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Molecular systematics and biogeography of the *Cardamine pratensis* complex (Brassicaceae)

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Abstract. Representatives of the *C. pratensis* complex were analysed for allozymes, ITS, non-coding cpDNA, and RAPDs to elucidate phylogenetic relationships and the historical biogeography of this species group. Our concepts differ in some important aspects from current ideas. Two diploid species from southeastern Europe form the Basal Group of the complex. A diploid from the Iberian Peninsula represents another old lineage. The phylogenetically younger Derived Group comprises diploid taxa and all known polyploid taxa. The two old lineages represent pleistocene relicts which were not involved in the formation of the Derived Group. All polyploids evolved in postglacial time from diploids of the Derived Group which may have survived the glaciations in refugia centered around and within the Alps. The arctic-circumpolar *C. nymanii* is of young age and migrated to Scandinavia in postglacial times from south to north.

Key words: *Cardamine pratensis* s.l., Cruciferae, molecular systematics, molecular biogeography.

Cardamine pratensis s.l. is a highly polymorphic species complex of mainly European and arctic-circumpolar distribution. It comprises diploid and polyploid taxa. Lövkvist (1956), in an influential paper, presented outlines of the cytogenetics and taxonomy of the complex. In the meantime many more important insights in this difficult species group have accumulated (i.e. Urbanska-Worytkiewicz and Landolt 1974, Marhold 1994a) but Lövkvist's treatment of the *C. pratensis* complex is still the only available concept which deals with the phylogeny and biogeography of the complex in its entirety.

Lövkvist (1956) divided *C. pratensis* s.l. into three groups: the Arctic Group, the Repent Rhizome Group, and the Temperate Group, reflecting as Lövkvist (1956) believed a phylogeny which is "evidently of high age". Lövkvist (1956) was convinced that the highly polyploid and arctic-circumpolar "Arctic Group showed the main features of its present distribution even in late Tertiary". The diploid Repent Rhizome Group from the Pyrenees and central Spain forms in Lövkvist's view an isolated unit that "may be a relic with its present distribution because of the Pleistocene glaciations". In the Temperate Group sensu Lövkvist (1956) postglacial polyploidisation played an important role.

The development of molecular methods has revolutionised evolutionary and systematic biology. An unprecedented array of variable genetic markers is now available to attack various problems of systematics. No molecular analyses are available for the *C. pratensis* complex, yet it seems feasible to unravel the evolutionary history of this extremely variable

complex by a multifaceted approach, using allozymes, ITS and cpDNA, and RAPDs as molecular markers. Previous studies in Brassicaceae have proven the significance of molecular markers for biogeographic analyses (e.g. *Arabidopsis*: Mummenhoff and Hurka 1995; *Cardamine*: Franzke et al. 1998, *Capsella*: Neuffer and Hurka 1999; *Cochlearia*: Koch et al. 1996, *Lepidium*: Mummenhoff et al. 1992). We expect that in the case of the *C. pratensis* complex molecular markers will also help to understand the history of its distribution area which is connected to Pleistocene/Holocene climatic changes. This is to our knowledge the first molecular-biogeographical analysis of an entire species complex of herbacous plant species in an European-wide scale.

Materials and methods

Plant material. Origin and numbers of individuals analysed for allozyme and DNA variation are given in Table 1. Voucher specimens are deposited at OSBU.

Allozymes. The following enzymes were assayed: aspartate aminotransferase (AAT, EC 2.6.1.1.); glutamate dehydrogenase (GDH, EC 1.4.1.2-4); leucine aminopeptidase (LAP, EC 3.4.11.1). Extracts were prepared from 0.7 g leaves of single plants. Electrophoresis was performed in a continuous system on 5.5% (w/v; only GDH) and 7.5% polyacrylamide gel slabs using different buffer systems (see Hurka et al. (1989) for AAT; Hurka and Düring (1994) for GDH; Neuffer and Hurka (1999) for LAP).

Allele frequencies within diploid taxa (*C. pratensis* s.str., *C. matthioli*, *C. rivularis*, *C. crassifolia*) were calculated, and genetic distances among species were determined according to Cavalli-Sforza and Edwards (1967). Based on the distance matrix, a phenogram was generated using the UPGMA algorithm (Sneath and Sokal 1973) supplied by the software package BIOSYS-1 version 1.7 (Swofford and Selander 1989).

DNA extraction for sequence and RAPD analysis. Genomic DNA was extracted from ca. 1 cm^2 leaf tissue using the CTAB method of Doyle and Doyle (1987). We modified the extraction for microcentrifuge tubes.

DNA Sequencing. The protocols for PCR amplification and sequencing of the ITS region, the *trn*T/L spacer, the *trn*L/F spacer, and the *trn*L intron are given in Franzke et al. (1998). The *trn*L/F spacer was amplified with the primers developed by Taberlet et al. (1991). The ITS sequences and the non-coding cpDNA regions (*trn*T/L spacer, *trn*L intron, *trn*L/F spacer) were aligned by hand. Regions with ambiguous alignment were eliminated from analysis. Gaps in the alignments (ITS1 and 2 combined; cpDNA intron and the two spacers combined) were treated as missing data. Both matrices were analysed by Fitch parsimony (Fitch 1971) using the heuristic search option in PAUP (version 3.1.1; Swofford 1993) with MULPARS, TBR (tree bisection-reconnection) branch swapping, and simple taxon addition. Accessions with identical sequences belonging to one taxon were merged. The evolutionary direction of sequence change was inferred by outgroup comparison. The consistency index (CI) of Kluge and Farris (1969) is presented to estimate the amount of homoplasy in the characters. Parsimony trees with equal length were summarized using the strict consensus method. Clade robustness was analysed by the bootstrap method (Felsenstein 1985) with search settings described above and 100 replicates. Pairwise sequence differences were calculated using PAUPs distance matrix option. Due to the fact that for *C. granulosa* only the ITS2 sequence could be obtained a second parsimony analysis with the same search settings but based on ITS2 only was performed for all taxa. The DNA alignments are available upon request. Sequences are published and GenBank accession numbers are given in Table 5.

RAPD analysis. RAPD reactions were carried out in 25 µl volumes with 25 ng DNA, 150 µM of each d-NTP (Biometra), 5 pmol 10-basepair primer (Operon) und 0.8 U DNA polymerase (Biotherm). The PCR program on a Trio-Thermoblock™ (Biometra) cycler was: 44 cycles at 94 °C/1 min (first cycle 4 min), 36 °C/1 min, 72 °C/2 min (last cycle 4 min). Products were separated on 1.5% agarose gels in TBE buffer together with a molecular weight ladder, stained with ethidium bromide and photodocumented on an UV bench.

We adopted a rather restrictive approach for RAPD character assessment and we concentrated on those bands only which would provide best resolving of the OTUs. Genetic similarities among OTUs may be underestimated but, on the other

Table 1. Collection data and chromosome numbers of *Cardamine* accessions examined for allozyme, RAPD marker, non-coding cpDNA and ITS sequence variation

Taxon and accession no.		Locality[a)]	Geographic coordinates N/E or W	Collector/ source[b)]	2n[c)]	Numbers of investigated individuals			
						Allozymes[d)]	RAPD	cpDNA[e)]	ITS
C. amara L. subsp. *amara*	UB	CH, Uri, Urnerboden	46°05′/09°05′E	Landolt et al.	16[1)]			1	3
C. amara subsp. *balcanica* Marhold et al.	MB41	B, Pirin Mts, near chalet Demjanica	42°15′/23°04′E	Marhold	(16)[2)]				1
C. amara subsp. *pyrenaea* Sennen	MS24	E, Gerona, near Núria	42°24′/02°11′E	Marhold	16[3)]			1	1
C. crassifolia Pourret	15	F, Pyrenees, Espoussouille	42°37′/02°03′E	OSBU	"16"			1	
	63	E, Ávila, Piedrahita, Puerto de la Peña Negra	40°25′/05°18′W	Franzke	16[4)]	3A,3L	5	2	2
	65	E, Ávila, Puerto de Chia	40°27′/05°09′W	Franzke	?		3		
	67	E, Guadalajara, near Orea	40°30′/01°43′W	Franzke	?		2		
	70	E, Guadalajara, Rio de la Hoz Seca	40°29′/01°42′W	Franzke	?		5	1	2
	101	E, Gerona, near Núria	42°24′/02°09′E	Franzke	"16"	2A,2G,2L	1		
	102	E, Gerona, near Núria	42°24′/02°09′E	Franzke	"16"		1		1
	103	E, Gerona, near Núria	42°24′/02°09′E	Franzke	"16"		1		
	104	E, Gerona, near Núria	42°24′/02°10′E	Franzke	"16"	2A,2G,2L	1		
	105	E, Gerona, near Núria	42°24′/02°09′E	Franzke	"16"	1A,1G,1L	1		
	106	F, Pyrenees, Lac des Bouillouses	42°34′/01°58′E	Plantholt	"16"		1		
	107	F, Pyrenees, Lac des Bouillouses	42°34′/01°58′E	Plantholt	"16"		1		
	MC18	E, Gerona, near Núria	42°24′/02°10′E	Marhold	16[5)]	1A,1G			
	MC25	E, Gerona, near Núria	42°24′/02°10′E	Marhold	16[5)]	1A,1G,1L	1		
	MC28	E, Gerona, near Núria	42°24′/02°10′E	Marhold	16[5)]	1A,1G,1L	1		
	MC55	E, Gerona, near Núria	42°24′/02°10′E	Marhold	16[5)]	1A,1G,1L	1		
	MC76	E, Gerona, near Núria	42°24′/02°10′E	Marhold	16[5)]	1A,1G,1L	1		
	MC87	E, Gerona, Pla dels Hospitalets	42°24′/02°10′E	Marhold/ Vicens	"16"	1A,1G,1L	1		1

Table 1 (continued)

Taxon and accession no.		Locality[a)]	Geographic coordinates N/E or W	Collector/ source[b)]	2n[c)]	Numbers of investigated individuals			
						Allozymes[d)]	RAPD	cpDNA[e)]	ITS
	MC88	E, Gerona, near Núria	42°24′/02°10′E	Marhold	"16"		1		
C. dentata Schultes	4	D, Thuringia, Plothen	50°40′/11°50′E	GFW	?			1	
	86	D, Lower Saxony, Dümmersee	52°29′/08°19′E	Bleeker	64[6)]	1A,1G,1L	1	1	1
	89	D, Bremen, Bremen-Huchting	53°41′/08°44′E	Spitzer	56[7)]	4A,4G,4L	1		
	90	D, Lower Saxony, Bramsche near Osnabrück	52°25′/08°00′E	Bleeker	56[7)]	1A,1G,1L	1		
	91	D, Lower Saxony, Boizenburg	53°21′/10°39′E	Bleeker	64[7)]	1A,1G,1L	1		
	92	D, Mecklenburg-Vorpommern, Greifswald	54°01′/13°02′E	Bleeker/ Mantey	64[7)]	3A,2G,3L	1	1	1
	94	D, Lower Saxony, Reitgaar near Neuenhaus	52°28′/07°00′E	Bleeker/ Franzke	64[7)]	5A,5G,5L	2		
C. granulosa Allioni	121	I, Turin, Boschi di Stupinigi	45°04′/07°02′E	BOLO: 19768	(16)[8)]				1
C. majovskii Marhold & Záborský	21	SK, Bukovské vrchy, Ulič	48°58′/22°24′E	Franzke	32	9A,6G,11L	5	1	1
	22	SK, Nízke Beskydy, L'ubiša	49°00′/21°56′E	Franzke	32	3A,4G,6L	2		1
	24	SK, Nízke Beskydy, Krásny Brod	49°13′/21°53′E	Franzke	32[9)]	6A,6G,7L	1		
C. matthioli Moretti	1	SK, Záhorská nížina/near Kátov	48°50′/17°09′E	Franzke	16[10)]	6A,5G,6L	1		
	2	SK, Záhorská nížina, Abrod	48°31′/16°59′E	Franzke	16[10)]	5A,5G,5L	1		1
	3	SK, Záhorská nížina, Abrod	48°31′/16°59′E	Franzke	16[10)]	6A,5G,6L			
	4	SK, Javorie Mts, near Kriváň	48°32′/19°28′E	Franzke	16[10)]	4A,4G,4L	1		
	9	SK, Slovenské rudohorie Mts, Látky	48°35′/19°38′E	Franzke	(16)[9)]	3A,4G,5L	1		
	10	SK, Slovenské rudohorie Mts, Klenovec	48°36′/19°52′E	Franzke	16[10)]	3A,5G,5L	1		
	11	SK, Slovenské rudohorie Mts, near Brezno	48°44′/19°51′E	Franzke	16[10)]	4A,7G,8L	1	1	1
	12	SK, Slovenské rudohorie Mts, near Brezno	48°46′/19°38′E	Franzke	16[10)]	7A,5G,7L	1		
	14	SK, Nízke Tatry Mts, Pohorelá	48°50′/20°01′E	Franzke	16[10)]	3A,3G,3L	1		

	MM17	SK, Nízke Tatry Mts, Hámor	48°44′/19°36′E	Marhold	(16)[9]		1		1
	MM32	SK, Slovenské rudohorie Mts, Drábsko	48°38′/19°44′E	Marhold	(16)[9]		1		
	MM34	SK, Nízke Tatry Mts, Hel'pa	48°51′/19°57′E	Marhold	16[10]		1		
	MM43	BG, Rila Mts, Borovec	42°05′/23°30′E	Marhold	(16)[9]		1	1	1
	MM47	SK, Nízke Tatry Mts, Beňuš	48°43′/19°47′E	Marhold	(16)[9]		1		
	MM77	SK, Muránska planina, Zbojská	48°40′/19°44′E	Marhold	16[10]		1		
	MM82	SK, Nízke Tatry Mts, Bujakovo	48°44′/19°36′E	Marhold	16[10]		1		
C. nymanii Gandoger	27	S, Lapland, near Abisco	68°20′/18°51′E	GOET: La-86-039	(63–90)[11]			1	1
	80	N, Dovrefjell	62°02′/09°44′E	Hurka/Neuffer	(63–90)[11]	5A,5G,2L	3	1	1
	108	N, Dovrefjell	62°02′/09°05′E	Bleeker	(63–90)[11]		1		
	109	N, Hadangervidda	60°02′/07°31′E	Bleeker	(63–90)[11]		1		
	110	IS, Heidmörk	64°04′/21°47′W	B.G. Reykjavik	(63–90)[11]	1A,1G	2		
	MW47	RU, Tajmyr peninsula	71°30′/89°20′E	MW	(63–90)[11]				1
C. penzesii Ančev & Marhold	MP11	B, Black Sea coast, river Veleka	27°05′/42°03′E	Marhold	16[12]	5A,4G,5L	4	1	1
	MP19	B, Black Sea coast, river Ropotamo	27°04′/42°02′E	Marhold	16[12]	2A,2G,2L	2		
	MP67	B, Black Sea coast, near Jasna Poljana	27°04′/42°02′E	Marhold	16[12]	5A,5G,1L	3	1	1
	MP70	B, Black Sea coast, river Ropotamo	27°04′/42°02′E	Marhold	16[12]	2A,2G,2L	1		
C. pratensis L. (from open habitats)	6	SK, Pol'ana, Mt Predná Pol'ana	48°37′/19°27′E	Franzke	44	5A,7G,8L	1	1	1
	7	SK, Pol'ana, Mt Predná Pol'ana	48°37′/19°27′E	Franzke	44	4A,2G,5L	1		
	11	D, Lower Saxony, Osnabrück (Harderberg)	52°13′/08°05′E	OSBU: 333	(30)[14]			1	
	25	SK, Nízke Tatry Mts, Vernár	48°55′/20°16′E	Franzke	30[9]	8A,4G,11L	1		
	26	SK, Nízke Tatry Mts, Vernár	48°54′/20°15′E	Franzke	30[9]	5A,4G,5L	1		
	28	SK, Spišské kotliny, Štrba	49°05′/20°02′E	Franzke	?	6A,6G,1L	1		
	29	SK, Liptovská kotlina, Važec	49°03′/19°57′E	Franzke	30[9]	4A,4G,1L	1		
	31	SK, Strážovské a Súľovské vrchy Mts, Považská Bystrica	49°11′/18°51′E	Franzke	?	5A,6G,5L			
	33	D, Baden-Württemberg, Tettnang	47°39′/09°35′E	Franzke	16	4A,3G,3L	1		

Table 1 (continued)

Taxon and accession no.		Locality[a]	Geographic coordinates N/E or W	Collector/ source[b]	2n[c]	Numbers of investigated individuals			
						Allozymes[d]	RAPD	cpDNA[e]	ITS
	34	D, Baden-Württemberg, Tettnang	47°39′/09°35′E	Franzke	16	1A,2G,2L	1	1	1
	36	CH, Thurgau, Amriswil	47°32′/09°16′E	Franzke	16[13]	2A,1G,3L	1		
	37	CH, Thurgau, Amriswil	47°31′/09°16′E	Franzke	30	2A, 4L	1		
	38	CH, Appenzell, Urnäsch	47°19′/09°17′E	Franzke	?		1		
	39	CH, Appenzell, Gonten	47°19′/09°21′E	Franzke	16	8A,,5G,10L	1		1
	40	CH, Appenzell, Gonten	47°19′/09°21′E	Franzke		3A,2G,2L	1		
	41	CH, Appenzell, Urnäsch	47°19′/09°19′E	Franzke	?	1A,1G,1L	1		
	42	CH, Appenzell, Gonten	47°19′/09°22′E	Franzke	16	3A,3G,3L	1		
	43	CH, Appenzell, Appenzell	47°20′/09°22′E	Franzke	?	2A,2G,2L	1		
	44	CH, Zurich, Zurich	47°22′/08°43′E	Franzke	30	2A,2G,2L	1		
	46	CH, Fribourg, St. Antoni	46°49′/07°16′E	Franzke	48[13]		1		
	55	CH, Agovia, Kaisten	47°32′/08°02′E	Franzke	30[13]	5A,5G,4L	1		
	60	E, Navarra, Portillo de Fraice	42°57′/00°47′W	Franzke	?	2A,2G,2L	1		
	61	E, Navarra, Sima de San Martin	42°58′/00°46′W	Franzke	?	2A,2G,2L	1		
	73	E, Zamora, Sierra Segundera	42°09′/06°74′W	Franzke	?		2		
	88	P, Białystok, Białowieża	23°05′/52°04′E	Stosiek	30	2A,2G,2L	1		
	117	D, Lower Saxony, Osnabrück (Rubbenbruchsee)	52°16′/07°59′E	Franzke	30		1		1
	118	D, Lower Saxony, Osnabrück (Nettetal)	52°19′/08°04′E	Franzke	30		1		
	119	D, Lower Saxony, Lingen	52°32′/07°22′E	Franzke	30		1		
C. pratensis L. (from forests)	53	CH, Argovia, Laubberg near Zurzach	47°36′/08°16′E	Franzke	?	2A,2G,2L	1		
	56	CH, Argovia, Sisseln	47°32′/07°59′E	Franzke	?	1A,1G,1L	1		
	57	F, Doubs, near Doubs	46°55′/06°21′E	Franzke	16	6A,5G,4L	1		
	58	F, Doubs, Onglières	46°48′/06°00′E	Franzke	16	5A,5G,5L	1		
	59	F, Doubs, Pont de la Chaux	46°39′/05°56′E	Franzke	16	1A	1		1
C. rivularis Schur	l.c.	R, Mtii Făgărasului, Valea Arpasului	24°02′/45°04′E	Marhold	16[9]		1	1	1
	MR9	B, Mt Vitoša, Železnica	23°02′/42°04′E	Marhold	16[5]	5A,5G,5L	5		

	MR39	B, Mt Vitoša, Aleko	23°02′/42°04′E	Marhold	16[5)]	5A,5G,5L	5	1	1
C. rivularis auct. non Schur	81	A, Carinthia, Turrach near Dieslingsee	46°57′/13°56′E	Franzke et al.	32	4A,2G	1		
	82	A, Carinthia, Nockberge near Falkertsee	46°51′/13°49′E	Franzke et al.	32	2A,6G,2L	1	1	1
	83	A, Styria, Koralpe near Speiksee	46°47′/14°59′E	Franzke et al.	16	3A,3G,6L	4	1	1
	85	U, Marmros'ki Al'py Mts, Mt Pop Ivan	24°04′/48°00′E	Letz	?	5L	2		1
	UB	CH, Uri, Urnerboden	46°05′/09°05′E	Landolt et al.	16[1)]		2	1	3
	MK71	R, Mtii Rodnei, near lake Lala	24°04′/47°02′E	Mráz/Turis	?	3A,3G,3L			
C. udicola Jordan	47	CH, Bern, Thun (Geistsee)	46°45′/07°32′E	Franzke	30	4A,4G	2		1
	48	CH, Bern, Gwatt	46°43′/07°37′E	Franzke	?	2A,2G,2L	1		1
	49	CH, Bern Neuhaus near Interlaken	46°40′/07°49′E	Franzke	?	1A,2G,2L	1		
	50	CH, Bern, Lütschera near Interlaken	46°40′/07°50′E	Franzke	?	3A,3G	1		

[a)] A = Austria, B = Bulgaria, CH = Switzerland, D = Germany, E = Spain, F = France, I = Italy, IS = Iceland, N = Norway, P = Poland, R = Romania, RU = Russia, S = Sweden, SK = Slovakia, U = Ukraine

[b)] Herbaria are indicated by their acronyms. B.G. = Botanical Garden

[c)] Chromosome numbers are from own counts and from literature; in the latter case source of information is cited. Numbers which do not refer to particular localities are given in brackets

[1)] Urbanska et al. (1997)

[2)] Marhold et al. (1996)

[3)] Lihová et al. (2000)

[4)] Rico (pers. communication)

[5)] Marhold (pers. communication)

[6)] Bleeker (pers. communication)

[7)] Bleeker (1997)

[8)] Urbanska-Worytkiewicz & Landolt (1974)

[9)] Marhold (1994)

[10)] Marhold (1984)

[11)] Lövkvist (1956)

[12)] Marhold & Ančev (1999)

[13)] Landolt (pers. communication)

[14)] Bartelheim (1995). Numbers in brackets " " mean that this number was counted for populations nearby

[d)] A = AAT (aspartate aminotransferase), G = GDH (glutamate dehydrogenase), L = LAP (leucine aminopeptidase)

[e)] cpDNA = *trn*T/L spacer-, *trn*L intron, *trn*L/F spacer sequences

hand, the risk of overestimating genetic distances by artificial enlarged distances is minimised. The more variable data are incorporated, the greater is the risk of conflicting data and hence the danger of artificially enlarged (or reduced) distances. In a preliminary survey 10 individuals (2 from 5 taxa) were screened for conservative primers that might produce characters with monomorphic character states within the OTUs (= *C. pratensis* s.l. species). Of 125 primers tested, 20 were finally used. Only bands that did not vary within species were scored as absence/presence characters. Due to the uncertain taxonomic status of *C. rivularis* auct. the putative *C. rivularis* auct. plants analysed were attributed to three separate OTUs according to their place of origin. Computing of pairwise distances following Nei and Li (1979), generating a Neighbor-Joining phenogram (Saitou and Nei 1987), and bootstrap analysis (100 replicates) for assessing group robustness were performed with TREECON Version 1.3b (Van de Peer and De Wachter 1994). The dendrogram was rooted using the MIDPOINT ROOTING option.

Results

Allozymes. Allozyme analyses were performed from representatives of 70 *Cardamine pratensis* s.l. populations (Table 1). For the enzymes analysed (i.e. AAT, GDH and LAP) 15 alleles attributed to five loci complexes were scored. The distribution of these alleles among the taxa of the *C. pratensis* group is given in Table 2. *Aat2-7* was detected exclusively in *C. matthioli* and *C. majovskii*. Unique alleles were detected for *C. matthioli* (*Aat2-5*), *C. crassifolia* (*Aat2-9*) and *C. penzesii* (*Lap3-2*). The frequencies for the detected alleles in the diploid taxa of the *C. pratensis* complex are presented in Table 3. Figure 1 shows the UPGMA phenogram based on these frequencies. The group is divided into a cluster consisting of *C. pratensis* s.str. and *C. matthioli* and a second cluster with *C. penzesii* and *C. rivularis*/*C. crassifolia*.

ITS sequences. The ITS1 and 2 regions from 39 *Cardamine pratensis* s.l. individuals from 32 populations were sequenced (see Table 1). For *C. granulosa* only the ITS2 sequence could be obtained (herbarium specimens). ITS sequences from three taxa of the *C. amara* group were used for outgroup comparison (Table 1). The ITS sequences of the following accessions/individuals were identical (labeling of the individuals refers to Table 1): *C. rivularis* auct. UB (3 individuals), *C. dentata* 86 and 92, *C. udicola* 47 and 48, *C. majovskii* 21 and 22, *C. matthioli* 2 and MM17, *C. crassifolia* 102 and MC87, *C. penzesii* MP67 and MP11, *C. crassifolia* 70 (2 individuals), *C. crassifolia* 63 (2 individuals). The alignment of ITS1 and 2 had 464 positions, 29 of which were potentially informative (4%). Thirteen of these 29 characters did not vary in the ingroup. No

Table 2. Distribution of alleles at five analysed loci complexes for aspartate aminotransferase (*Aat*), glutamate dehydrogenase (*Gdh*), and leucine aminopeptidase (*Lap*) among taxa of *Cardamine pratensis* s.l.

Locus complex	*Aat*	*Aat*					*Gdh*		*Gdh*	*Lap*					
	1	*2*					*1*		*2*	*3*					
allele	*1*	*1*	*2*	*5*	*7*	*9*	*1*	*4*	*1*	*1*	*2*	*3*	*4*	*5*	*6*
C. matthioli (2x)	×	×	×	×	×		×		×	×			×		×
C. majovskii (4x)	×	×	×		×			×	×	×		×	×		×
C. pratensis (2x/4x/6x)	×	×	×				×	×	×	×		×	×	×	×
C. rivularis auct. (2x/4x)	×	×	×				×		×	×			×		×
C. udicola (?x/4x)	×	×	×					×	×	×		×	×		×
C. dentata (7x/8x)	×	×					×		×	×			×		
C. nymanii (8x–12x)	×	×						×	×	×		×	×		×
C. crassifolia (2x)	×	×	×			×		×	×	×					
C. rivularis (2x)	×	×						×	×	×					
C. penzesii (2x)	×	×						×	×	×	×	×			

Table 3. Allele frequencies for the polymorphic enzyme loci *Aat2*, *Gdh1* and *Lap3* in *Cardamine matthioli* (2x), *C. pratensis* s.str. (2x), *C. crassifolia* (2x), *C. rivularis* (2x), and *C. penzesii* (2x). The species samples were fixed for the same allele at *Aat1* and *Gdh2* (see Table 2)

Locus-Alleles	*C. matthioli*	*C. pratensis*	*C. crassifolia*	*C. rivularis*	*C. penzesii*
Aat2	45 ind./10 pop.	21 ind./7 pop.	14 ind./10 pop.	9 ind./2 pop.	14 ind./4 pop.
Aat2-1	0.356	0.381	0.500	1.000	1.000
Aat2-2	0.178	0.619	–	–	–
Aat2-5	0.022	–	–	–	–
Aat2-7	0.444	–	–	–	–
Aat2-9	–	–	0.500	–	–
Gdh1	45 ind./10 pop.	21 ind./7 pop.	11 ind./10 pop.	10 ind./2 pop.	13 ind./4 pop.
Gdh1-1	1.000	0.286	–	–	–
Gdh1-4	–	0.714	1.000	1.000	1.000
Lap3	57 ind./10 pop.	27 ind./7 pop.	13 ind./10 pop.	10 ind./2 pop.	10 ind./4 pop.
Lap3-1	0.123	0.204	1.000	1.000	0.050
Lap3-2	–	–	–	–	0.300
Lap3-3	–	0.093	–	–	0.650
Lap3-4	0.535	0.370	–	–	–
Lap3-5	–	0.019	–	–	–
Lap3-6	0.342	0.315	–	–	–

Note: n ind./n pop. = number of analysed individuals and populations

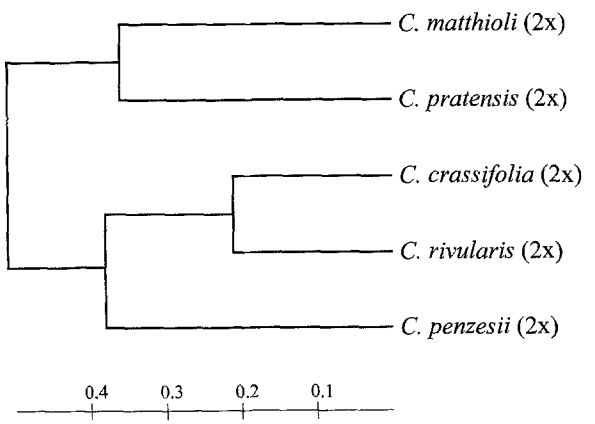

Fig. 1. Phenogram calculated from allozyme frequencies among diploid taxa of *C. pratensis* s.l. using chord distances and the UPGMA method

multiple character states in the ITS sequences have been observed. The strict consensus tree of the 66 shortest cladograms and its statistics are shown in Fig. 2. A basal clade with 63% bootstrap support consists of *C. rivularis* and *C. penzesii*. The remaining taxa are nested in a weakly supported polytomy (50% bootstrap value). Within this clade all *C. crassifolia* accessions form a highly supported group (93% bootstrap value). In addition *C. crassifolia* accessions from central Spain and Pyrenees are clearly separated with 68% bootstrap support. Although weakly supported by bootstrap analysis (ca. 50%) *C. matthioli* along with *C. majovskii* seem to represent a separate lineage. There are no evidences in the ITS sequences for recognising *C. rivularis* auct., *C. nymanii* and *C. pratensis* s.str. as distinct units. The analysis on the basis of the ITS2 sequences that additionally includes *C. granulosa* revealed a similar topology (Fig. 2).

The sequence difference for the ingroup ranged from 0 to 4.6%. The sequence differences between the taxa that later will be called the Derived Group (*C. rivularis* auct., *C. nymanii*, *C. dentata*, *C. udicola*, *C. pratensis* s.str., *C. majovskii* and *C. matthioli*) were in a range of ca. 0.9%. Differences of ITS sequences between outgroup and ingroup taxa ranged from 3.7% to 6.6%.

cpDNA sequences. Sequences from *trn*T/L spacer, *trn*L intron and *trn*L/F spacer for 22 *Cardamine pratensis* s.l. individuals representing 23 populations and from two individuals of

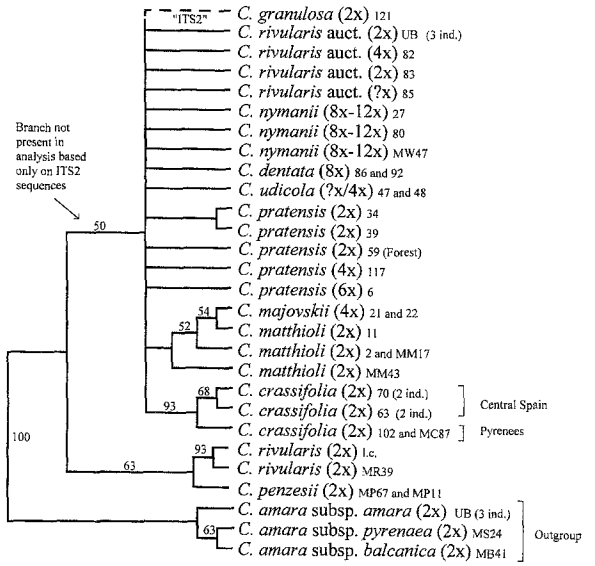

Fig. 2. Strict consensus of 66 most parsimonious trees based on *Cardamine pratensis* s.l. ITS1 and 2 sequence data. Gaps were treated as missing data. The bootstrap support is shown above branches. Tree length: 72, consistency index (CI): 0.86 with autapomorphies excluded. For accession numbers see Table 1. The broken line refers to a strict consensus tree based only on ITS2 sequence data. The topological difference between both analyses is indicated. For accession numbers see Table 1. Ind. = individuals

C. amara group taxa (outgroup) were determined (Table 1). The *trn*T/L spacer has a length of approximately 750 bp. It was not possible to sequence the entire fragment. Starting from primer A we could only read about 240 base pairs because an adjacent poly T region prevented accurate further reading. With primer B, we were able to read ca. 210 bp per reaction. The sequencing of the *trn*L intron yielded ca. 380 nucleotide positions, and from sequencing the *trn*L/F spacer with primer E we obtained ca. 240 positions for the cpDNA alignment. The length of the *trn*L/F spacer fragment ranged from 500 to 900 bp. The 3′ region of the *trn*L/F spacer sequenced with primer F was omitted from phylogenetic analysis because of alignment ambiguities. The alignment of the combined cpDNA data mentioned above has 1077 positions, 19 of which are potentially informative (1.8%). The two individuals of *C. penzesii* analysed were identical in sequences and, therefore, merged. The two identical sequences of *C. rivularis* auct. (82 and 83) were not merged because they belong to two cytotypes (4x, 2x). The pairwise sequence differences for the ingroup ranged from 0 to 1.7%. Most ingroup accessions showed pairwise sequence differences of roughly 1%. The strict consensus tree of the 90 shortest cladograms and its statistics are shown in Fig. 3. The two *C. crassifolia* individuals from central Spain appear as sister-group to the remainder taxa analysed. These remaining taxa are nested in a strongly supported polytomy (98% bootstrap value). Within this clade all analysed *C. matthioli* and *C. majovskii* accessions form a weakly supported group (56% bootstrap value).

RAPD analysis. 135 *C. pratensis* s.l. individuals from 104 populations (Table 1) were analysed with 20 RAPD primers selected from a 125 primer screening (see material and methods). Eleven primers revealed a total of 13 RAPD markers which are in accordance with our character assessment principles (see above). The resulting presence/absence data

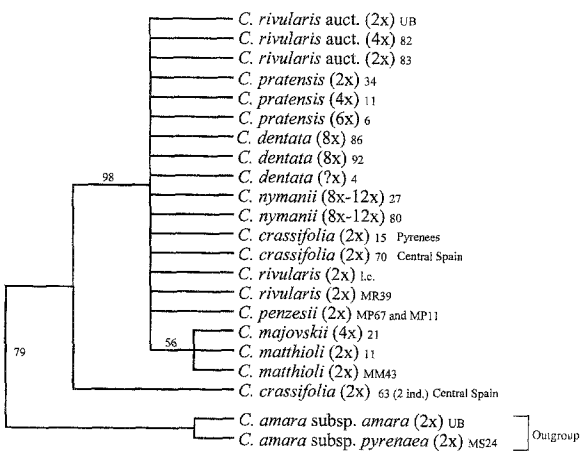

Fig. 3. Strict consensus of 90 most parsimonious trees based on *Cardamine pratensis* s.l. *trn*T/L spacer, *trn*L intron, and *trn*L/F spacer sequence data. Gaps were treated as missing data. The bootstrap support is shown above branches. Tree length: 65, consistency index (CI): 0.83 with autapomorphies excluded. For accession numbers see Table 1. Ind. = individuals

matrix is given in Table 4. Three of the thirteen characters showed unique states for each of two taxa (two for *C. crassifolia*, one for *C. penzesii*). There were no additive character states in the polyploids. The generated Neighbor-Joining phenogram is shown in Fig. 4. Only groupings supported by bootstrap values higher than 70% are given. A basal group consists of *C. rivularis* and *C. penzesii*. The remaining taxa are divided in *C. crassifolia* and a poorly resolved group. In the latter group the following pairs of taxa are clustered with bootstrap values ≥76%: 1.) *C. nymanii*/*C. dentata*, 2.) *C. matthioli*/*C. majovskii*, 3.) *C. pratensis* s.str./*C. udicola*, and 4.) *C. rivularis* auct. (Ukraine)/*C. rivularis* auct. (Switzerland).

Discussion

1. Taxonomy of the Cardamine pratensis complex

Numerous contributions during the last four decades have extended our knowledge about the *Cardamine pratensis* group considerably. They focus on certain taxa and/or concentrate on restricted geographic regions: Belgium and North France: Vyvey and Stieperaere (1984); the Asiatic part of the former Soviet Union: Khatri (1989); England: Dale and Elkington (1974); The Netherlands: Berg (1967); Swiss Alps and Jura Mountains: Landolt and Urbanska-Worytkiewicz (1971); Switzerland: Landolt (1984); diploid taxa from central Europe: Urbanska-Worytkiewicz and Landolt (1974), Dersch (1969); Carpathians and Pannonia: Marhold (1994a). However the most influential paper remains that of Lövkvist (1956). It is still the only coherent systematic concept for the whole complex available. Nonetheless, we have to consider the progress in systematic knowledge of the *C. pratensis* complex achieved in the meantime when discussing our results and conclusions in context with the systematic and biogeographical outlines of Lövkvist. We shortly comment on the present taxonomic status of the *Cardamine pratensis* s.l. taxa.

Table 4. Distribution of taxonspecific RAPD markers in *Cardamine pratensis* s.l. *Cardamine rivularis* auct. plants analysed were attributed to three separate OTUs according to their place of origin

Taxon	n ind./ n pop.	Character no.[a)]												
		1	2	3	4	5	6	7	8	9	10	11	12	13
C. matthioli (2x)	16/16	0	1	1	0	1	0	0	0	0	1	1	1	0
C. majovskii (4x)	8/3	0	1	1	0	1	0	0	0	0	1	1	1	0
C. pratensis (2x/4x/6x)	33/32	0	1	0	1	1	0	0	1	0	1	1	1	0
C. udicola (?x/4x)	5/4	0	1	0	1	1	0	0	1	0	1	1	1	0
C. rivularis auct. (2x/4x) A	6/3	0	1	0	1	1	0	0	0	0	1	1	1	0
C. rivularis auct. (2x) CH	2/1	0	0	0	1	1	1	0	0	0	1	1	1	0
C. rivularis auct. (?x) UKR	2/1	0	0	0	0	1	1	0	0	0	1	1	1	0
C. nymanii (8x–12x)	7/4	0	1	1	1	1	0	0	0	0	1	1	1	0
C. dentata (7x/8x)	7/6	0	1	1	1	1	0	0	0	0	1	1	1	0
C. crassifolia (2x)	28/17	1	0	0	0	0	0	0	1	0	1	0	0	0
C. penzesii (2x)	10/4	0	0	0	0	0	0	1	1	1	0	1	1	1
C. rivularis (2x)	11/3	0	0	0	0	0	0	0	0	1	0	0	1	1

Note: A = Austria, CH = Switzerland, UKR = Ukraine; n ind./n pop. = number of analysed individuals and populations

[a)] Characters 1–3 are RAPD markers of the Operon primer B3, character 4 is a RAPD marker of Operon primer H5, character 5 of H14, character 6 of L1, character 7 of N1, character 8 of R6, character 9 of R17, character 10 of R19, character 11 of S9, character 12 of Y8, and character 13 of Y20. Primer designation according to the supplier "Operon"

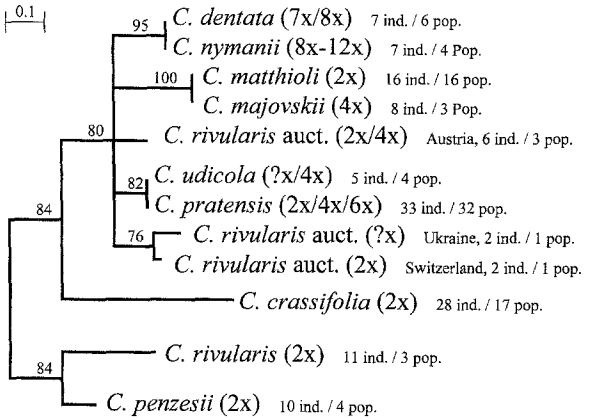

Fig. 4. Neighbor-Joining phenogram of RAPD characters in *Cardamine pratensis* s.l. taxa. Numbers above branches are bootstrap values. Groups with bootstrap support ≤70% are drawn as unresolved. n ind./n pop. = number of analysed individuals and populations

***Cardamine penzesii* Ančev & Marhold.** *Cardamine penzesii* was not known to Lövkvist (1956). In 1960 Pénzes and Vida (Pénzes 1965) collected diploid plants (2n = 16) in Bulgaria and described them as *C. tuberosa* Pénzes & Vida, a new species of the *C. pratensis* complex. The taxon remained forgotten in the following time until it was rediscovered by Marhold and Ančev in the years 1996 and 1997 at localities from the Bulgarian Black Sea Coast (Marhold and Ančev 1999). The taxon was renamed *C. penzesii* Ančev & Marhold because the name *C. tuberosa* Pénzes & Vida is illegetimate. The chromosome counts of Marhold and Ančev confirmed a diploid cytotype with 2n = 16 for *C. penzesii*. The presence of globular tubers on the rhizomes seems to be a unique character of *C. penzesii*. This taxon is like other central and/or southeast European taxa (*C. rivularis* Schur, *C. matthioli*, *C. majovskii*) characterised by appressed hairs on the rhachis.

***Cardamine rivularis* Schur and *Cardamine rivularis* auct. non Schur.** Lövkvist (1956) "provisionally" named plants of the *Cardamine pratensis* complex occurring in the subalpine belt of the Alps as *C. rivularis* Schur as was done before by Kerner (1883) and Lindmann (1914) and later by Jones (1964), Urbanska-Worytkiewicz and Landolt (1974), and Landolt (1984). The description of Schur (1852), however, referred to plants from Transsilvania. The taxonomic studies of Marhold and Rayner (1994) and Marhold (1994a, 1995, 1996) revealed that *C. rivularis* Schur is restricted to the South Carpathians and Bulgaria and differs from the plants of the Alps in having purplish (*versus* yellow) anthers and appressed (*versus* patent) hairs on the rhachis. The mountain populations previously treated as *C. rivularis* were provisionally named *C. rivularis* auct. non Schur by Marhold (1994a). Plants of the *C. rivularis* auct. type occur in the Alps, the Carpathians, the Apennines, the Massiv Central, the Vosges, and possibly in the Pyrenees (Marhold 1993). It is unsettled whether these populations should be treated as one or several taxa or whether they should be included in *C. pratensis* s.str. given the fact that they do not have "any reliable differential character" (Marhold 2000).

***Cardamine crassifolia* Pourret.** *Cardamine crassifolia* is a long known species, and was described in 1788 by Pourret. It has a disjunct distribution area occurring in the Pyrenees and central Spain. It is a diploid species with 2n = 16 and has a repent rhizome. This morphological character is unique within the *C. pratensis* complex. We analysed plants from both parts of the disjunct distribution of *C. crassifolia*.

***Cardamine matthioli* Moretti and *Cardamine majovskii* Marhold & Záborský.** According to Marhold 1994a the diploid *C. matthioli* occurs in central and southern Europe, the Carpathians, Pannonia and south of the Alps (from Piemont in the west to Romania and Bulgaria in the east). Apart from 2n = 16, also an aneuploid chromosome number with 2n = 18 was occasionally reported (Czechia: Javůrková (1986); Slovakia, Ukraine: Marhold (1994b); Hungary: Marhold (1991); Austria: Marhold (2000)). The tetraploid *C. majovskii* (2n = 32) was not known to Lövkvist (1956). This species was described in 1986 by Marhold and Záborský based on populations from

Table 5. List of GenBank accession numbers

Taxa*	ITS 1	ITS 2	GenBank accession numbers			
			*trn*T/L spacer 5′ end	*trn*T/L spacer 3′ end	*trn*L intron	*trn*L/F spacer 5′ end
C. amara subsp. *amara* UB	AF077993	AF077994	AF078485	AF078486	AF079334	AF266582
C. amara subsp. *balcanica* MB41	AF265184	AF265204	–	–	–	–
C. amara subsp. *pyrenaea* MS24	AF265183	AF265203	AF266619	AF266617	AF266633	AF266583
C. crassifolia 102	AF265180	AF265200	–	–	–	–
C. crassifolia 15	–	–	AF266625	AF266613	AF266639	AF266592
C. crassifolia 63	AF265179	AF265199	AF266626	AF266612	AF266640	AF266593
C. crassifolia 70	AF077983	AF077984	AF078475	AF078476	AF079329	AF266594
C. crassifolia MC87	AF265674	AF265680	–	–	–	–
C. dentata 4	–	–	AF266620	AF266618	AF266634	AF266584
C. dentata 86	AF265169	AF265188	AF266621	AF266616	AF266635	AF266586
C. dentata 92	AF265672	AF265677	AF078481	AF078482	AF079332	AF266585
C. granulosa 121	–	AF265196	–	–	–	–
C. majovskii 21	AF077987	AF077988	AF078479	AF078480	AF079331	AF266599
C. majovskii 22	AF270701	AF270702	–	–	–	–
C. matthioli 11	AF077985	AF077986	AF078477	AF078478	AF079330	AF266597
C. matthioli 2	AF265673	AF265679	–	–	–	–
C. matthioli MM17	AF265176	AF265195	–	–	–	–
C. matthioli MM43	AF265175	AF265194	AF266628	AF266605	AF266642	AF266598
C. nymanii 27	AF265177	AF265197	AF266624	AF266607	AF266638	AF266590
C. nymanii 80	AF077991	AF007992	AF078483	AF078484	AF079333	AF266591
C. nymanii MW74	AF265178	AF265198	–	–	–	–
C. penzesii MP11	AF265675	AF265681	AF266630	AF266610	AF266644	AF266601
C. penzesii MP67	AF265182	AF265202	AF266629	AF266611	AF266643	AF266600
C. pratensis 11	–	–	AF266631	AF266609	AF266645	AF266602
C. pratensis 117	AF265171	AF265190	–	–	–	–
C. pratensis 34	AF077977	AF077978	AF078469	AF078470	AF079326	AF266603
C. pratensis 39	AF265170	AF265189	–	–	–	–
C. pratensis 59	AF265173	AF265192	–	–	–	–
C. pratensis 6	AF265172	AF265191	AF266632	AF266608	AF266646	AF266604
C. rivularis auct. 83	AF077979	AF077980	AF078471	AF078472	AF079327	AF266588
C. rivularis l.c.	AF077981	AF077982	AF078473	AF078474	AF079328	AF266595
C. rivularis MR39	AF265181	AF265201	AF266627	AF266606	AF266641	AF266596
C. udicola 47	AF265174	AF265193	–	–	–	–
C. udicola 48	AF265676	AF265678	–	–	–	–
C. rivularis auct. 82	AF265167	AF265186	AF266623	AF266614	AF266637	AF266589
C. rivularis auct. 85	AF265168	AF265187	–	–	–	–
C. rivularis auct. UB	AF265166	AF265185	AF266622	AF266615	AF266636	AF266587

* Note: For accession numbers see Table 1

eastern Slovakia. *Cardamine majovskii* is morphologically more similar to *C. matthioli* than to *C. pratensis* (Marhold and Záborský 1986). Meanwhile *C. majovskii* was also reported from Hungary, Romania, and Austria (Marhold 1991, 1996, 2000). Based on extensive morpho-

metric analyses *C. majovskii* was supposed to be an autotetraploid derivative that arose directly from *C. matthioli* (Marhold 1996).

***Cardamine granulosa* Allioni.** *Cardamine granulosa* was described by the Italian botanist Allioni in 1789. The description referred to populations in wet meadows from the surroundings of Torino. Reports from other regions of Italy are regarded doubtful. *Cardamine granulosa* is taxonomically characterised by its basal leaves being simple or with only 3–5 leaflets. A second morphological feature are roots bearing occasionally granules at their distal parts. Lövkvist (1956) could not obtain living material and speculated that *C. granulosa* had died out. Our serious attempts to find living plants at formerly known localities were also without success. The last documented discoveries were reported by Urbanska-Worytkiewicz and Landolt (1974). Cytological investigations of these authors revealed a diploid cytotype with 2n = 16 as was previously supposed by Lövkvist (1956) based on pollen diameter measurements. The plants investigated by Urbanska-Worytkiewicz and Landolt, however, did not have the above mentioned granules. We analysed herbarium material and could only sequence the ITS2 region.

***Cardamine nemorosa* Lejeune.** In 1813 Lejeune described *C. nemorosa* and *C. latifolia* from forests in the surroundings of Malmedy (Belgium). Later in 1831 Lejeune and Courtois treated these taxa as *C. pratensis* var. *nemorosa* and *C. pratensis* var. *latifolia*. Lövkvist (1956) applied these names to forest plants from the French Jura. The name *C. pratensis* var. *nemorosa* was used by Lövkvist (1956) for plants which were determined to be diploid by Guinochet (1946). These forms grew in forests but also in meadows. The name *C. pratensis* var. *latifolia* was used by Lövkvist (1956) for tetraploid forest plants. He supposed the tetraploids to be autopolyploid derivatives of the diploids. He stressed that these tetraploids are a doubtful taxon, being not able to separate them morphologically from other tetraploids. Later, diploid *C. pratensis* taxa from forests of the Jura Mountains were treated as *C. nemorosa* (Landolt and Urbanska-Worytkiewicz 1971, Urbanska-Worytkiewicz and Landolt 1974), as were even diploids from forests of Lower Saxony and Hesse in Germany (Dersch 1969). Urbanska-Worytkiewicz and Landolt (1974) and Dersch (1969) emphasised that it is hardly ever possible to distinguish *C. nemorosa* from *C. pratensis* morphologically. Consequently Landolt (1984) proposed to include this taxon in *C. pratensis* s.str.. We also analysed taxa formerly treated as *C. nemorosa* and were not able to characterise these plants by any molecular marker. Following Landolt (1984) we included these plants in *C. pratensis* s.str.. In Table 1, the plants concerned are indicated.

***Cardamine pratensis* L.** *Cardamine pratensis* s.str. is the most widespread taxon of the *C. pratensis* group. It occurs throughout most of Europe except the extreme North and South. "South of the Pyrenees, the Alps, and the Carpathians it is rare. Its distribution extends eastwards to Siberia and the Far East and southwards to North Africa" (Marhold 1994a). It was also introduced to North America (Fernald 1920). The taxon comprises three cytotypes: diploids, tetraploids, and hexaploids. Diploid taxa from forests ("*C. nemorosa*") are already discussed. Diploid populations from meadows were reported by Urbanska-Worytkiewicz and Landolt (1974) from the surroundings of Lake Constance. Lövkvist (1956) analysed a diploid population from Portugal. DeLanghe and D'Hose (1976) described diploid populations from Belgium characterised by a sharp taste as *C. pratensis* subsp. *picra*. Further diploids from Belgium and northern France were reported by Vyvey and Stieperaere (1984). The most common cytotype of *C. pratensis* s.str. is the hypotetraploid with 2n = 30 which was probably the type material for *C. pratensis* L. (Marhold 1994a). Hexaploid cytotypes are rather rare. We will not list the numerous reports of aneuploid counts which also showed intrapopulational variation. Berg (1967) and Vyvey and Stieperaere (1984) even reported intraindivid-

ual variability of chromosome numbers. There is no obvious correlation between cytotypes and morphology but the ploidy level may be influenced by ecological factors. Based on investigations by Guinochet (1946), Lövkvist (1947), Banach (1950), and Berg and Segal (1966) it would appear that high chromosome numbers might be correlated with the water content of the soil. Lövkvist (1956) detected populations with variable chromosome numbers in disturbed habitats. We analysed representatives from all three cytotypes.

***Cardamine udicola* Jordan.** *Cardamine udicola* was described by Jordan (1860) from the vicinity of Lyon. Urbanska-Worytkiewicz and Landolt (1974) transferred this name to plants from Switzerland, Upper Bavaria, Poland, and former Czechoslovakia that are morphologically characterised by a terminal leaflet of the second uppermost cauline leaf that is 3/4–1(1/4) as long as the rest of the leaf. Chromosome counts by Urbanska-Worytkiewicz and Landolt (1974) revealed diploid populations (2n = 16) and mixed stands with 2n = 16 and 2n = 32 plants. The taxon *C. udicola* is not generally accepted (see Marhold 1994a). We analysed plants from localities in the surroundings of Lake Thun (Switzerland) that were identified by Urbanska-Worytkiewicz and Landolt (1974) as *C. udicola*.

***Cardamine dentata* Schultes.** Schultes (1809) when describing *C. dentata* separated it from *C. pratensis* s.str. because of the stalked leaflets of the cauline leaves and because of alleged stolons. However, he mistook rooting rosette leaves for stolons. This caused some confusion and led to multiple descriptions of this taxon (Marhold 1994a). Lövkvist (1956) used the description of Petermann (1846) for *C. palustris* Petermann who distinguished two varieties of *C. pratensis* s.l. plants with stalked leaflets of the cauline leaves: *Cardamine palustris* var. *isophylla* characterised by cauline leaves with dentate leaflets, and *C. palustris* var. *heterophylla* with entire leaflets. Chromosome counts by Lövkvist (1956) revealed 56, 64, 72, 76, 80, and 96 as somatic numbers. Plants with 2n = 76 were of the var. *isophylla* type. Lövkvist did not find sterility barriers between octo- and decaploids and regarded the whole aggregate of octo-, deca- and dodecaploid cytotypes as "one wide species". In the wild, the different cytotypes often grow together. This broad concept is adopted in the Flora Europaea (Marhold 1993). *Cardamine dentata* Schultes grows in very wet places, and "occurs in central and north-western Europe, to the north of the River Po and the Danube Basin; it extends eastwards to Siberia, the Far East, the Kamčatka Peninsula and Kuril Islands" (Marhold 1994a). We analysed plants with 2n = 56 and 2n = 64, and one plant with an unknown chromosome number.

***Cardamine nymanii* Gandoger.** *Cardamine nymanii* is a highly polyploid complex of arctic circumpolar distribution. Cytotypes with 2n = 64 and 2n = 80 chromosome numbers are widespread and cannot be distinguished from each other by their morphology. Plants in populations with varying chromosome numbers (reported are 60, 72, 75, 80, 90) differ in their shape of rosette leaves from the octoploids (leaflets of basal leaves lanceolat *versus* orbicular). Lövkvist (1956) supposed that these plants go back to hybrid events between octo- or decaploid plants and a (postulated) dodecaploid cytotype which, however, was never reported. Our analysed accessions belonged to the most common morphotype with orbicular leaflets.

2. *Molecular systematics of the* Cardamine pratensis *complex*

Observed differences of the noncoding cpDNA sequences (*trn*T/L spacer, *trn*L intron, *trn*L/F spacer) were in the range of 1%, and not enough mutations in cpDNA had accumulated yet to resolve the phylogenetic relationships within the complex (Fig. 3). Variation of the ITS sequences was ca. fourfold higher (0–4.6%), and the ITS phylogeny therefore was better resolved than the cpDNA tree. The best resolution was finally achieved by using RAPD-markers.

ITS and RAPD data clearly support a close relationship between *C. rivularis* and *C. penz-*

esii whereas in the allozyme-based phenogram *C. rivularis* was placed next to *C. crassifolia* but still clustered with *C. penzesii* in a separate clade (Fig. 1). It would appear that these different placements in the allozyme phenogram are not of major significance, at least they should not be overemphasised since allele frequencies may depend on single population histories and may be affected by sampling errors. The different positions of the individual *C. crassifolia* accessions in the cpDNA tree (Fig. 3), however, seem to pose more serious implications and will be discussed later on. A synopsis of our molecular data evaluation resulted in the phylogenetic concept presented in Fig. 5. It is compared with the concept of Lövkvist (1956).

2.1 Two old lineages: the Basal Group and the Cardamine crassifolia Lineage

2.1.1 The Basal Group: *Cardamine rivularis* and *C. penzesii*. *Cardamine rivularis* and *C. penzesii* are closely related and form the Basal Group of the *C. pratensis* complex which is clearly supported by ITS (Fig. 2), RAPD (Fig. 4), and allozyme data (Fig. 1). Both species were not known to Lövkvist (1956) as discussed above. *Cardamine penzesii* was discovered only later (Pénzes 1965, Marhold and Ančev 1999). Our molecular data clearly provide evidence that *C. rivularis* sensu Marhold is different from plants of the *C. rivularis* auct. type (Fig. 5). *Cardamine rivularis* and *C. penzesii* are characterised by appressed hairs on the leaf rhachis (Marhold and Ančev 1999). As this character is shared with *C. matthioli* and *C. majovskii*, Marhold and Ančev (1999) discussed a common origin of the four species. This hypothesis, however, is not supported by our molecular data. *Cardamine matthioli* and *C. majovskii* are members of the Derived Group and are not closer related to *C. rivularis* and *C. penzesii* (Figs. 1, 2 and 4). Appressed hairs, therefore, seem to have evolved convergently.

2.1.2 The *Cardamine crassifolia* Lineage. According to Lövkvist's concept, the diploid *C. crassifolia* (2n = 16), which is the only representative of his Repent Rhizome Group (see Fig. 5), is phylogenetically older

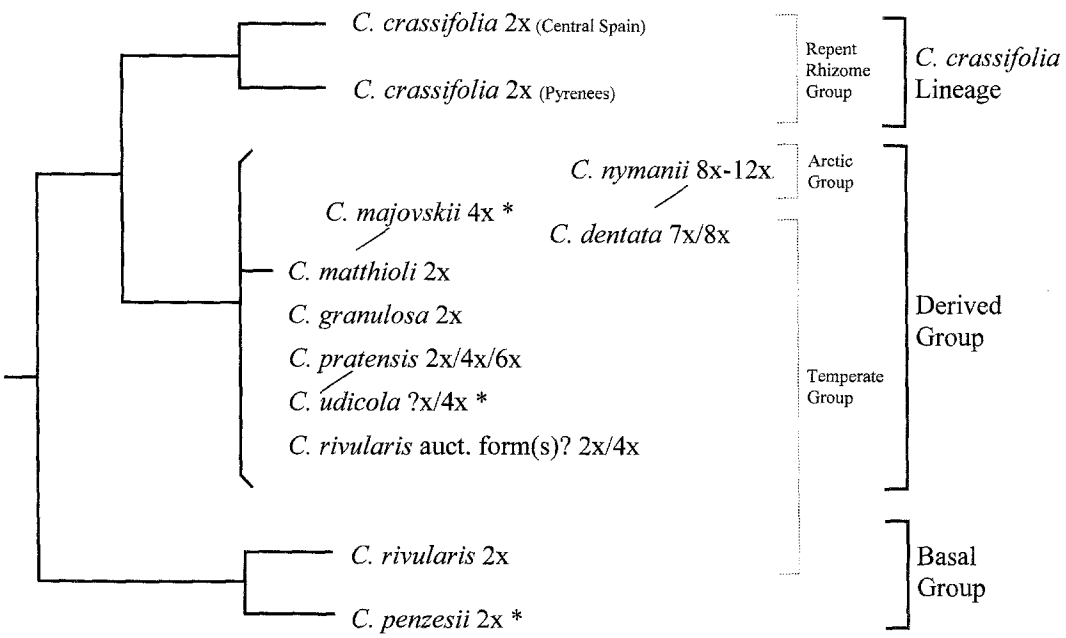

Fig. 5. Phylogenetic concept of the *Cardamine pratensis* group based on molecular data. The systematic concept of Lövkvist (1956) is indicated by broken lines. Taxa with an asterik (*) were not recognised by Lövkvist (1956). For details see text

than all taxa of his Temperate Group. Today's disjunct distribution of *C. crassifolia* (Pyrenees and central Spain) was interpreted by him as a result of range fragmentation due to Pleistocene glaciation events.

Cardamine crassifolia is well characterised by our molecular data. RAPD and allozyme analyses revealed unique characters (Tables 4 and 2), and *C. crassifolia* was monophyletic with 93% bootstrap support in the ITS tree (Fig. 2). However, the individual accessions were divided into two groups with 68% bootstrap support in the ITS tree (Fig. 2). These groups correspond to the disjunct distribution areas of this species (Pyrenees and central Spain) and are in favour of a relatively high age of this taxon, thus supporting the view of Lövkvist (1956). In our phylogenetic concepts, therefore, *C. crassifolia* represents a basal lineage of the *C. pratensis* group (Fig. 5) which we will call the *Cardamine crassifolia* Lineage.

In contrast to the ITS data, *C. crassifolia* is not monophyletic in the cpDNA consensus tree (Fig. 3). Both individuals analysed from population 63 (central Spain) were separated with 98% bootstrap support from all the other individuals analysed, and even from those of another population from central Spain. We explain the "foreign" cpDNA by "chloroplast capture" as a result of an (unknown) hybridization event (Rieseberg and Soltis 1991).

2.2 The Derived Group

The Derived Group includes diploid and all polyploid taxa (see Fig. 5). Diploids with 2n = 16 chromosomes are *Cardamine granulosa* and *C. matthioli*, cytotypes of *C. rivularis* auct. non Schur, cytotypes of *C. pratensis* s.str. and cytotypes of *C. udicola*. The polyploid taxa are *C. majovskii*, *C. dentata* and *C. nymanii*, as well as cytotypes of *C. pratensis* s.str., of *C. udicola*, and of *C. rivularis* auct. (Fig. 5). The affiliation of *C. granulosa* with the Derived Group is undisputed though confirmed by ITS2 sequences only.

The diploid *C. matthioli* and the tetraploid *C. majovskii* are closely related (ITS, Fig. 2 and cpDNA, Fig. 3). This corresponds to the findings of Urbanska-Worytkiewicz and Landolt (1974) who confirmed fertility barriers between this lineage and other diploid taxa (*C. granulosa*, *C. rivularis* auct. and *C. nemorosa*). Based on morphometric analyses, Marhold (1996) thought *C. majovskii* to be an autotetraploid of *C. matthioli*. Interestingly, *C. matthioli* and *C. majovskii* form a weakly supported clade (< 50% bootstrap value) in the ITS tree (Fig. 2), with the former taxon being paraphyletic with respect to the latter. This would be expected from an ideal progenitor-derivative species pair. Additionally the allele *Aat2-7* was exclusively shared by these two species (Table 2).

Cardamine pratensis s.str. and *C. udicola* are very close. It was not possible to recognise different cytotypes of *C. pratensis* by using molecular markers nor were the molecular data in favour of a separate taxon *C. udicola*. Even the RAPD data showed high similarities between *C. pratensis* s.str. and *C. udicola* (Fig. 4, Table 4).

Strong affinity between the two highly polyploid taxa *C. nymanii* and *C. dentata* is evidenced by the RAPD data (Fig. 4). This supports the view of Khatri (1989) who stated that *C. nymanii* and *C. dentata* are "undoubtly direct derivatives of *C. pratensis*".

The phylogenetic position of *C. nymanii* is crucial when comparing our concept with that of Lövkvist (1956). Lövkvist was convinced that *C. nymanii* represents a distinct basal lineage within the whole *C. pratensis* complex which he named the Arctic Group and which "showed the main features of its present distribution even in late Tertiary". There is no evidence from our molecular data that *C. nymanii* is a distinct and/or basal lineage of the *C. pratensis* complex. *Cardamine nymanii* clearly is a representative of our Derived Group which consists of very closely related taxa (Fig. 5).

2.3 Differentiation in the Derived Group

Cardamine matthioli and its polyploid derivative species *C. majovskii* represent a well

characterised lineage within the Derived Group. The widespread *C. dentata* aggregate (7x to 12x) has affinities to the *C. nymanii* aggregate (8x, 10x) as supported by our RAPD data and by morphological characters (Khatri 1989). Both taxa may be descendants of the *C. pratensis* s.str. aggregate. In England hexaploid plants occur, which by their cytotype have to be treated as *C. pratensis* s.str. in the sense of Lövkvist (1956) but which by their morphology and ecology resemble *C. dentata* (Dale and Elkington 1974). *Cardamine pratensis* s.str. is the most widespread aggregate with diploid, tetraploid and hexaploid cytotypes. Diploid taxa like *C. nemorosa*, *C. udicola*, and *C. rivularis* auct. non Schur can hardly be separated from *C. pratensis* s.str.. Cytotaxonomic studies of different cytotypes of *C. pratensis* s.l. in the Netherlands lead to the assumption of gene flow across ploidy levels (Berg 1967).

No clear-cut morpho-, eco-, and cytological patterns can be recognised for the taxa within and around the *C. pratensis* s.str. aggregate. We rather deal with a continuum of forms whose characters more or less blend. This argues for a relatively young age of this association of cyto-, morpho- and ecotypes. None of the molecular markers provide evidence for hybridisation between distinct units. This is opposite to the findings in other polyploid *Cardamine* taxa which reported additive isozyme patterns (Urbanska et al. 1997), additive RAPD markers (Neuffer and Jahncke 1997), and multiple character states in ITS sequences (Franzke and Mummenhoff 1999). Knowing the problems of terminology, we nevertheless suggest to characterise the polyploid cytotypes of the *C. pratensis* complex as "autopolyploids".

3. Historical biogeography of the Cardamine pratensis complex

Climatic changes during and/or after the ice ages have played a major role in vegetation history and evolution of plant species, particularly in Europe (Lang 1994). Vegetation belts shifted from north to the south and from higher to lower altitudes and *vice versa*. The ranges of species were fragmented. Species died out, migrated and/or survived in refuge areas from where they expanded their distribution range or where they still live as relicts.

Southern and southeastern Europe were refuge areas for many higher plants, mainly trees (Lang 1994, Gliemeroth 1995). The ice-free parts of northern and middle Europe were covered by a tundra or cold steppe vegetation. "Massifs de refuge" may have existed in glaciated mountain ranges of the Alps as revealed by the works of Gams (1936), Merxmüller (1952, 1953, 1954), and Niklfeld (1972). Contrasting hypotheses of either glacial survival (nunatak hypothesis) or postglacial immigration (tabula rasa hypothesis) have been put forward for Scandinavia (Dahl 1987), and are currently in the interest of studies on molecular phylogeography (e.g. Gabrielsen et al. 1997 for *Saxifraga oppositifolia*). Reconstruction of the history of migration patterns is traditionally based on palaeobotanical records and paleoecological investigations (e.g. Gliemeroth 1995 for European tree species) but molecular data are gaining equal importance (see references in Comes and Kadereit 1998).

Late Quaternary climatic changes had great influence on the diversification and geographical distribution pattern of the *Cardamine pratensis* complex. This view was already expressed by Lövkvist (1956), Urbanska-Worytkiewicz and Landolt (1974), and by Khatri (1989). We recorded ITS sequence differences in the *C. pratensis* complex of 0–4.6%. This is comparable to the range reported by Hungerer and Kadereit (1998) and Comes and Kadereit (1998) for *Gentiana* species (1.3–3%). Based on the magnitude of the ITS sequence differences, these authors hypothesised that diversification of *Gentiana* Section *Ciminalis* took place during the late Quaternary. Adopting these estimation procedures for *Cardamine pratensis* s.l., what certainly is problematic, would also point to a rather young phylogenetic age (late Quaternary).

The southeastern European *C. rivularis* and *C. penzesii* represent a relatively old lineage within the *C. pratensis* group which we called the Basal Group (Fig. 5). We assume that during (early) glaciations southeastern Europe provided refuge areas for some *C. pratensis* s.l. populations from which the two extant species may have evolved. We have no evidence for gene flow from the Basal Group to the Derived Group. This is also true for the *C. crassifolia* Lineage, the other basal lineage (Fig. 5). It probably evolved from refugial populations on the Iberian Peninsula. The present day disjunct distribution in the Pyrenees and central Spain may point to a formerly wider distribution, and the molecular differentiation between the two parts of the area argues for a relatively high age of this lineage. It appears from our molecular data that *C. crassifolia* just like the Basal Group was not involved in the evolution of the Derived Group. The Derived Group includes all polyploid taxa of the whole *C. pratensis* complex and a number of diploid taxa (Fig. 5) reported from different geographic regions as discussed above. Partly, the distributions of the diploids coincide with supposed glacial refuge areas: localities of *C. matthioli* with refugia (south) east of the Alps (Gams 1936), localities of *C. rivularis* auct. with nunataks in the eastern Alps (Koralpe, Van Husen 1987), *C. granulosa* south of the Alps. We assume that the diploids of the Derived Group survived the glaciations in different refugia in central Europe centered within and round the Alps. It were only these diploids which gave rise to the polyploid taxa of the whole *C. pratensis* complex as evidenced by our molecular data. Polyploidisation occurred several times and with all probability by a process which is best described as "autopolyploidisation" as discussed above. The highly polyploid taxa *C. dentata* and *C. nymanii* are in all probability of postglacial origin, biotypes in the sense of Ehrendorfer (1962) that colonised newly available habitats opened up by the retreating glaciers. Lövkvist (1956) considered *C. nymanii* as a basal lineage of the *C. pratensis* complex, that "showed the main features of its present distribution even in late Tertiary". He ruled out a postglacial colonisa-

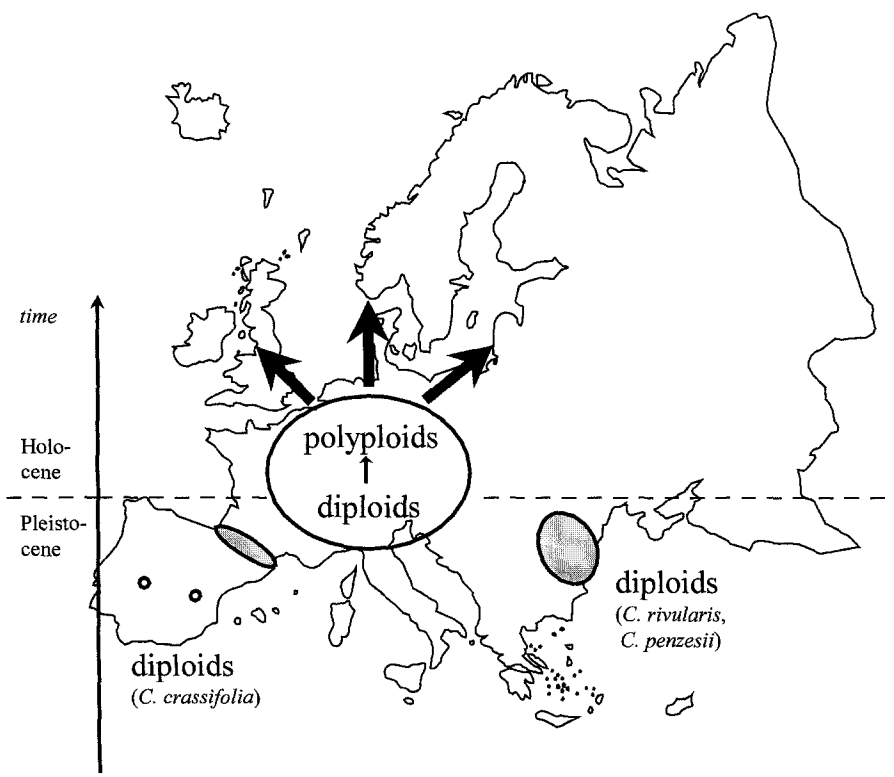

Fig. 6. Historical biogeographic concept of the *Cardamine pratensis* complex: Taxa with grey shaded distribution areas represent diploid species of Pleistocene origin which were *not* involved in the formation of polyploid *Cardamine* s.l. taxa. The polyploids arose from another group of diploids in postglacial times and colonised newly available habitats. For details see text

tion of Scandinavia from the south. This is not in agreement with our molecular data. We conclude that *C. nymanii* immigrated to Scandinavia and to the whole arctic-circumpolar region in postglacial times from south to north. Our biogeographic concept for the *C. pratensis* complex is outlined in Fig. 6.

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