Asian flexuosa MEX
Asian flexuosa US35
Asian flexuosa US34
0.81
flexuosa DE3
flexuosa BG1
flexuosa BG2
0.91
scutata JP107
scutata JP81
0.55
1.00
pensylvanica CAN1
pensylvanica US39
flexuosa ES5
niigatensis JP97
scutata JP66
scutata JP50
scutata JP103
scutata JP68
scutata JP52
scutata JP46
pensylvanica US40
pensylvanica US40
pensylvanica US41
Asian flexuosa CHI11
flexuosa Tu4
flexuosa UC
flexuosa ES3
flexuosa ES6
flexuosa IT3
flexuosa RO3
flexuosa RO2
flexuosa SK4
flexuosa RO1
flexuosa US31
flexuosa US32
flexuosa ES4
flexuosa US32
European C. flexuosa, Asian C. flexuosa,
C. pensylvanica,
C. scutata (and related Japanese endemics)
Europe, Asia, North America
IVb. (2n = nx)
0.84
Asian flexuosa JP28
fallax CHI14
fallax JP139
fallax JP137
fallax JP138
fallax JP138
0.79
fallax JP138
fallax JP19
fallax JP19
fallax JP19
Asian flexuosa VIE
Asian flexuosa CHI2
Asian flexuosa JP1
C. fallax, Asian C. flexuosa
Asia
1.00
0.73
paucijuga AUS6
paucijuga AUS7
C. paucijuga, Australia
0.91
amara CH(AM-E2)
amara IT(AM-S4)
amara IT(AM-S6)
amara IT(AM-S5)
C. amara, Europe
1.00
umbellata US20
umbellata US19
1.00
debilis NZ
cf. corymbosa CAN6
corymbosa AF100678
IVa. (2n = nx)
C. umbellata, C. corymbosa, C. debilis
amphi-Beringia, New Zealand
introduced to North America
0.69
hirsuta JP17
hirsuta DE2
hirsuta AUS12
hirsuta ETH3
hirsuta CR3
hirsuta CR1
hirsuta MN2
hirsuta MN1
hirsuta DE1
hirsuta IT2
hirsuta Tu1
hirsuta Tu3
hirsuta CH
hirsuta SK1
hirsuta NO1
hirsuta ES1
hirsuta AU
hirsuta IT1
hirsuta JP12
hirsuta JP19
hirsuta JP13
1.00
hirsuta JP10
hirsuta JP29
hirsuta GE1
hirsuta EQ
hirsuta VEN
hirsuta US7
hirsuta US12
hirsuta US43
hirsuta US11
hirsuta US10
hirsuta US9
hirsuta US8
hirsuta US4
hirsuta US3
hirsuta US2
hirsuta US1
hirsuta US6
hirsuta US5
hirsuta AUS4
hirsuta AUS3
hirsuta AUS2
hirsuta AUS11
hirsuta AUS1
C. hirsuta
Europe
introduced to
North America,
Asia, Australia,
Africa
1.00
1.00
1.00
III. (2n = 2x)
1.00
0.99
hirsuta ETH1
hirsuta ETH1
hirsuta ETH2
hirsuta ES2
0.99
oligosperma US13
oligosperma US14
oligosperma CAN1
C. oligosperma
North America
0.91
0.73
arenicola US26
arenicola US22
arenicola CAN2
arenicola US27
0.52
arenicola US25
arenicola US23
arenicola US30
arenicola US29
arenicola US28
0.89
arenicola US44
arenicola US24
C. parviflora var. arenicola
North America
0.97
II. (2n = 2x)
1.00
1.00
parvifora SK3
parvifora SK2
parvifora CZ
C. parviflora var. parviflora
Europe
1.00
0.99
impatiens DE4
impatiens CR2
1.00
impatiens RO4
impatiens SK5
I. (2n = 2x)
1.00
1.00
0.95
pectinata BG3
pectinata BG5
pectinata BG4
C. impatiens, C. pectinata
Europe, Asia
impatiens JP4
1.00
Barbarea vulgaris
Barbarea vulgaris
Rorippa palustris
Rorippa heterophylla
0.1 substitution/site

populations traditionally treated as *C. flexuosa* represent an independent lineage that should be treated as a distinct taxon. We sequenced accessions from Japan, mainland China, Taiwan, Thailand, and Vietnam, and all of them displayed a single cpDNA haplotype that was strongly divergent from the haplotypes found in *C. flexuosa* from Europe. Apart from apparently different ecology of *C. flexuosa* in Europe, Asian populations also seem to be high polyploids. Preliminary investigations have suggested an octoploid level, in contrast to the tetraploid one in Europe (Table 1). However, morphological differentiation of Asian *C. flexuosa* is complicated by the enormous variability present in Asian populations. European *C. flexuosa* has a tendency toward having smaller flowers and fewer basal branches compared with Asian plants, which often do not retain basal leaf rosettes and differ in stem hairness, but the variation overlaps between both groups of plants. More detailed morphometric investigations are needed.

Molecular data shed some light on the possible polyploid origin of Asian *C. flexuosa*. In most of the Asian accessions that were analyzed, multiple ITS copy types were inferred from intra-individual polymorphisms that aligned to alternative nucleotides across the taxa sampled (Table 2). This revealed an additive pattern for most polymorphic nucleotide sites, indicating that the Asian polyploids, hexaploid *C. fallax*, and tetraploid *C. scutata,* or their diploid progenitors may have been involved in the polyploid origin of Asian *C. flexuosa*. The cpDNA gene tree also supports *C. fallax* as the putative maternal parent, because it shares its haplotype with the Asian *C. flexuosa*. Preliminary results from single-copy gene investigations (K. K. Shimizu, University of Zürich, unpublished data) are in congruence with this proposed pattern. Although both *C. fallax* and *C. scutata* are polyploids, there is no evidence to date as to their diploid progenitors. *Cardamine parviflora* and *C. amara* are two closely related widespread species meriting consideration, but we have no data on other potential diploids from Asia. Asia harbors an enormous diversity of *Cardamine* species, which are very poorly known. In Japan, most of them are polyploids, but we are not aware of any chromosome count for *Cardamine* from mainland China (Kučera et al., 2005). It is difficult to ascertain the exact place of its origin, whether in Japan, China (rice was introduced from China to Japan), or other Asian countries, but it is probable that its polyploid evolution, establishment, and spread are associated with the establishment of suitable man-made habitats. Examples of other polyploid taxa that are successful colonizers of man-made habitats are already known both from *Cardamine* (*C. schulzii*, Urbanska-Worytkiewicz, 1977) and the related *Rorippa* (*R. palustris* and *R. sylvestris*, Bleeker et al., 2002b). Indeed, hybridization and polyploidy have been proposed as important mechanisms prompting the evolution of invasiveness more generally (Ellstrand and Schierenbeck, 2000).

Given that *C. flexuosa* was described from Europe and that the illustrations cited in the protologue (in the absence of the extant original herbarium material) are based on and correspond to *C. flexuosa* in the European sense, this name is applicable only to the European populations. Throughout this paper, we have referred to the Asian populations informally as Asian *C. flexuosa*, but a search for the correct name for these Asian plants will be required.

Cardamine flexuosa has also been sporadically reported from other continents as an introduced alien species (e.g., from America: Al-Shehbaz, 1988; Australia: Hewson, 1982; southern Africa: Dreyer and Jordaan, 2000) usually as a weed in flowerbeds, flowerpots, gardens, greenhouses, and lawns. Here we have shown that these weedy populations include both European and Asian *C. flexuosa*. Several accessions of both taxa have been discovered and sequenced across North America (Appendix, Table 2). The name *C. debilis* Don (later homonym of here studied *C. debilis* DC.) has been applied in North America to an introduced weed (Rollins, 1993) and apparently refers to Asian *C. flexuosa*. These populations have been often identified as *C. flexuosa* or misidentified and confused with *C. parviflora*. In Australia, another taxon of unknown origin and taxonomic position has been referred to as *C.* aff. *flexuosa* (Thompson, 1996). Material of this was sequenced by Franzke et al. (1998), and this sequence clearly matches those of Asian *C. flexuosa*. Alongside the Australian accession by Franzke et al. (1998), we analyzed two other Australian samples from herbarium specimens, one determined as *C. flexuosa* and another misidentified as *C. paucijuga*. Both corresponded to the Asian *C. flexuosa* (Appendix).

Several previous attempts have been made to explain the polyploid origin of *C. flexuosa* in Europe (Banach, 1950; Ellis and Jones, 1969). Molecular phylogenetic analyses by Franzke et al. (1998) did not show high support for *C. impatiens* and *C. hirsuta* as parental species, proposed by Ellis and Jones (1969). Nevertheless, the hypothesized increased sequence evolution in *C. hirsuta* (see the next subsection) might have led to the current distant position of this species, and under this hypothesis this pattern does not exclude it from further considerations as a putative parental species. The origin of *C. flexuosa*, however, remains intriguing. Our cpDNA gene tree indicates close affinities to the Eurasian diploid *C. amara*, while in the ITS gene tree it is placed among other polyploids. *Cardamine amara* is thus the most likely maternal parent, but the paternal parent remains unknown.

Taxonomic and phylogenetic positions of other Cardamine taxa, introductions to non-native areas—*Cardamine hirsuta* is an annual or rarely biennial weed occurring in ruderal habitats across all continents except Antarctica. Its origin and native range are assumed to be in Europe, but the eastern limit in western parts of Asia is not clear. From central Asia, it is

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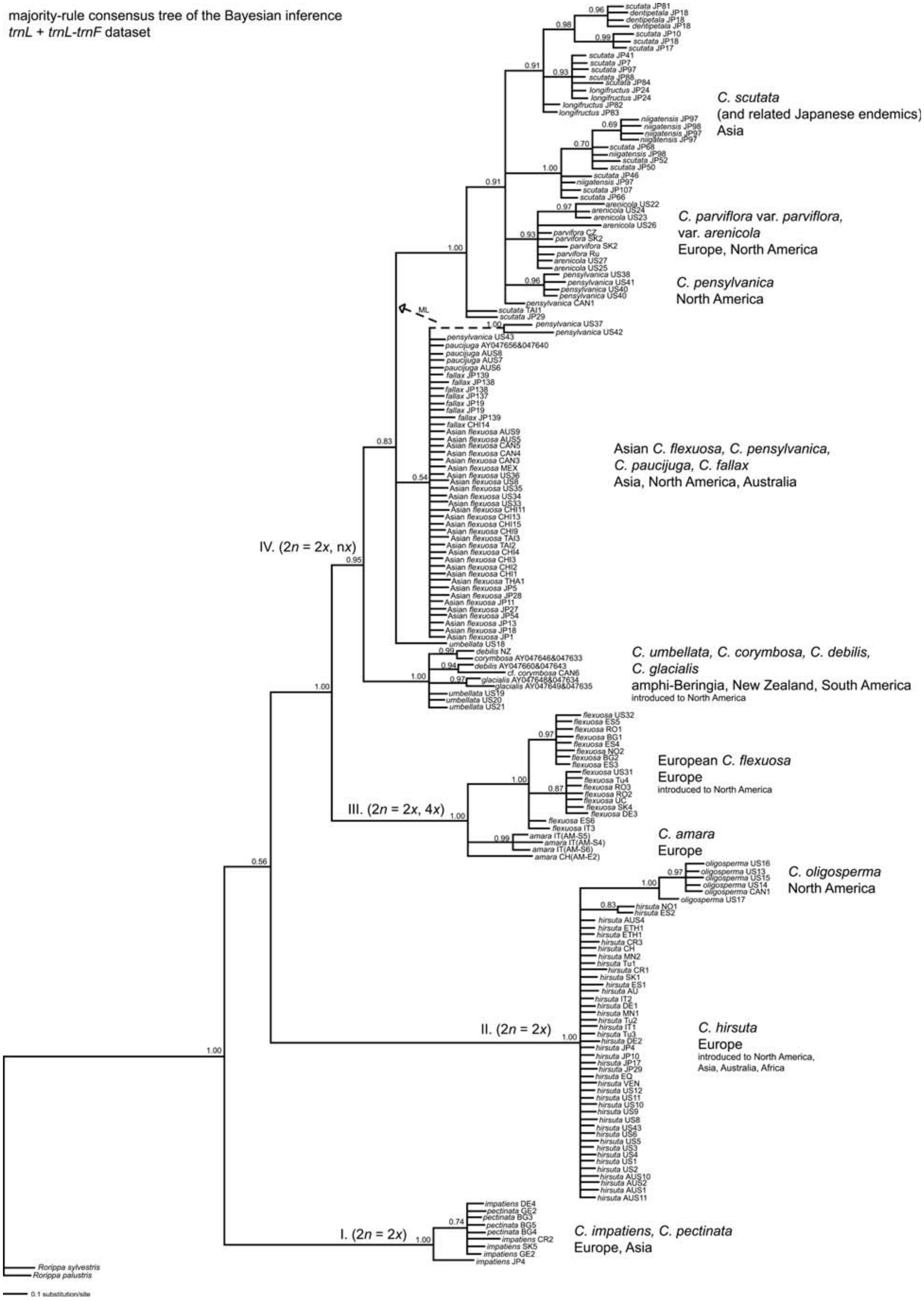
←

Fig. 1. Majority-rule consensus tree of the Bayesian inference based on the reduced data set (excluding accessions with numerous intra-individual polymorphisms) of nrDNA ITS sequences of *Cardamine* taxa. Posterior probability values (averaged across three independent runs) of the nodes are indicated above the branches. *Rorippa palustris* (X98639), *R. heterophylla* (X98638), and *Barbarea vulgaris* (AJ232915, AJ232914) were used as outgroups. Clades are denoted as discussed in the text, including indication of the ploidy level (2*x* stands for diploids, n*x* for polyploids). Distribution ranges are given for material used in this analysis. Dashed lines indicate branches that collapsed in the tree based on the complete ITS data set. Accessions codes follow the Appendix and indicate accession origins: AU = Austria, DE = Germany, CH = Switzerland, NO = Norway, IT = Italy, ES = Spain, CZ = Czech Republic, SK = Slovak Republic, CR = Croatia, MN = Monte Negro, RO = Romania; BG = Bulgaria, Tu = Turkey, UC = Ukraine, Ru = Russia, GE = Georgia, US = USA, CAN = Canada, MEX = Mexico, EQ = Equador, VEN = Venezuela, TAI = Taiwan, CHI = China, THA = Thailand, JP = Japan, AUS = Australia, NZ = New Zealand, and ETH = Ethiopia.


majority-rule consensus tree of the Bayesian inference
trnL + trnL-trnF dataset
scutata JP81
dentipetala JP18
dentipetala JP18
dentipetala JP18
scutata JP10
scutata JP18
scutata JP17
scutata JP41
scutata JP7
scutata JP97
scutata JP88
scutata JP84
longifructus JP24
longifructus JP24
longifructus JP82
longifructus JP83
niigatensis JP97
niigatensis JP98
niigatensis JP97
niigatensis JP97
scutata JP68
niigatensis JP98
scutata JP52
scutata JP50
scutata JP46
niigatensis JP97
scutata JP107
scutata JP66
arenicola US22
arenicola US24
arenicola US23
arenicola US26
parvifora CZ
parvifora SK2
parvifora SK2
parvifora Ru
arenicola US27
arenicola US25
pensylvanica US38
pensylvanica US41
pensylvanica US40
pensylvanica US40
pensylvanica CAN1
scutata TAI1
scutata JP29
pensylvanica US37
pensylvanica US42
pensylvanica US43
paucijuga AY047656&047640
paucijuga AUS8
paucijuga AUS7
paucijuga AUS6
fallax JP139
fallax JP138
fallax JP138
fallax JP137
fallax JP19
fallax JP19
fallax JP139
fallax CHI14
Asian flexuosa AUS9
Asian flexuosa AUS5
Asian flexuosa CAN5
Asian flexuosa CAN4
Asian flexuosa CAN3
Asian flexuosa MEX
Asian flexuosa US36
Asian flexuosa US8
Asian flexuosa US35
Asian flexuosa US34
Asian flexuosa US33
Asian flexuosa CHI11
Asian flexuosa CHI13
Asian flexuosa CHI15
Asian flexuosa CHI9
Asian flexuosa TAI3
Asian flexuosa TAI2
Asian flexuosa CHI4
Asian flexuosa CHI3
Asian flexuosa CHI2
Asian flexuosa CHI1
Asian flexuosa THA1
Asian flexuosa JP5
Asian flexuosa JP28
Asian flexuosa JP11
Asian flexuosa JP27
Asian flexuosa JP54
Asian flexuosa JP13
Asian flexuosa JP18
Asian flexuosa JP1
umbellata US18
debilis NZ
corymbosa AY047646&047633
debilis AY047660&047643
cf. corymbosa CAN6
glacialis AY047648&047634
glacialis AY047649&047635
umbellata US19
umbellata US20
umbellata US21
flexuosa US32
flexuosa ES5
flexuosa RO1
flexuosa BG1
flexuosa ES4
flexuosa NO2
flexuosa BG2
flexuosa ES3
flexuosa US31
flexuosa Tu4
flexuosa RO3
flexuosa RO2
flexuosa UC
flexuosa SK4
flexuosa DE3
flexuosa ES6
flexuosa IT3
amara IT(AM-S5)
amara IT(AM-S4)
amara IT(AM-S6)
amara CH(AM-E2)
oligosperma US16
oligosperma US13
oligosperma US15
oligosperma US14
oligosperma CAN1
oligosperma US17
hirsuta NO1
hirsuta ES2
hirsuta AUS4
hirsuta ETH1
hirsuta ETH1
hirsuta CR3
hirsuta CH
hirsuta MN2
hirsuta Tu1
hirsuta CR1
hirsuta SK1
hirsuta ES1
hirsuta AU
hirsuta IT2
hirsuta DE1
hirsuta MN1
hirsuta Tu2
hirsuta IT1
hirsuta Tu3
hirsuta DE2
hirsuta JP4
hirsuta JP10
hirsuta JP17
hirsuta JP29
hirsuta EQ
hirsuta VEN
hirsuta US12
hirsuta US11
hirsuta US10
hirsuta US9
hirsuta US8
hirsuta US43
hirsuta US6
hirsuta US5
hirsuta US3
hirsuta US4
hirsuta US1
hirsuta US2
hirsuta AUS10
hirsuta AUS2
hirsuta AUS1
hirsuta AUS11
impatiens DE4
pectinata GE2
pectinata BG3
pectinata BG5
pectinata BG4
impatiens CR2
impatiens SK5
impatiens GE2
impatiens JP4
Rorippa sylvestris
Rorippa palustris
0.1 substitution/site
0.96
0.98
0.99
0.91
0.93
0.69
0.70
1.00
0.91
0.97
0.93
0.96
1.00
ML
1.00
0.83
0.54
0.95
0.99
0.94
1.00
0.97
1.00
0.97
1.00
0.87
1.00
0.99
0.56
1.00
0.97
0.83
1.00
1.00
0.74
1.00
IV. (2n = 2x, nx)
III. (2n = 2x, 4x)
II. (2n = 2x)
I. (2n = 2x)
C. scutata
(and related Japanese endemics)
Asia
C. parviflora var. parviflora,
var. arenicola
Europe, North America
C. pensylvanica
North America
Asian C. flexuosa, C. pensylvanica,
C. paucijuga, C. fallax
Asia, North America, Australia
C. umbellata, C. corymbosa, C. debilis,
C. glacialis
amphi-Beringia, New Zealand, South America
introduced to North America
European C. flexuosa
Europe
introduced to North America
C. amara
Europe
C. oligosperma
North America
C. hirsuta
Europe
introduced to North America,
Asia, Australia, Africa
C. impatiens, C. pectinata
Europe, Asia

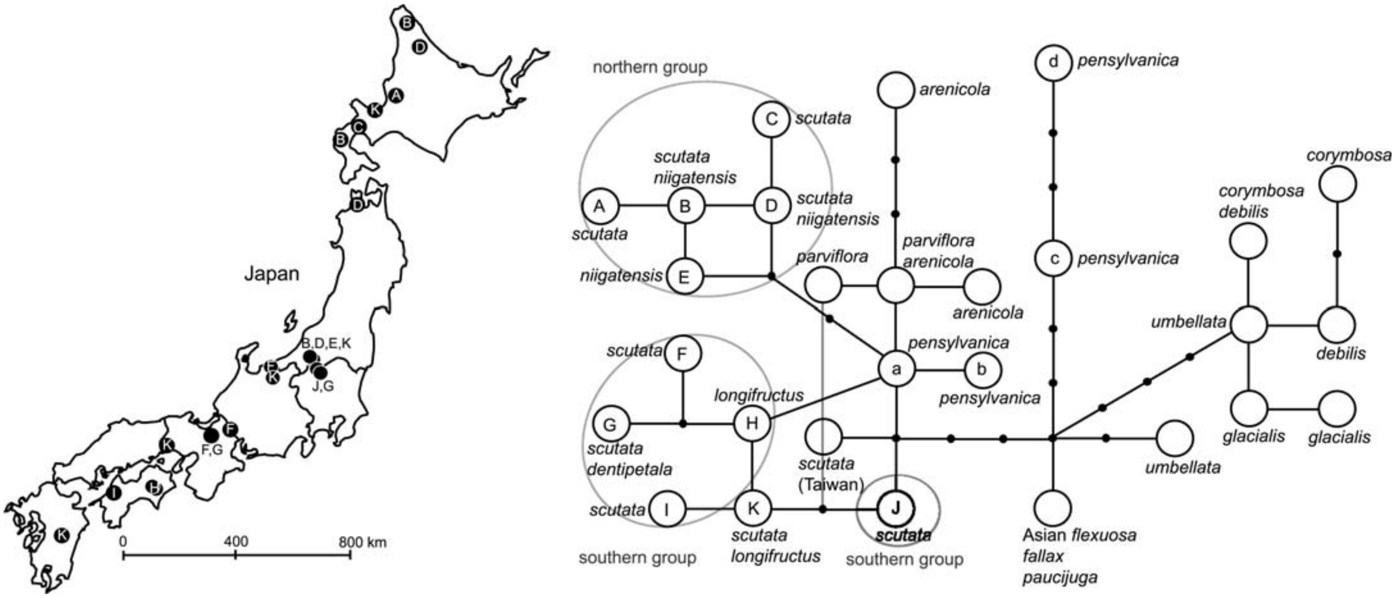


Fig. 3. Parsimony network of haplotypes of clade IV (see Fig. 2) defined on the basis of *trnL* and *trnL-F* sequences. Lines are single mutational steps; small black circles indicate haplotypes not found in any accession. Geographic distribution of haplotypes found in closely related taxa *Cardamine scutata, C. niigatensis, C. dentipetala*, and *C. longifructus* in the area of Japan is shown (haplotypes A–K). Four haplotypes identified in North American *C. pensylvanica* are related to the geographic origin of its accessions: haplotype a, British Columbia; haplotype b, Washington and Oregon; haplotype c, Arkansas; haplotype d, New York.

reported as an adventive weed in fields (Khatri, 1989), and there are no records at all from Siberia (Doron'kin, 1994), Russian Far East (Berkutenko, 1988), or Korea (Pak, 2005). In Japan, it has been recorded only in the last few decades. This species was probably not introduced to Japan before the 1950s (Kudoh et al., 1992), but now it is spreading rapidly across Honshu and Hokkaido (Kudoh et al., 2006; Yatsu et al., 2003). Just as in Japan, *C. hirsuta* has spread and naturalized in other parts of the world. It has been reported as an introduced weed in America, Africa, Australia, and New Zealand (Hewson, 1982; Jonsell, 1982; Webb et al., 1988; Rollins, 1993), growing on disturbed sites such as lawns, pavement cracks, roadsides, or ditches. In Australia, it has been sometimes confused with the native *C. paucijuga*; several accessions from Australian herbarium material sequenced have been originally determined as *C. paucijuga* (Appendix). Although *C. hirsuta* has also been reported from China (Zhou et al., 2001), we have not seen any Chinese herbarium material that corresponds to this species. Specimens determined as *C. hirsuta* from this country, as well as the illustration of *C. hirsuta* in *Flora of China* (Cheo et al., 1987; Zhou et al., 2003) are different from European *C. hirsuta*, and some at least can be referred to as Asian *C. flexuosa*. Very low genetic variation was found in *C. hirsuta* in both ITS and cpDNA data. Despite our extensive sampling, lack of variation precluded identification of the areas of its original occurrence or detailed tracking of its spread and introduction to other continents. More variable markers such as AFLPs (amplified fragment length polymorphism) will be needed to address these questions.

Cardamine oligosperma and *C. umbellata* were considered to be very close to or even conspecific with *C. hirsuta*. *Cardamine hirsuta* and *C. oligosperma* are both diploid annuals or biennials, and based on our survey of herbarium material from MO, they have been often confused and misidentified. *Cardamine oligosperma* was described from Oregon (Torrey and Gray, 1838) and seems to be confined to western North America. We consulted the type specimen at NY to interpret this name correctly and conclude that *C. oligosperma* is indeed closely related to, but clearly distinct and genetically differentiated from *C. hirsuta*. The long branch supporting *C. hirsuta* and *C. oligosperma* (Figs. 1, 2) reflects the high number of synapomorphic substitutions shared between them. This pattern has been observed (for *C. hirsuta*) previously by Franzke et al. (1998), who suggested a faster rate of molecular evolution in this species. The disjunct distributions of these two species (setting aside current contact following introduction of *C. hirsuta* to North America) are consistent with allopatric speciation following fragmentation of a previously widespread ancestor. Alternatively, migration to North America from Eurasia via Beringia could also explain this distribution. As for the amphi-Beringian species *C. umbellata*, in both the cpDNA and ITS gene trees it was

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Fig. 2. Majority-rule consensus tree of the Bayesian inference based on the combined *trnL* and *trnL-trnF* sequence data of *Cardamine* taxa. Posterior probability values (averaged across three independent runs) of the nodes are indicated above the branches. *Rorippa palustris* (AF079351, AF362669) and *R. sylvestris* (AF079352, AF0362644) were used as outgroups. Clades are denoted as discussed in the text, including indication of the ploidy level (2*x* stands for diploids, n*x* for polyploids). Distribution ranges are given for material used in this analysis. Accessions codes follow the Appendix and indicate accession origins (see legend of Fig. 1). The dashed line and arrow indicate the different placement of two accessions of *C. pensylvanica*, as resolved in the maximum likelihood tree (ML).

placed in a distinct lineage more closely related to taxa from New Zealand than to *C. oligosperma* or *C. hirsuta*. Although *C. hirsuta* or *C. oligosperma* might have been involved in its polyploid origin, current genetic differentiation of the polyploid is apparent. Analyses of other nuclear markers may provide more insights into the polyploid origin and evolution of that group including *C. umbellata, C. debilis*, and *C. corymbosa*.

Cardamine pensylvanica is a morphologically variable tetraploid species native to North America and probably one of the most widespread *Cardamine* species on that continent (Al-Shehbaz, 1988). Nuclear and cpDNA data indicated different phylogenetic patterns, but did not elucidate its polyploid origin. Two groups of cpDNA haplotypes separated by several mutations are inferred for this species perhaps reflecting variation of its progenitor(s) and/or multiple independent origins. Two haplotypes were very close to the haplotypes of diploid *C. parviflora* and Asian polyploids, while the remaining ones were differentiated from any other species. In the ITS gene tree, on the other hand, we did not find differentiation between *C. pensylvanica* and European *C. flexuosa*.

In recent literature, populations of *C. parviflora* from North America have been treated as distinct from Eurasian ones and classified either as a separate species *C. arenicola* or as *C. parviflora* var. *arenicola* (Al-Shehbaz, 1988). In the absence of detailed morphometric studies, the morphological distinction between Eurasian and North American populations seems to be problematic and limited to differences in the leaf shape and some quantitative characters (Al-Shehbaz, 1988; Rollins, 1993). Our analyses showed them to be closely related with overlap in cpDNA haplotypes favoring infraspecific treatment in line with the almost continuous northern hemisphere distribution of *C. parviflora* (s.l.) apart from minor gaps in Yukon, Alaska (Warwick et al., 2005) and northeast Asia (Berkutenko, 1988).

Tetraploid *C. scutata* and the Japanese endemics *C. niigatensis, C. longifructus*, and *C. dentipetala* are clearly closely related based on morphology. So far, knowledge of these taxa is very limited; *C. scutata* and *C. niigatensis* are tetraploids, but there are no data on the ploidy of the other two taxa. The distinction of *C. niigatensis,* with its conspicuous leaf morphology, has not been questioned, but field observations on the locality where both *C. scutata* and *C. niigatensis* co-occur, suggest almost continuous morphological variation between these two species. Recognition of *C. dentipetala* and *C. longifructus* as separate taxa, on the other hand, has not been accepted in the recent *Flora of China* (Zhou et al., 2001), nor in the forthcoming *Flora of Japan* (I. A. Al-Shehbaz, Missouri Botanical Garden, St. Louis, USA, personal communication) with both names placed in synonymy under *C. scutata*. Final decisions about the taxonomic status of these endemics must await more detailed analyses, including thorough morphological and karyological investigation, as well as molecular studies using more variable markers.

Populations corresponding to *C. fallax*, which occur in Japan, Korea, and sporadically also in China (in spite of the fact that this taxon is not recognized in the most recent *Flora of China* by Zhou et al., 2001; K. Marhold, J. Lihová, H. Kudoh, and I. A. Al-Shehbaz [Missouri Botanical Garden], unpublished data) show similarities to Asian *C. flexuosa* in morphology and adaptation to moist habitats, but differs in leaf morphology, growth form, and habitat preferences to more natural sites (Kimata, 1983; Kudoh et al., 1993). We assume that morphological delimitation of *C. fallax* is complicated by variation and plasticity present in Asian *C. flexuosa*, which blurs morphological distinction of the former. Here we hypothesize that *C. fallax* may represent one of parental taxa of Asian *C. flexuosa*: it is of lower ploidy level (Table 1), shares the cpDNA haplotype with Asian *C. flexuosa*, and posesses a single and distinct ITS sequence variant, in contrast to the additive pattern in Asian *C. flexuosa*.

cpDNA haplotype diversity and biogeographic patterns—Contrasting patterns of genetic variation (particularly in cpDNA) among different species and lineages have been revealed in our study. Very little haplotype diversity was found in *C. hirsuta* and European *C. flexuosa*. Despite the large number of accessions analyzed, only two and three haplotypes were detected, respectively. We postulate that low diversity in Europe, their assumed area of origin, reflects pronounced bottleneck effects following migration and population extinction during the Pleistocene. Moreover, even among the few distinct haplotypes that are resolved, there is no obvious geographic pattern that could shed some light on their glacial survival and colonization routes. In weedy species as *C. hirsuta* and *C. flexuosa*, we might expect that historical migration patterns could be lost. There are, however, examples of weedy species in which clear phylogegraphic structure has been detected (e.g., *Arabidopsis thaliana,* Sharbel et al., 2000; *Microthlaspi perfoliatum,* Koch and Bernhardt, 2004). We assume that more variable DNA markers may provide more resolution of the species studied here. Both species, especially *C. hirsuta*, are characterized by high dispersal and colonization ability, and weedy behavior. Apparently, they have been introduced by man to areas where they are now spreading rapidly in ruderal habitats (as documented for *C. hirsuta* in Japan). Multiple introductions of *C. flexuosa* to North America were expected, judging only from its occurrence there, reported from both Atlantic and Pacific coast areas (Rollins, 1993; USDA, NRCS, 2005) and this is apparent also from our data.

Completely different patterns were found in the Asian tetraploid *C. scutata* and related Japanese endemics. Within just a relatively small area of Japan, enormous diversity showing clear phylogeographic structure was found. Two groups of northern and southern haplotypes were identified, respectively, with the main diversity concentrated in central Honshu. The Japanese archipelago is characterized by a wide range of climatic zones along its northeast to southwest axis and a complex topography with several mountain chains running parallel to the coasts. In addition, the Pacific Ocean and the Sea of Japan sides of the islands show differences in climate conditions over short distances, resulting in different floristic compositions (Miyawaki et al., 1994). This is reflected also in a few phylogeographic studies centered on the area of Japan that have been published in recent years (e.g., Fujii et al., 2002; Ohi et al., 2003; Tsuda and Ide, 2005; Fujii and Senni, 2006). *Cardamine scutata* shows wide ecological amplitude with respect to climatic conditions and cold tolerance. Genetic diversity patterns in this species strongly suggest that central Honshu as well as part of southern Japan acted as refugia during colder periods, with subsequent northward migrations. Most genetic variation is now found in the putative refugial region of central Honshu, which might also have been enriched from populations that expanded postglacially from southern refugia. Postglacial expansion from the proposed refugial areas was apparently associated with the loss of genetic variation, as the populations north of central Honshu display only a subset of total genetic variation present in central regions of Honshu.

Conclusions—Our study based on cpDNA and ITS sequence data aimed to elucidate phylogenetic relationships within a group of taxa taxonomically related to *C. flexuosa* sampled throughout the world. The results provided unequivocal evidence that Asian weedy populations traditionally assigned to *C. flexuosa* form an independent evolutionary lineage and represent a distinct taxon from European *C. flexuosa*. Inevitable nomenclatural changes, however, remain to be done. Two taxa usually considered to be very close or even conspecific with *C. hirsuta*, *C. oligosperma*, and *C. umbellata* (= *C. oligosperma* var. *kamtschatica*) showed distinct phylogenetic positions. While the former is sister to *C. hirsuta*, the latter seems to be more closely related to New Zealand *C. debilis* and *C. corymbosa*. *Cardamine parviflora* var. *parviflora* and *C. parviflora* var. *arenicola* from Eurasia and North America, respectively, indeed represent genetically well-defined sister taxa. Contrasting patterns of genetic variation (particularly in cpDNA) between widespread *C. hirsuta* and *C. flexuosa* on one side, and eastern Asian *C. scutata* on the other, were found. While very little variation was detected in *C. hirsuta* and *C. flexuosa*, which has been suggested to reflect pronounced bottleneck effects following migration and population extinction, *C. scutata* had a distinct phylogeographic structure.

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Appendix. Plant material and DNA sequences of the genus *Cardamine* used in this study. Sequences were either (A) acquired in the present study or (B) used in our previous studies or downloaded from GenBank. Abbreviations of most common collectors: *JL* = J. Lihová, *KM* = K. Marhold, *JK* = J. Kučera, *MP* = M. Perný, *MK* = M. Kolník, *PZ* = Peter Zika, and *HK* = H. Kudoh. Herbarium abbreviations follow Holmgren et al. (1990).

A) Sequences acquired in the present study.

***Taxon*—Accession code**; Source, *Voucher*; GenBank accessions: ITS, *trnL, trnL-trnF*.

***Cardamine amara* (subsp. *amara*)—IT (AM-S5)**; Italy, prov. Piemont, S of Frabosa Soprana, *JL et al.* (SAV); —, DQ278898, —. **IT (AM-S4)**; Italy, prov. Piemont, Garessio, Cla. di Casotto, *JL* (SAV); —, DQ278900, —. **IT (AM-S6)**; Italy, prov. Piemont, Gias del Roccassone, *JL et al.* (SAV); —, DQ278899, —. **IT (AM-E2)**; Switzerland, Graubünden, NE of Brusio, *JL et al.* (SAV); —, DQ278897, —. ***Cardamine* cf. *corymbosa*—CAN6**; Canada, British Columbia, Vancouver, UBC Botanical Garden, *F. Lomer* (UBC); DQ268393, DQ268060, DQ268227. ***Cardamine debilis*—NZ**; New Zealand, Wellington, Otari-Wiltons Bush Forest Reserve, *M. Tsiantos* (SAV); DQ268392, DQ268059, DQ268226. ***Cardamine dentipetala*—JP18**; Japan, Shiga Pref., Katsuragawa, Mt. Minako, *JL et al.* (SAV); DQ268499-DQ268501, DQ268149-DQ268151, DQ268316-DQ268318. ***Cardamine fallax*—JP19**; Japan, Shiga Pref. , Takashima-gun, Shin-ashahi-cho, Harie, *JL et al.* (SAV); DQ268456-DQ268458, DQ268117-DQ268119, DQ268284-DQ268286. **JP137**; Japan, Fukushima Pref., Sohma-gun, Fukuda Pass, *K. Yonekura & T. Fukuda 95503* (MO 0508811); DQ268462, DQ268120, DQ268287. **JP138**; Japan, Mie Pref., Matsusaka-shi, Kushida River, *HK* (SAV); DQ268459-DQ268461, DQ268121-DQ268122, DQ268288-DQ268289. **JP139**; Japan, Kanagawa Pref., Aikawa-cho, Hanbara, *HK* (SAV); DQ268463, DQ268123, DQ268290. **CHI14** (as *C. flexuosa*); China, Xiuning Xian, Anhui prov., *Liu Qixin* (MO 04509180); DQ268464, DQ268103, DQ268270 [morphological identification of this specimen is not unequivocal]. ***Cardamine flexuosa* (European)—DE3**; Germany, Heidelberg, Heiligenberg, *JL* (SAV); DQ268411, DQ268082, DQ268249. **SK4**; Slovakia, E Carpathians, Miroľa, *MK & JK* (SAV); DQ268415, DQ268083, DQ268250. **ES3**; Spain, prov. Palencia, Embalse de Camporredondo, *JL* (SAV); DQ268420, DQ268072, DQ268239. **ES4**; Spain, prov. León, Pto. de Pajares, *JL* (SAV); DQ268410, DQ268075, DQ268242. **ES5**; Spain, Sierra Nevada Mts., Capilleira, *JL* (SAV); DQ268408, DQ268078, DQ268245. **ES6**; Spain, prov. Lérida, Val d' Aran, Refugi Eth Santet, *JL* (SAV); DQ268419, DQ268080, DQ268247. **IT3**; Italy, prov. Piemont, Garessio, Cla. di Casotto, *JL & KM* (SAV); DQ268418, DQ268081, DQ268248. **UC**; Ukraine, Ivano-Frankivska obl., Nadvirianskyi raion, Hoverla, *MK* (SAV); DQ268421, DQ268084, DQ268251. **BG1**; Bulgaria, Vasiliovska planina-Tetevenski Balkan, Ribaritsa, *JK* (SAV); DQ268424, DQ268076, DQ268243. **BG2**; Bulgaria, Rila Mts., Ch. Iskar stream, *MK & JK* (SAV); DQ268422, DQ268073, DQ268240. **RO1**; Romania, Timişu de Sus, Mt. Postâvarul, *MK & JK* (SAV); DQ268414, DQ268077, DQ268244. **RO2**; Romania, Mt. Rodna, Borşa, Pietroşul Mt., *MK & JK* (SAV); DQ268416, DQ268085, DQ268252. **RO3**; Romania, Mt. Bucegi, Zanoaga, *MK & JK* (SAV); DQ268417, DQ268086, DQ268253. **NO2**; Norway, Furuneset, Fjaler, Sogn & Fjordane, *K. Hansen* (SAV); —, DQ268074, DQ268241. **Tu4**; Turkey, Istanbul, *M. Slovák* (SAV), DQ268423, DQ268087, DQ268254. **US31**; USA, Washington, Snohomish Co., Bryant, *PZ 18441, F. & A. Weinmann* (SAV); DQ268413, DQ268079, DQ268246. **US32**; USA, Washington, King Co., Shoreline Center Park, Richmond Heights, *PZ 18347* (SAV); DQ268409 and DQ268412, DQ268071, DQ268238. **'Asian *Cardamine flexuosa*'—JP1**; Japan, Yamanashi Pref., Nirasaki-shi, Miyakubo, *JL et al.* (SAV); DQ268429, DQ268088, DQ268255. **JP5**; Japan, Nagano Pref., Minamiazumi-gun, Minamiazumi-mura, Ariake, *JL et al.* (SAV); DQ268428, DQ268095, DQ268262. **JP11**; Japan, Toyama Pref. Shimoniikawa-gun, Nyusen-machi, Yoshiwara, *JL et al.* (SAV); DQ268430, DQ268093, DQ268260. **JP13**; Japan, Toyama Pref., Tonami-shi, Yagi, *JL et al.* (SAV); DQ268425, DQ268090, DQ268257. **JP18**; Japan, Shiga Pref., Ohtsu-shi, Katsuragawa, Mt. Minako, *JL et al.* (SAV); DQ268431, DQ268089, DQ268256. **JP27**; Japan, Gumma Pref., Tone-gun, Shirasawa-mura, *JL et al.* (SAV); DQ268426, DQ268092, DQ268259. **JP28**; Japan, Gumma Pref., Tone-gun, Katashina-mura, *JL et al.* (SAV); DQ268427, DQ268094, DQ268261. **JP54**; Japan, Hokkaido, Ishikari-gun, Tohbetsu-cho, Aoyamaokunibangawa, *JL et al.* (SAV); —. DQ268091, DQ268258. **CHI1** (as *C. hirsuta*); China, Yunnan, Nujiang Lisu Aut. Pref., Lushui Co., from Pain Ma to Liuku, *Li Heng 10015* (MO 5151560); DQ268438, DQ268097, DQ268264. **CHI2** (as *C. hirsuta*); China, Yunnan, Nujiang Lisu Aut. Pref., Gongshan Co., Gongshan suburbs, *Li Heng 8680* (MO 5306051); DQ268437, DQ268098, DQ268265. **CHI3** (as *C. hirsuta*); China, Liuyang, Hunan Prov., *Chen Dehuan* (MO 04745232); —, DQ268099, DQ268266. **CHI4** (as *C. hirsuta*); China, Ziyuan Xian, Guangxi Prov., *Li Ming* (MO 04723770); DQ268432, DQ268100, DQ268267. **CHI5** (as *C. flexuosa*); China, Hubei Prov., Hefeng, *Zhang 419* (MO 04515568); —, DQ268105, DQ268272. **CHI6** (as *C. flexuosa*); China, Sichuan, Emei Shan, Mugua, *Xu Honggui 0112* (MO 04545485); DQ268435, —, —. **CHI7** (as *C. flexuosa*); China, Jiangxi, Wuynan Xian, *Lai & Shan 4512* (MO 04564479); DQ268451, —, — [morphological identification of this specimen is not unequivocal]. **CHI9** (as *C. flexuosa*); China, Shaanxi Prov., Yang xian, *Li 8* (MO 04521808); —, DQ268104, DQ268271. **CHI10** (as *C. flexuosa*); China, Si-chuan, Datsian lu city, *G. N. Potanin* (MO 04969045); DQ268440, —, —. **CHI11** (as *C. flexuosa*); China, Wu-lao-feng, Lushan, Jiangxi Prov., *S. S. Lai & H. R. Shan113* (MO 04558088); —, DQ268124, DQ268291. **CHI13** (as *C. flexuosa*); Hong Kong, *Hu & But 20161* (MO 0463702); DQ268436, DQ268106, DQ268273. **TAI2** (as *C. flexuosa*); Taiwan, Nantou Hsien, Jenai Hsiang, Meifeng, *C. M. Wang 02615* (MO 0495782); DQ268439, DQ268101, DQ268268. **TAI3** (as *C. flexuosa*); Taiwan, Taoyuan Hsien, Kuanyin Hsiang, Hsinpo, 8 Dec 1999, *Ching-I Peng 17875*

(MO5312726); DQ268441, DQ268102, DQ268269. **THA1**; Thailand, National ark Doi Inthanon, *M. Koch* (HEID); DQ268434, DQ268096, DQ268263. **THA2** (as *C. flexuosa*); Thailand, Chiang Mai, Chiang Dao, Huay Nam Ru, Doi Sahm Meun, *J. F. Maxwell 90–149* (MO 4041946); DQ268433, **—, —.** **VIE** (as *C. flexuosa*); Vietnam, Ninh Binh Province, Sanh village, *N. M. Cuong, D. T. Kien & M. V. Sinh NMC1311* (MO 5599888); DQ268450, **—, —.** **AUS5** (as *C. flexuosa*); Australia, Western Australian Herbarium grounds, Kensington, Perth WA, *B. J. Lepschi* (PERTH 04232569); DQ268452, DQ268113, DQ268280. **AUS9** (as *C. paucijuga*); Narrogin Townsite WA, *G. J. Keighery & N. Gibson 7093* (PERTH 06760732); DQ268454, DQ268128, DQ268295. **CAN3**; Canada, Meadow Avenue, Burnaby, B.C., E of Vancouver, *F. Lomer* (UBC); DQ268442, DQ268114, DQ268281. **CAN4**; Canada, Surrey, B.C., *F. Lomer* (UBC); DQ268443, DQ268115, DQ268282. **CAN5**; Canada, Cariboo Hill Salvation Army Temple, Burnaby, *F. Lomer* (UBC); DQ268444, DQ268116, DQ268283. **US33** (as *C. parviflora*); USA, California, San Bernardino Valley, Yucaipa, *A. C. Sanders* (MO 04890589); DQ268445, DQ268107, DQ268274. **US34** (as *C. flexuosa*); USA, California, Riverside, UCR campus, *B. Pitzer 2935* (MO 04890590); DQ268446, DQ268108, DQ268275. **US35** (as *C. parviflora*); USA, California, Riverside, UCR campus, *A. Sanders 15915* (MO 04924769); DQ268447, DQ268109, DQ268276. **US8** (as *C. flexuosa*); USA, North Carolina, Guilford Co., Browns Summit, *Dan Tenaglia* (MO 5737873); DQ268449, DQ268110, DQ268277. **US36** (as *C. flexuosa*); USA, Rhode Island, Washington Co., Perryville, *G. C. Tucker 13473* (MO 5709457); **—,** DQ268111, DQ268278. **MEX** (as *C. hirsuta*); Mexico, Rancho El Jabali, Volcán de Colima, *A. S. Sanders 10245, w/B. L. Phillips & B. M. Rothschild* (MO 09464960); DQ268448, DQ268112, DQ268279. ***Cardamine hirsuta*—DE1**; Germany, Freiburg, *JL* (SAV); DQ268377, DQ268035, DQ268202. **DE2**; Germany, Heidelberg, Schlierbach, *JL* (SAV); **—,** DQ268030, **—.** **AU**; Austria, E of Gleisdorf, Arnwiesen Raststätte, *JL et al.* (SAV); DQ268369, DQ268037, DQ268204. **CH**; Switzerland, Lake Geneva, Nyon, *M. Tsiantis* (SAV); DQ268373, DQ268043, DQ268210. **IT1**; Italy, prov. Trentino, Val di Breguzzo, *JL et al.* (SAV); **—,** DQ268032, **—.** **IT2**; Italy, prov. Piemont, Botanical Garden of Torino, *JL & KM* (SAV); DQ268376, DQ268036, DQ268203. **ES1**; Spain, prov. Lérida, Val d' Aran, Val de Valarties, *JL* (SAV); DQ268370, DQ268038, DQ268205. **ES2**; Spain, prov. Zamora, Vigo de Sanabria, *JL & MP* (SAV); DQ268368, DQ268046, DQ268213. **NO1**; Norway, Jeløya, Moss, Østfold, *S. Kjølner & T. Carlsen* (SAV); DQ268371, DQ268045, DQ268212. **SK1**; Slovakia, Botanical Garden of Blatnica (origin on Mt. Borišov, Veľká Fatra Mts.), *J. Kochjarová* (SAV); DQ268372, DQ268039, DQ268206. **CR1**; Croatia, Zagreb, Zool. Garden, *JK* (SAV); DQ268380, DQ268040, DQ268207. **CR3**; Croatia, Plitvicka jezera lakes, *MK & JK* (SAV); DQ268381, DQ268044, DQ268211. **MN1**; Monte Negro, Čevo, *MK & JK* (SAV); DQ268378, DQ268034, DQ268201. **MN2**; Monte Negro, Maglic Mts., Plužine, *MK & JK* (SAV); DQ268379, DQ268042, DQ268209. **Tu1**; Turkey, Giresum Dağlari Mts., Kümbet, *MK & JK* (SAV); DQ268375, DQ268041, DQ268208. **Tu2**, Turkey, Ilgaz Mts., *MK & JK* (SAV); **—,** DQ268033, DQ268200. **Tu3**; Turkey, Rize, Doğ Karageniz Mts., Çamlik, *MK & JK* (SAV); DQ268374, DQ268031, DQ268199. **GE1**; Georgia, Majacowsky distr., S-E from Majacowsky, *L. Krupkina et al.* (MO 04674069); DQ268361, **—, —.** **JP10**; Japan, Niigata Pref., Nishikubiki-gun, Oumi-cho, Hashidate, *JL et al.* (SAV); DQ268363, DQ268028, DQ268197. **JP29**; Japan, Gumma Pref., Tone-gun, Katashina-mura, Higashiogawa, *JL et al.* (SAV); DQ268362, DQ268026, DQ268195. **JP4**; Japan, Nagano Pref., Minamiazumi-gun, Azumi-mura, Shimashima-dani, *JL et al.* (SAV); **—,** DQ268029, DQ268198. **JP17**; Japan, Gifu Pref., Fuwa-gun, Sekigahara-cho, Odaka, *JL et al.* (SAV); DQ268364, DQ268027, DQ268196. **JP13**; Japan, Toyama Pref., Tonami-shi, Yagi, *JL et al.* (SAV); DQ268365, **—, —.** **JP19**; Japan, Shiga Pref., Takashima-gun, Shinashahi-cho, Harie, *JL et al.* (SAV); DQ268366, **—, —.** **JP12**; Japan, Toyama Pref., Toyama-shi, Hamakurosaki Kourai, *JL et al.* (SAV); DQ268367, **—, —.** **US1**; USA, Washington, San Juan Co., Friday Harbor, *PZ 18344* (SAV); DQ268348, DQ268013, DQ268182. **US43** (as *C. oligosperma*); USA, Washington, Grays Harbor Co., Grayland, *G. C. Tucker 12636* (MO 5688347); DQ268356, DQ268018, DQ268187. **US2**; USA, California, Alameda county, Berkeley, *PZ 16765* (SAV); DQ268349, DQ268012, DQ268181. **US3**, USA, Washington, Clark Co., Kevanna Park, Burnt Bridge Creek, *PZ 18393* (SAV); DQ268350, DQ268015, DQ268184. **US4**; USA, Oregon, Clatsop Co., Clatsop Spit, *PZ 18408* (SAV); DQ268351, DQ268014, DQ268183. **US5** (as *C. oligosperma*); USA, Washington, Grays Harbor Co., Highland Golf Course, *PZ 18241* (SAV); DQ268346, DQ268016, DQ268185. **US6** (as *C. oligosperma*); Oregon, Columbia Co., St. Helens, *PZ 18224* (SAV); DQ268347, DQ268017, DQ268186. **US7**; USA, Maryland, Kent Island, Crab Alley Road, *C. F. Reed* (MO 5428171); DQ268358, **—, —.** **US8**; USA, North Carolina, Guilford Co., Browns Summit, *D. Tenaglia* (MO 5737872); DQ268352, DQ268019, DQ268188. **US9**; USA, Florida, Leon Co., W of Tallahassee, *R. K. Godfrey 56250* (MO 5582753); DQ268353, DQ268020, DQ268189. **US10;** USA, Illinois, Lawrence Co., Indiana Robeson Hills Nature Preserve, *B. Edgin 2033* (MO 5171397); DQ268354, DQ268021, DQ268190. **US11**, USA, Connecticut, New London Co., Groton, *G. C. Tucker 10486* (MO 04998220); DQ268355, DQ268022, DQ268191. **US12**; USA, California, Los Angeles Co., Claremont, Rancho Santa Ana Botanic Garden, *A. C. Sanders 17956* (MO 5706308); DQ268357, DQ268023, DQ268192. **VEN**; Venezuela, Estado Aragua, Maya, *J. A. Steyermark & R. Liesner* (MO 2795440); DQ268359, DQ268024, DQ268193. **EQ**; Ecuador, Pichincha, Quito Cantón, Bosque Seco Montano Bajo, *C. Cerón & M. Montesdeoca 12116* (MO 04990843); DQ268360, DQ268025, DQ268194. **AUS1**, Australia, WA, Manjimup, *V. L. Tunsell 223* (PERTH 06147070); DQ268341, DQ268009, DQ268178. **AUS2**; Australia, Lowlands, Serpentine River, *G. J. Keighery 3474* (PERTH 04181700); DQ268343, DQ268010, DQ268179. **AUS3;** Australia, Woodvale Nature Reserve, *G. J. Keighery 14390* (PERTH 04832086); DQ268344, **—, —.** **AUS4**; Australia, Mt. Chudalup, WA, *A. R. Annels, R. W. Hearn ARA 4399* (PERTH 04440773); DQ268345, DQ268007, DQ268176. **AUS10** (as *C. paucijuga*); Australia, Lake Muir Nature Reserve, WA, *G. J. Keighery 15413* (PERTH 05275113); **—,** DQ268011, DQ268180. **AUS11**; Australia, Lovett Street, Scarborough, WA, *K. F. Kenneally* (PERTH 06147070); DQ268342, DQ268008, DQ268177. **AUS12** (as *C. paucijuga*); Australia, Rockingham Lakes Regional Park, WA, *G. J. Keighery 16265* (PERTH 06330339); DQ268340, **—, —.** **ETH1;** Ethiopia, Simea Mt. National Park, *E. Ermias* (SAV); DQ268382 and DQ268385; DQ268047-DQ268048, DQ268214-DQ268215. **ETH2**; Ethiopia, Choke Mt., *E. Ermias* (SAV); DQ268383, **—, —.** **ETH3**; Ethiopia, Addis Ababa Univ., *E. Ermias* (SAV); DQ268384, **—, —.** ***Cardamine impatiens*—GE2**; Georgia, Borjomis Raioni, Bakuriani, *KM* (SAV); **—,** DQ268169, DQ268335. **JP4**; Japan, Nagano Pref., Minamiazumi-gun, Azumi-mura, Shimashima-dani, *JL et al.* (SAV); DQ268503, DQ268168, DQ268334. **SK5**; Slovakia, Východ. nížina lowland, Boťany, *KM & HK* (SAV); DQ268504, DQ268170, DQ268336. **DE4**; Germany, Heidelberg, Schlierbach, *JL* (SAV), **—,** DQ268167, **—.** **CR2**; Croatia, Velebit, Oštarje, *JK* (SAV); DQ268505, DQ268171, DQ268337. ***Cardamine longifructus*—JP24**; Japan, Hyogo Pref., Ibo-gun, Shingu-cho, Okugoya, *JL et al.* (SAV); DQ268496-DQ268498, DQ268152-DQ268153, DQ268319-DQ268320. **JP82**; Tokushima Pref., Mima-gun, Koyadaira-mura, Mt. Tsurugisan, Gyójyó, *KM & HK* (SAV); DQ268495, DQ268154, DQ268321. **JP83**; Tokushima Pref., Miyoshi-gun, Higashiiyayama-mura, Mt. Miune, *KM & HK* (SAV); DQ268494, DQ268155, DQ268322. ***Cardamine niigatensis*—JP97**; Japan, Niigata Pref., Kitauonuma-gun, Yunotani-mura, Tochiomata, *KM & HK* (SAV); DQ268490-DQ268493, DQ268159 and DQ268161-DQ268163, DQ268326 and DQ268328-DQ268330. **JP98**; Japan, Niigata Pref., Kitauonuma-gun, Yunotani-mura, Ginzandaira, *KM & HK* (SAV); DQ268479-DQ268482, DQ268164-DQ268165, DQ268331-DQ268332. ***Cardamine oligosperma*—US13**; USA, Oregon, Washington Co., Tualatin Hills Nature Park, Beaverton, *PZ 18232* (SAV); DQ268386, DQ268053, DQ268220. **US14** (as *C. flexuosa*); USA, Oregon, Washington Co., Tualatin Hills Nature Park, Beaverton, *PZ 18234* (SAV); DQ268388, DQ268051, DQ268218.

US15; USA, Washington, San Juan Co., Lopez Island, Point Colville, *PZ 18343* (SAV); **—,** DQ268052, DQ268219. **US16**; USA, Oregon, Capses, East Side Hill, *L. F. Henderson 399/1924* (MO 911084); DQ268389, DQ268049, DQ268216. **US17**; USA, California, Butte Co., Oroville, *M. S. Taylor 3498* (MO 3207800); —, DQ268054, DQ268221. **CAN1**; Canada, 1 km W of Bridal Falls, *F. Lomer* (UBC); DQ268387, DQ268050, DQ268217. ***Cardamine parviflora* var. *parviflora*—SK2**; Slovakia, Východ. nížina lowland, Strážne, *KM* (SAV); DQ268407, DQ268068-DQ268069, DQ268235-DQ268236. **SK3**; Slovakia, Východ. nížina lowland, Leles, *KM* (SAV); DQ268405, **—, —. Ru**, Russia, prov. Rjasan, district Spassk, Brykin Bor, *V. Tichomirov et al.* (MO 2402212); DQ268067, DQ268234. **CZ**; Czech Republic, Dolnomoravský úval, Zbrod, *KM* (SAV); DQ268406, DQ268070, DQ268237. ***Cardamine parviflora* var. *arenicola*—US22**; USA, Oklahoma, Mayes Co., E of Locust Grove, *R. C. & K. W. Rollins 8753* (MO 3665047); DQ268403, DQ268061, DQ268228. **US23**; Missouri, St. Genevieve Co., Terre Blue Creek, NW of Sprott, *J. A. Steyermark 806* (MO1027030); DQ268404, DQ268064, DQ268231. **US24**; USA, Florida, Leon Co., Tallahassee, *R. K. Godfrey 62647* (MO 5168086); DQ268400, DQ268066, DQ268233. **US25**; USA, New York, Suffolk Co., Fisher's Island, Southold, *G. C. Tucker & E. H. Horning 5989* (MO 3898808); DQ268395, DQ268063, DQ268230. **US26;** USA, New York, Suffolk Co., Fisher's Island, Southold, *G. C. Tucker & E. H. Horning 5977* (MO 3898807); DQ268398, DQ268062, DQ268229. **US27**; USA, Illinois, Sullivan Co., First Old Field North, *W. McClain* (MO 5414442); DQ268399, DQ268065, DQ268232. **US28**; USA, Ohio, Butler Co., Hamilton city, Gilmore Ponds Preserve, *A. W. Cusick 32,936 & D. C. Dister* (MO 05017273); DQ268396, **—, —. US29**; USA, Missouri, Pulaski Co., Falls Hollow Sandstone Glade, Ft. Leonard Wood, *J. Hays 352* (MO 04614587); DQ268397, **—, —. US30**; USA, Howell Co., N of Brandsville, *B. Summers 4123* (MO 4235073); DQ268402, **—, —. US44**; USA, North Carolina, Madison Co., Co. road 1304, *D. Boufford 12946* (MO2401083); DQ268394, **—, —. CAN2**; Canada, Essex Co., Pelee Island, W of Stone Rd, S of East West Rd, *A. W. Cusick #33,770 & M. J. Oldham* (MO 04898922); DQ268401, **—, —. *Cardamine paucijuga*—AUS6**; Australia, Layman Block, Tuart Forest WA, *G. J. Keighery 14120* (PERTH 04475410); DQ268453, DQ268125, DQ268292. **AUS7**; Australia, Layman Block, Tuart Forest, E of Busselton, *G. J. Keighery 14851* (PERTH 05017653); DQ268455, DQ268126, DQ268293. **AUS8**; Australia, Simmonds Block, Tuart Forest, *G. J. Keighery 13,633* (PERTH 04533208); —, DQ268127, DQ268294. ***Cardamine pectinata*—BG3**; Bulgaria, Tsarichina natural reserve, St. Planina, Vezhen, *JK* (SAV); DQ268502, DQ268174, DQ268338. **BG4**; Bulgaria, Pirin Mts., Banderitsa, *JK* (SAV); **—,** DQ268172, **—. BG5**; Bulgaria, Tetevensky Balkan, Zavodna River valley, *JK* (SAV); —, DQ268173, **—. GE2**; Georgia, Borjomis Raioni, Bakuriani, *KM* (SAV); **—,** DQ268175, DQ268339. ***Cardamine pensylvanica*—US37**; USA, Arkansas, Pope Co., Atkins, *R. C. & K. W. Rollins 8755* (MO 3665046);—, DQ268132, DQ268299. **US38**; USA, Washington, Pierce Co., SE of Frederickson, *R. R. Halse 4130* (MO 05037131); —, DQ268133, DQ268300. **US39**; USA, New York, Hamilton Co., Whitaker Lake, *G. C. Tucker 6044* (MO 04998197); DQ268466, —. **US40**; USA, Washington, Clark Co., Vancouver, Columbia River, *PZ 18321* (SAV); DQ268468-DQ268469, DQ268134-DQ268135, DQ268301-DQ268302. **US41**; USA, Oregon, Yamhill Co., Cozine Creek, *PZ 18183* (SAV); DQ268467, DQ268136, DQ268303. **US42**; USA, New York, Orange Co., Harriman State Park, Summer Hill, *G. C. Tucker & J. G. Barbour 8663* (MO 04998214); —, DQ268137, DQ268304. **US43**; USA, South Carolina, Richland Co., Congaree Swamp National Monument, *J. N. Nelson 20407* (MO 5706300); **—,** DQ268130, DQ268297. **CAN1**; Canada, British Columbia, Bridal Falls, *F. Lomer* (UBC); DQ268465, DQ268131, DQ268298. ***Cardamine scutata*—TAI1** (as *C. flexuosa*); Taiwan, Miaoli Hsien, Nanchuang Hsiang, Fengmeihsi, *Tsui-Ya Liu 375 et al.* (MO 04594130); —, DQ268129, DQ268296. **JP7**; Japan, Nagano Pref., Oomachi-shi, Kurosawa, *JL et al.* (SAV); DQ268483, DQ268166, DQ268333. **JP10**; Japan, Niigata Pref., Nishikubiki-gun, Oumi-cho, Hashidate, *JL et al.* (SAV); DQ268470, DQ268142, DQ268309. **JP17**; Japan, Gifu Pref., Fuwa-gun, Sekigahara-cho, Odaka, *JL et al.* (SAV); DQ268488, DQ268143, DQ268310. **JP18**; Japan, Shiga Pref., Ohtsu-shi, Katsuragawa, Mt. Minako, Ado river, *JL et al.* (SAV); **—,** DQ268144, DQ268311. **JP29**; Japan, Gumma Pref., Tone-gun, Katashina-mura, Higashiogawa, *JL et al.* (SAV); DQ268471, DQ268145, DQ268312. **JP41**; Japan, Hokkaido, Otaru-shi, S of Otaru, Tenjin, *JL et al.* (SAV); DQ268486, DQ268138, DQ268305. **JP46**; Japan, Hokkaido, Isoya-gun, Rankoshi-cho, Isotani-gun, Tachikawa, *JL et al.* (SAV); DQ268472, DQ268146, DQ268313. **JP50**; Japan, Hokkaido, Setana-gun, Setana-cho, Kariba-keikoku, *JL et al.* (SAV); DQ268484, DQ268141, DQ268308. **JP52**; Japan, Hokkaido, Ishikari-gun, Tohbetu-cho, Aoyamaokunibangawa, *JL et al.* (SAV); DQ268473, DQ268147, DQ268314. **JP66**; Japan, Hokkaido, Nakagawa-gun, Bifuka-cho, Panke, *JL et al.* (SAV); DQ268485, DQ268148, DQ268315. **JP68**; Japan, Hokkaido, Teshio-gun, Horonobe-cho, Kamitoikan, *JL et al.* (SAV); DQ268474, DQ268140, DQ268307. **JP81**; Japan, Tochigi Pref., Nikko-shi, Chyuzenji lake, *JL et al.* (SAV); DQ268477, DQ268139, DQ268306. **JP84**; Japan, Ehime Pref., Matsuyama-shi, Minamitakai-cho, Tsuenofuchi-kohen Park (in cultivation), *KM & HK* (SAV); DQ268489, DQ268156, DQ268323. **JP88**; Japan, Miyazaki Pref., Higasiusuki-gun, Kitakata-cho, Nakasuiryu, Kiunadani valley, *KM & HK* (SAV); DQ268487, DQ268157, DQ268324. **JP97**; Japan, Niigata Pref., Kitauonuma-gun, Yunotani-mura, Tochiomata, *KM & HK* (SAV); DQ268478, DQ268160, DQ268327. **JP103**; Japan, Iwate Pref., Shimohei-gun, Iwaizumi-cho, Hitsutori-shitsugen, *KM & HK* (SAV); DQ268475, **—, —. JP107**; Japan, Aomori Pref., Kitatsugaru-gun, Kanagi-cho, Takahashi-zawa river, *KM & HK* (SAV); DQ268476, DQ268158, DQ268325. ***Cardamine umbellata*—US18**; USA, Alaska, Mt. McKinley National Park, *A. Nelson & R. A. Nelson* (MO 1200653); **—,** DQ268055, DQ268222. **US19**; USA, Alaska, Rainy Creek, Hyder, *K. Whited* (MO 925629); DQ268391, DQ268056, DQ268223. **US20**; USA, Aleutian Islands, Attu Island, Massacre Bay area, *G. W. Soule* (MO 1705457); DQ268390, DQ268058, DQ268225. **US21**; USA, Aleutian Islands, Atka, *Lt.Cl.York* (MO 1294537); **—,** DQ268057, DQ268224.

B) Sequences used in our previous studies or downloaded from GenBank. ***Taxon*—Accession code** (if specified); GenBank accessions: ITS, *trnL*, *trnL-trnF*.

***Cardamine amara* (subsp. *amara*)—IT(AM-S5)**; DQ209070, —, DQ209184. **IT(AM-S4)**; DQ209069, —, DQ209183. **IT(AM-S6)**; DQ209067, —, DQ209181. **IT(AM-E2)**; DQ209066, —, DQ209180. ***Cardamine debilis*—**; **—,** AY047643, AY047660. ***Cardamine corymbosa*—**; AF100678, AY047633, AY047646. ***Cardamine glacialis*—**; —, AY047634- AY047635, AY047648-AY047649. ***Cardamine hirsuta*—DE2**; DQ209131, —, DQ209142. **IT1**; DQ209132, —, DQ209143. ***Cardamine impatiens*—RO4**; DQ209127, **—, —. DE4**; DQ209128, —, DQ209201. ***Cardamine parviflora* (var. *parviflora*) —SK2**; DQ209133, —, DQ209134. ***Cardamine paucijuga*—**; —, AY047640, AY047656. ***Cardamine pectinata*—BG4**; DQ209129, —, DQ209200. **BG5**; DQ209130, —, DQ209199.