Are there any trees in the Arctic? Reconstruction of evolutionary histories in a young biome

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Guard: Found them? In Mercia? The coconut's tropical!

King Arthur: What do you mean?

G: Well, this is a temperate zone

KA: The swallow may fly south with the sun or the house martin or the plover may seek warmer climes in winter, yet these are not strangers to our land?

G: Are you suggesting coconuts migrate?

KA: Not at all. They could be carried.

G: What? A swallow carrying a coconut?

KA: It could grip it by the husk!

Monty Python on plant dispersal

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Summary

Reconstructing molecular phylogenies and unraveling biogeographic histories of arctic plants are needed to obtain better insights into the processes of evolution, dispersal and colonization in this young biome. Studies of dispersal into, and speciation within, the Arctic are important to obtain better knowledge of the source areas for arctic biodiversity. Unraveling the history of recently diverged lineages such as those typical for the young arctic biome is challenging, because it is difficult to find molecular markers with sufficient variation and to handle the problem of incomplete lineage sorting and hybridization. Thus several different molecular marker systems for many potentially suitable model groups were tested and developed. Three genera which are represented in Beringia (the Asian and American land masses surrounding the Bering Strait from Lena River to Mackenzie River) and which have assumed phylogenetic connections to plants living in East Asia and North America were selected. Two of these genera are also good candidates for studying polyploidization as a mode of speciation in the Arctic, as they show large variation in chromosome number within and among the species. This study was also intended to contribute to the PanArctic Flora project by providing data to help resolving the taxonomy of some challenging species complexes.

- The history of the genus *Smelowskia* was reconstructed based on microsatellite loci combined with sequences of nuclear and plastid regions. An Asian origin of the genus and two independent dispersal events into the Beringian and North American regions were inferred. We also found evidence for merging the Beringian *S. porsildii, S. spathulatifolia,* and *S. jurtzevii* into one species; *S. porsildii*.

- The biogeography and phylogeny of the large genus *Cardamine* were inferred based on nuclear and plastid sequences. The phylogenetic trees showed limited resolutions, supporting a hypothesis of recent and rapid speciation in the genus. We found evidence for several extremely long-distant dispersal events. Dispersal into the Southern Hemisphere and the Arctic has occurred repeatedly, and we identified at least three phylogenetically distinct arctic lineages. Polyploidization has occurred independently many times during the evolution of *Cardamine*. Rapid divergence combined with widespread polyploidization offer an explanation for the complex evolutionary history of the genus. Two species complexes within this genus were selected for more detailed studies.

- Six microsatellite loci originally developed for the *Arabidopsis* genome, were used to identify evolutionary and taxonomic units within the Beringian *Cardamine digitata* aggregate. Molecular groups corresponding to morphological differences suggested recognition of four species in this complex; *C. blaisdellii*, *C. digitata*, *C. microphylla*, and *C. purpurea*. Each species included at least two ploidy levels, indicating recurrent polyploidizations.

- As a first step towards addressing the origin of the circumarctic *Cardamine bellidifolia*, we conducted a study with main focus on its two putatively most closely related European alpine species (*C. alpina* and *C. resedifolia*) using AFLPs. Surprisingly, the arctic species C. *bellidifolia* was distinctly differentiated from its putative alpine ancestral lineages. Contrasting phylogeographies were inferred between the two alpine species *C. alpina* and *C. resedifolia*. A high degree of genetic distinction was found between the Alpine and Pyreneean populations of *C. alpina*. In addition, a high level of diversity was found within Pyreneean populations compared to Alpine populations. In contrast, *C. resedifolia* showed more genetic variation among populations in the Alps than between the Alps and distant areas such as Corsica, the Carpatians and the Pyrenees. These results show that the two species have very different histories of glacial survival and recolonization.

- To facilitate these and future studies of recently diverged taxa, we developed 72 new microsatellite loci and tested 15 previously published loci for the Brassicaceae. We found them to provide variation among and within three distantly related genera: *Cardamine, Smelowskia,* and *Draba*. Of these 87 loci, 18 were variable within *Cardamine*, while ten were variable within *Smelowskia*. Seventy-one of these primers were variable within *Draba*, and 50 were variable within *Draba nivalis*. The markers amplifying across these genera are potentially suitable for studying other genera in Brassicaceae as well.

- A phylogeographic analysis of *Cassiope tetragona* including both Central Asian and Beringian relatives revealed that the circumpolar ssp. *tetragona* was well separated from the North American ssp. *saximontana*. A Beringian origin of *C. tetragona* ssp. *tetragona* was inferred, and the levels and geographical patterns of differentiation and gene diversity suggested that the latest expansion from Beringia into the Circumarctic was recent, possibly during the current interglacial. The results were in accordance with a recent leading-edge mode of colonization, particularly towards the east throughout Canada/Greenland and across the North Atlantic into Scandinavia and Svalbard.

Sammendrag

Få detaljerte studier av spredning til og artsdannelse innen arktis er gjort. Slektskap og biogeografiske mønstre må analyseres for å forstå hvordan spredning og evolusjon foregår innen det arktiske området, og for å finne opphavet til den arktiske biodiversiteten. Å finne de evolusjonære historiene i arktis er utfordrende siden det ofte er lite variasjon i vanlige molekylære markører, og hybridiseringer og ufullstendig lineage sorting skaper ytterligere problemer. Flere ulike molekylære markørsystemer for mange potensielt passende modellgrupper ble derfor testet og utviklet. I dette studiet er det valgt ut tre planteslekter som alle har utbredelse i Beringia i tillegg til andre deler av Øst-Asia og Nord-Amerika. To av de valgte slektene har stor variasjon i kromosomtall innen og mellom arter og er derfor også gode kandidater for å studere polyploidisering som artsdannelsesprosess. Ved å løse opp i noen artskomplekser i de utvalgte slektene, har dette studiet videre bidratt til arbeidet med Den panarktiske floraen.

En fylogeni av slekten *Smelowskia* ble konstruert basert på mikrosatellittområder kombinert med nukleære sekvenser og plastidsekvenser. Resultatene tilsier at slekten har en asiatisk opprinnelse og at den har spredt seg til Beringia og Nord-Amerika to uavhengige ganger. Vi fant også støtte for å slå sammen de beringiske artene *S. porsildii, S. spathulatifolia* og *S. jurtzevii* til én art: *S. porsildii*.

Biogeografien og fylogenien til den store slekten *Cardamine* ble skissert basert på nukleære sekvenser og plastidsekvenser. De fylogenetiske trærne hadde liten oppløsning, noe som støtter en rask og nylig artsdannelse i slekten. Studien viser at slekten har en asiatisk opprinnelse og har spredt seg til den sørlige halvkule og til arktis flere ganger, og vi fant minst tre forskjellige arktiske linjer. Polyploidisering har skjedd mange ganger i *Cardamine*, og i kombinasjon med rask divergens, kan dette forklare hvorfor den evolusjonære historien til slekten er så kompleks. To artskomplekser i slekten ble valgt ut for mer detaljerte studier.

Seks mikrosatellittloci som originalt ble utviklet for *Arabidopsis*-genomet ble brukt til å finne evolusjonære og taksonomiske enheter innen det Beringiske *Cardamine digitata*-komplekset. Vi fant fire molekylære grupper som korresponderte med morforlogiske forskjeller, noe som støtter at dette komplekset deles inn i fire arter: *C. blaisdellii*, *C. digitata*, *C. microphylla* og *C. purpurea*. Hver av disse inneholdt minst to ploidinivåer, noe som tyder på gjentatte polyploidiseringer.

Som et første steg i en prosess for å finne opprinnelsen til den sirkumarktiske *Cardamine bellidifolia*, utførte vi et studium med de to antatt nærmest beslektede europeiske alpine artene (*C. alpina* og *C. resedifolia*) basert på AFLP. Overraskende nok viste det seg at den arktiske C. *bellidifolia* var klart adskilt fra sitt sannsynlige alpine opphav. I tillegg hadde de to alpine artene veldig ulike fylogeografiske historier. For *C. alpina* fant vi stor genetisk forskjell mellom populasjonene fra Alpene og Pyreneene og større genetiske forskjeller innen de pyreneiske populasjonene enn innen de alpine populasjonene. I motsetning til dette hadde *C. resedifolia* større genetiske forskjeller mellom populasjoner innen Alpene enn mellom Alpene og fjerntliggende områder som Corsika, Karpatene og Pyreneene. Resultatene viser at de to artene har veldig ulike historier når det gjelder istidsoverlevelse og tilbakespredning.

For å tilrettelegge disse og fremtidige studier av nylig evolverte grupper, utviklet vi 72 nye mikrosatellittloci og testet 15 loci tidligere publisert for Brassicaceae. Disse viste seg å gi variasjon innen og mellom tre fjerntstående slekter: *Cardamine, Smelowskia* og *Draba*. Av 87 loci, hadde 18 variasjon i *Cardamine* og ti variasjon i *Smelowskia*. Sytti-en av primerene ga variasjon i *Draba*, og 50 ga variasjon innen *Draba nivalis*. Områdene som var variable mellom disse tre slektene er sannsynligvis også nyttige for andre slekter i Brassicaceae.

En fylogeografisk analyse av *Cassiope tetragona* som også inkluderte sentralasiatiske og beringiske slektninger, viste at den sirkumpolare underarten *tetragona* var godt adskilt fra den nord-amerikanske underarten *saximontana*. Det er sannsynlig at *C. tetragona* ssp. *tetragona* har et beringisk opphav, og det geografiske mønsteret i genetisk diversitet tyder på at ekspansjonen ut av Beringia til hele sirkumarktis er ung, kanskje etter den siste istiden. Resultatene stemmer overens med en ”leading-edge” måte å kolonisere. Dette er spesielt tydelig østover mot Canada/Grønland og over Nord-Atlanteren til Skandinavia og Svalbard.

List of papers

This dissertation is based on the following papers and they will be referred to in the text by their roman numerals:

I - Carlsen T., Elven R., and Brochmann C. Combined data from microsatellites and DNA sequences resolves the evolutionary history of Beringian *Smelowskia* (Brassicaceae). Manuscript.

II - Carlsen T., Bleeker W., Hurka H., Elven R., and Brochmann C. Biogeography and phylogeny of *Cardamine* (Brassicaceae). Submitted.

III - Jørgensen M.H., Carlsen T., Skrede I., and Elven R. Microsatellites resolve the taxonomy of the polyploid *Cardamine digitata* aggregate (Brassicaceae). Submitted.

IV – Lihova J.C., Carlsen T., Marhold, K. Contrasting phylogeographies inferred for two alpine sister species, *Cardamine resedifolia* and *C. alpina*. Manuscript.

V - Skrede I., Carlsen T., Rieseberg L.H., and Brochmann C. Microsatellites for three distantly related genera in the Brassicaceae. Submitted.

VI - Eidesen P.B., Carlsen T., Molau U., and Brochmann C. (2007) Repeatedly out of Beringia: *Cassiope tetragona* embraces the Arctic. *Journal of Biogeography*, 34, 1559–1574.

Introduction

The Arctic is a relatively young biome on a biogeographic timescale. The current arctic tundra replaced a more or less continuous forest following the climatic shift in the late Tertiary (Lafontaine & Wood 1988; Bennike & Böcher 1990; Matthews & Ovenden 1990; Murray 1995; Lear *et al.* 2000). In addition, the multiple Pleistocene glaciations wiped out the vegetation in large parts of the Arctic region, and each glaciation was followed by waves of recolonization from surrounding unglaciated regions. To fully understand the origin and evolution of the Arctic flora, there is a need for combining fossil evidence and biogeographic and phylogeographic evidence at different time scales. Murray (1995) suggested that the arctic flora of today is composed of a mixture of survivors from the arctic Tertiary forest, Pleistocene immigrants from various mountain areas, and in-situ evolved Pleistocene taxa. Molecular case studies addressing one or more of these alternatives are still scarce.

Beringia (The region from Lena River in Siberia to Mackenzie River in Canada) is proposed to be a hotspot for biodiversity in the Arctic as the larger parts of the region remained ice free during all of the Pleistocene glaciations and may have served as the main arctic region for in situ presence of taxa since the late Tertiary (Hultén 1937; Weider & Hobæk 2000; Abbott & Brochmann 2003; Geml *et al.* 2006). Savile (1972) and Billings (1974) postulate that there was no true lowland arctic flora in North America until late Pliocene or the onset of Pleistocene, but that alpine floras were present in the region from the mid-Tertiary. This may point to Beringia being a very important region both in shaping and maintaining the arctic flora.

The evolution of a PhD project

This PhD project consisted of considerable initial testing work with many results that are not presented in the papers. In the initial phase of the project, a range of genera were tested for their suitability as case studies for making biogeographic inferences for the Arctic and especially the Beringian region. The main testing work was carried out with the genera *Vaccinium* L., *Dryas* L., and *Tephroseris* Rchb., in addition to the species *Ranunculus glacialis* L.*, Cardamine obliqua* Hochst. ex A.Rich., *Arabis alpina* L., and *Oxyria digyna* (L.) Hill. All these projects were abandoned at different stages due to lack of suitable molecular marker systems showing sufficient variation, and/or problems with obtaining sufficient material of required quality, and in some cases because similar project already

had been initiated in other labs. At an early stage, a microarray analysis of gene expression of cultivated plants originating from wild populations of *Draba* L. and *Arabis* L. was initiated. The pilot experiments were successful, but were discarded because a full analysis would exceed the available funding. Our final selection of study groups included three genera that are distributed in Beringia and on the Asian and North American continents. They were the best available candidates for studies of colonization into and diversification within the Arctic.

The genus *Smelowskia*

Smelowskia C.A. Mey. is a taxonomically complex genus with both diploid and polyploid species (Al-Shehbaz & Warwick 2006). It is disjunctly distributed in mountains and arctic areas in northern and central Asia to western Himalaya, north-eastern Asia, north-western North America, and Cordilleran North America south to California (Berkutenko 1988; Ovchinnikova 2004; Elven *et al.* 2006). The scattered distribution of *Smelowskia* south of the Arctic is probably caused by its preference for high mountain scree slopes, rock crevices, and unturfed rubble and also a distinct preference for base-rich substrates (Ovchinnikova 2004).

Based on molecular evidence, the genus *Smelowskia* s. lat. is monophyletic when including the former genera *Ermania* Cham. ex. Botch., *Gorodkovia* Botch. & Karav., *Hedinia* Ostenf., *Hediniopsis* Botch. & V.V. Petrovsky, *Melanidion* Greene, *Redowskia* Cham. & Schltdl., *Sinosophiopsis* Al-Shehbaz, and *Sophiopsis* O.E. Schulz (Warwick *et al.* 2004; Al-Shehbaz & Warwick 2006). This has expanded the number of species in the genus from 8-10 to 25 (Al-Shehbaz & Warwick 2006). *Redowskia* is the oldest published name, but since it is a very rare Siberian endemic and not well known, the name *Smelowskia* was conserved at the Botanical Congress in Vienna 2005 (Brummitt 2005). This has reduced the number of nomenclatural changes needed and retained the use in horticulture (Al-Shehbaz 2003).

There have been extensive differences in opinion as to the delimitation of species within *Smelowskia*. *Smelowskia calycina* (Stephan) C.A.Mey. was treated by Drury & Rollins (1952) as a widespread, polymorphic species with five varieties (var. *americana*, var. *media*, var. *calycina*, var. *porsildii*, and var. *integrifolia*). This has been the most common treatment in North American floras, as opposed to Russian authors considering *S. calycina* as a Central Asian species absent from North America (Velichkin 1979; Ovchinnikova 2004). Rydberg (1902) and Velichkin (1979) treated these five varieties as

separate species. The variety *integrifolia* was given the new name *S. spathulatifolia* (Velichkin 1974). Warwick *et al.* (2004) treated *S. spathulatifolia* as a synonym for *S. americana*, with a note that the taxon in Velichkin's delimitation includes material that belongs to *S. porsildii*. However, Al-Shehbaz and Warwick (2006) suggested that *S. spathulatifolia* should be merged with *S. porsildii* into one highly variable species, but they called for further studies to conclude whether these entities represent one or two taxa. Velichkin (1979) also described the new species *S. jurtzevii* and noted its close relationship to *S. spathulatifolia* and *S. porsildii*. This taxon was treated as conspecific with *S. porsildii* by Czerepanov (1995) and Al-Shehbaz & Warwick (2006).

Drury & Rollins (1952) assumed the present-day distribution of *Smelowskia* s. str. and *Melanidion* to be a fragmented pattern of an earlier continuous distribution throughout Siberia and North America. They also stated that the most probable place of origin is in North America with a spread into Siberia and Altai mountains (Drury & Rollins 1952). This conclusion was based on the present distribution of the genus, where no representatives are found west of the Ural Mountains, and the assumption that more species are located in the North American region than in the Siberian region. Thus, they assumed an Asian origin and a subsequent eastwards spread into and speciation within North America to be unlikely.

The genus *Cardamine*

Cardamine L. is a taxonomically complex, cosmopolitan genus with 160-200 mostly arctic, alpine, and boreal species, and is thus one of the most species-rich genera of the Brassicaceae (Sjöstedt 1975; Hewson 1982; Al-Shehbaz 1988; Webb *et al.* 1988; Al-Shehbaz *et al.* 2006). The number of species accepted varies considerably among different authors, illustrating the notorious taxonomic complexity of this genus. The centre of diversity is located in Eurasia. According to conservative estimates (mainly based on Al-Shehbaz, 1988), approximately 95 species are Eurasian (~48 in China and ~25 in Europe including the Caucasus). There are also many species in North and Central America (~40), and at least nine species extend into arctic areas. Some species are invasive cosmopolitan weeds, such as *C. hirsuta* L.*, C. impatiens* L.*, C. flexuosa* With.*,* and *C. parviflora* L. The number of native species in the Southern Hemisphere is much lower: ten in Australia and New Zealand, five in South America (likely underestimated), three in Africa, and four in New Guinea.

Cardamine is probably a fairly young genus; molecular data indicate that a clade comprising the genera *Barbarea* R. Br., *Armoracia* P. Gaertn., B. Mey. & Scherb. and *Rorippa* Scop. is sister to a *Cardamine* – *Nasturtium* R. br. clade (Franzke *et al.* 1998; Yang *et al.* 1999; Koch *et al.* 2001). *Rorippa* pollen are not found in sediments older than from the Pliocene (2.5-5 MYA; Mai 1995). Koch *et al.* (2000) used this time span to estimate that the lineages that gave rise to *Cardamine* and *Barbarea* diverged 6.0 MYA. This was suggested to be an underestimate by Heads (2005). However, based on the nuclear data set of Koch *et al.* (2000), Haubold and Wiehe (2001) performed a more thorough study under various evolutionary rate assumptions, all resulting in a divergence time of 6.2 MYA.

Most species of *Cardamine* are polyploid, and up to five basic chromosome numbers have been suggested (Al-Shehbaz 1988). The most probable basic number for the majority of species is $x = 8$ (Kucera *et al.* 2005). For some species, such as the Beringian taxa in section *Cardaminella* Prantl., the most probable basic number is $x = 7$ (Elven et al., 2006). Diploids are only known with $2n = 16$, and the highest recorded number is $2n = 32x = 256$ (C. concatenata and C. diphylla; Kucera *et al.* 2005).

Schulz (1903; 1936) considered section *Cardaminella* to be one of the main sections in the genus *Cardamine*. However, there has been a long-time suspicion that section *Cardaminella* is polyphyletic, with the circumpolar and alpine *C. bellidifolia* L. and some of its European alpine relatives constituting a distinct branch, separate from, e.g., the Beringian *Cardaminella* species. The connection between *C. bellidifolia* and the morphological similar *C. alpina* Willd. and *C. resedifolia* L. is of particular interest as this may represent a European phylogenetic connection between arctic and alpine lineages. In the Beringian branch, we find the *C. digitata* Richardson aggregate where the nomenclature and circumscription of some of the species have been disputed. This is an interesting case for studying probable recent speciation and polyploidization events within the arctic region.

The seeds of *Cardamine* are actively spread by the curling of the silique walls, a typical short-distance mode of dispersal (Kimata 1983). *Cardamine* is nevertheless found on all continents except Antarctica. Under moist conditions the seeds can become mucilaginous and adhere to animals (Al-Shehbaz 1988). As the majority of *Cardamine* species occur in moist habitats, this may be a common mode of dispersal, also across vast areas by adhering to birds. Dispersal between Eurasia and North America may have occurred stepwise via the Tertiary Beringian land bridge that existed until 5.4 - 5.5 MYA

(Marincovich & Gladenkov 1999, 2001; Gladenkov *et al.* 2002), but dispersal over longer distances must have occurred between these *Cardamine*-rich continents and Oceania, South America, and Africa.

The genus *Cassiope*

Cassiope D. Don (Ericaceae) comprises 15 small shrubby species, eleven of which occur in and adjacent to the Chinese Himalayas. The other four species are restricted to northern alpine and arctic areas: *Cassiope ericoides* (Pallas) D. Don (Pacific Asian), *C. lycopodioides* (Pallas) D. Don (amphi-Pacific), *C. mertensiana* (Bong.) D. Don (North American) and *C. tetragona* (L.) D. Don (circumpolar). *Cassiope tetragona* is a diploid (2*n* = 26), evergreen dwarf shrub forming coarse, freely branching mats (vegetative propagation through layering) with white, campanulate flowers producing numerous, small seeds. *Cassiope tetragona* is an important component of dwarf shrub, and mixed heath communities in the Arctic. The species is xeromorphic, but depends on snow protection during winter. Most of the annual water uptake in this species takes place during snowmelt, and the xeromorphic habit is beneficial during summer, as *C. tetragona* is capable of inhabiting sites in the High Arctic with low precipitation totals, e.g. Melville Island (see Molau 2001 and references therein). The widespread arctic plants all belong to ssp. *tetragona* (Elven et al. 2006). This subspecies is partly replaced by and partly sympatric with ssp. *saximontana* (Small) A.E. Porsild in northern Cordillera. Subspecies *saximontana* differs from ssp. *tetragona* in having very short pedicels. The prevailing nutrition mode in the Ericaceae is ericoid mycorrhiza (Smith & Read 1997), but *C. tetragona* ssp. *tetragona* is exceptional in its ability to form both ericoid and ectomycorrhiza (Hesselmann 1900; Kohn & Stasovski 1990; Väre *et al.* 1992; Gardes & Dahlberg 1996; Michelsen *et al.* 1996).

The ancestor of the four northern *Cassiope* species probably came from Central Asia (Good 1926) and diversified in or near Beringia, where the current ranges of the four species, and the two subspecies of *C. tetragona*, overlap. The first fossil record of *C. tetragona* from the Beaufort Formation on Meighen Island, Nunavut, Canada is about 3 Ma old, but this finding requires confirmation (reviewed by Matthews & Ovenden 1990). A later, but confirmed fossil find of *C. tetragona* is from the Kap København formation in North Greenland (2.5-2.0 Ma old; Bennike & Böcher 1990). Fossils of *C. tetragona* are also found in several sediments from the previous interglacial (about 120 000 years ago.) in East Greenland (Bennike & Böcher 1994). Thus, *C. tetragona* probably originated in

Beringia and expanded into the Circumarctic during the late Tertiary, prior to the Pleistocene glaciations.

Polyploidy

Polyploidy has played a major role in the evolution of the arctic flora (Brochmann *et al.* 2004). Detailed studies of polyploid complexes have shown that both allopolyploidization and autopolyplodization events occur frequently within recently evolved lineages (Scheen *et al.* 2002; Brysting *et al.* 2004; Jørgensen *et al.* 2006; Brysting *et al.* 2007). Two of the three selected genera for this PhD study are very relevant for studying polyploidization as they show variation in ploidy level within and among species. In *Cardamine*, the arctic representatives varies from diploid $2n = 16$ in *C. bellidifolia* to dodecaploid $2n = 96$ in *C. purpurea*, and even higher ploidy numbers have been reported for arctic subspecies of *C. pratensis* (Kucera *et al.* 2005). In *Smelowskia*, the chromosome numbers varies from diploid $2n = 12$ to hexaploid $2n = 36$, with many taxa showing multiple cytotypes, such as *S. jacutica* (Botsch. & Karav.) Al-Shehbaz & Warwick where both diploid and hexaploid individuals are reported (cf. Warwick *et al.* 2004; Al-Shehbaz & Warwick 2006).

Treebuilding methods

Establishing sister group relationships are essential for inference of source areas and dispersal events. This implies that treebuilding methods must be applied and that resulting, resolved phylogenetic trees must be obtained, which typically can be problematic when studying recently diverged lineages. Island radiations and human domestications are comparable to the recently evolved arctic region, as rapid morphological diversification has occured without giving sufficient time for evolution of differences in commonly used molecular marker systems (Ribesell 1982; Fondon & Garner 2004). Typically, studies of groups that have rapidly radiated often identify large morphological divergence in spite of little molecular variation, insufficient for rigorous phylogenetic inference (e.g. Kapralov & Filatov 2006). Additional challenges to the traditional statistical methods are presented by polyplodization events and different evolutionary histories of genes and genomes within the plants.

The main objectives of this PhD study were:

1. To find possible colonization routes into the arctic region for the selected taxa by inferring phylogenies including arctic representatives and presumptive close relatives from potential source areas in various alpine and boreal regions south of the arctic region.
2. To contribute to the PanArctic Flora Project on species delimitation in the *Cardamine digitata* and *Smelowskia porsildii* complexes.
3. To infer a phylogeny of the large genus *Cardamine* to evaluate whether the arctic species, and in particular the section *Cardaminella*, constitute a monophyletic group.
4. To construct phylogeographic histories of Holocene dispersal and colonization in the arctic *Cassiope tetragona* and the alpine *Cardamine alpina* and *Cardamine resedifolia*.
5. To find suitable molecular marker systems for recently diverged lineages to be able to delimit species and to identify sister group relationships for studying the systematic and biogeographic objectives described above.

Methods

Laboratory methods

In this thesis, sequencing, amplified fragment length polymorphisms AFLPs, and microsatellites (SSRs) have been applied, often in combination. Sequencing provides datasets easily comparable between labs, and they can be stored in databases for future use and critical review. Universal primers that amplify across the plant kingdom or more specific primers for selected taxa are available. The method has some drawbacks; sequencing is costly and provides few characters compared to time and lab-costs of other molecular methods. In addition, sequencing of nuclear regions is difficult where duplication of genes or genomes has occurred. Cloning has to be applied, which is an expensive and time-consuming method. Sequencing of the plastid genome is much easier, as it is haploid, presumed not to be recombining, and available in high numbers in each cell. However, concern has been raised as to analyzing the plastid genome without analyzing supplementary data sets in parallel (Rieseberg & Soltis 1991; Rieseberg *et al.* 1996; Avise 2004; Smissen *et al.* 2004). In some cases the evolution of organellar lineages are only loosely linked to the evolution of the organisms in which they are symbionts (Neigel & Avise 1986; Rieseberg & Soltis 1991; Rieseberg *et al.* 1996). Recent studies have also shown that the plastid genome may be recombining, and that it can switch between maternal and paternal inheritance (Bendich 2004; Hansen *et al.* 2007). Direct sequencing of both nuclear and plastid regions has been performed in this PhD study (Papes I, II, and IV). In paper I, cloning was performed to design taxon and subunit specific primers before direct sequencing of the presumed single copy *RPA*2 region.

AFLPs are very cost-efficient and provide a large amount of information without prior knowledge of primer sites and ploidy level (Vos *et al.* 1995). On the other hand, the markers are dominant and consistent scoring may be difficult (Bonin *et al.* 2004). The reliability of the AFLP analyses is also highly dependent on the quality and concentration of the DNA extractions (Bonin *et al.* 2004). Material rapidly dried in silica gel is recommended. Reproducibility tests were performed in the initial stages of this study on silica dried material and the corresponding herbarium vouchers of the same specimens in the genus *Tephroseris*. As these results were discouraging, AFLPs were not used on studies that relied on herbarium specimens for several taxa.

Microsatellites are costly to develop, but each locus provide much information. In a diploid organism the marker can be scored as codominant. In polyploids the statistical analyses become much more complex, and there is no reliable way to check for null-alleles, heterozygosity, or dosage effects. In this PhD study, SSRs were scored as independent dominant alleles in the same manner as scoring of AFLPs. Finding cross-species transfer of primers developed for closely related taxa is a time- and cost-efficient alternative to finding new primers amplifying polymorphic microsatellite loci within a study group. In this PhD study, SSRs developed for *Arabidopsis* (DC.) Heynh, *Brassica* and *Draba* were tested for amplification and applicability and used in the genera *Smelowskia* and *Cardamine* (Papers I and III).

Treebuilding methods

Parsimony and Bayesian analyses are commonly utilized methods for treebuilding. Which of the two analyses that performs better has been subject to debate. Bayesian analyses is considered to be better for reconstructing phylogenies since it is not so susceptible to long branch attraction (Huelsenbeck *et al.* 2001; Bergsten 2005; Philippe *et al.* 2005). On the other hand, Bayesian analyses have been shown to overestimate confidence on phylogenies (Suzuki *et al.* 2002; Cummings *et al.* 2003; Simmons *et al.* 2004). Performing both methods on the same datasets will assure that these methodological errors are discovered and hopefully avoided. Both methods have been used in this study on datasets in papers I and II.

Parsimony has been shown to be an appropriate method for analyzing AFLP fragments in *Lactuca* L. s. lat. and *Arabidopsis* (Koopman & Gort 2004; Koopman 2005), but Bussell *et al.* (2005) recommended that treebuilding methods should only be applied when at least 20% of fragments were monomorphic, as more divergent lineages would have accumulated too much homoplastic fragments providing spurious results and phylogenetic trees. Treebuilding methods have been used on AFLPs and SSRs in papers III, IV, and VI. In all cases parsimony has been used (in addition to Neighbor Joining in paper IV).

Results & discussion

Main results from the three genera

Our molecular analyses of *Smelowskia* showed a structure corresponding to the former subdivision into several genera (Paper I). The analyses based on ITS, *RPA*2 and SSRs all identified *Smelowskia* s. str. as a group separate from *Melanidion* and *Ermania*. The results of the two nuclear regions and the SSR were congruent and separated *Smelowskia* s. str. from *Melanidion* as sisters. With an assignment of *S. inopinata* (Kom.) Kom to *Melanidion*, the old genus subdivision thus seems to be justified. The sequences also suggested *Ermania* as sister to the *Melanidion*/*Smelowskia* clade, and *Redowskia* as sister to this group. However, as our plastid data suggested gene transfer between the lineages, we recommended retaining one large genus until supplementary studies are performed. An Asian origin of the genus and two independent dispersal events into the Beringian and North American regions were inferred. We also found evidence for merging the Beringian *S. porsildii, S. spathulatifolia,* and *S. jurtzevii* into one species; *S. porsildii*.

The phylogenetic analysis of *Cardamine* showed limited resolution, supporting a hypothesis of recent and rapid speciation in the genus (Paper II). Rapid divergence combined with widespread polyploidization offer an explanation for the complex evolutionary history of the genus. There were two distinct examples of European origin of arctic *Cardamine*, including two different species (*C. bellidifolia* and *C. pratensis*) which have become broadly distributed in the Arctic without further diversification. In addition, there was one example of a probable North American origin followed by diversification into many species in Beringia, but without further expansion into the circumarctic.

Four approximately equidistant units were recognized within the Beringian *Cardamine digitata* aggregate (Paper III). The separation of *C. purpurea* Cham. & Schtdl. and *C. microphylla* Adams from the remaining units was supported in both principal coordinate (PCO) and parsimony analyses, and the isolation of the groups got Bremer support of 2 and 1, respectively. *Cardamine digitata* Richardson and *C. blaisdellii* Eastw. were separated by both STRUCTURE and PCO analyses. The resolution in the parsimony analyses gave neither support to, nor contradicted the separation of the two groups. As the four groups correspond to morphologically defined and distinct units, we suggested to acknowledge the groups as four taxa at the rank of species: *C. blaisdellii, C. digitata, C. microphylla,* and *C. purpurea*.

The arctic species C. *bellidifolia* displayed extremely low genetic variation, and was distinctly differentiated from its putative alpine ancestral lineages at AFLP loci (Paper IV). A high degree of genetic separation was found between the Alpine and Pyreneean populations of *C. alpina*, as well as more diversity within Pyreneean populations than among Alpine populations. In contrast, *C. resedifolia* showed more genetic variation among populations in the Alps than between the Alpine and distant areas such as Corsica, the Carpatians and the Pyrenees. This suggested the existence of a largely widespread and continuous gene pool along with several geographically more restricted lineages, and also indicated quite common secondary contacts between them. The results showed that the two species have very different histories of glacial survival and recolonization.

A phylogeographic analysis of *Cassiope tetragona* including both Central Asian and Beringian relatives revealed that the circumpolar ssp. *tetragona* was well separated from the North American ssp. *saximontana*, and a Beringian origin of *C. tetragona* ssp. *tetragona* was inferred. The genetic structure within *C. tetragona* ssp. *tetragona* was in agreement with Hultén's hypothesis of expansion from Beringia (Hultén 1937). However, the levels and geographical patterns of differentiation and gene diversity suggested that the latest expansion into the circumarctic occurred during the Mid- to Late Pleistocene, possibly during the current interglacial. The results were in accordance with a recent leading-edge mode of colonization, particularly towards the east throughout Canada/Greenland and across the North Atlantic into Scandinavia and Svalbard.

Origins of the Arctic flora

We have provided examples of colonization histories into the Arctic that fits well with the scenario suggested by Murray (1995); that the present arctic flora is a mixture of survivors of the arctic tertiary forest, Pleistocene migrants, plants that recolonized from glacial refugia south of the ice sheets, in situ suvival in northern refugia, and newly evolved taxa. We have found evidence for evolution of new species in the Arctic within the *Cardamine digitata* complex and the Beringian *Smelowskia* species. Immigration histories from various mountain areas are likely: *Cardamine bellidifolia* originating from the European Alps, and *Smelowskia* and *Cassiope* from Central Asian mountains. *Cardamine* might also provide examples of either immigration from the Rocky Mountains or survival of plants that inhabited the continuous tertiary forest. *Cassiope tetragona* is a probable *in situ* survivor in Beringia.

Are there any trees in the Arctic?

It seems to be a trend in studies of arctic groups of the Brassicaceae that the phylogenies are to a large extent unresolved. *Smelowskia, Cardamine, Draba,* and *Arabis* all show a lack of resolution not necessarily due to lack of sequence variation (Koch & Al-Shehbaz 2002; Ehrich *et al.* 2007; papers I and II). This is what we would expect if there had been a rapid diversification of lineages. Fishbein *et al.* (2001) stated that lack of resolution in recently radiated lineages could be mended by sequencing more regions, but this should be tested more rigidly with model-generated datasets. Looking for a candidate gene or intron that will have mutated in the short time window between where the different lineages diverged, will be unfruitful as this region probably will have continued to evolve rapidly, and the region will have reached a level of saturation with homoplasy swamping the phylogenetic signal, as it has been shown in other studies of rapid radiations (Lovette & Bermingham 1999; Morrison *et al.* 2004). In paper II, we have a nuclear and a plastid dataset, both with some sequence variation, however none neither result in resolved trees. This is similar to *Draba*, which also remained unresolved in DNA sequence-based phylogenies (Koch & Al-Shehbaz 2002). Both these genera are rich in species and widespread both in arctic and tropical environments. The sequences do not lack variation, but the variation does not define clades and branches in the backbone of the phylogenetic trees. This is discussed in paper II, and the conclusion is that *Cardamine* is a young genus that rapidly diversified, and the sequence variation found only defines clades and lineages that have evolved after this short time period.

In this thesis I did not find any distinct differences between results from the two methods of treebuilding. The Bayesian posterior probabilities seem to be higher than jackknife and bootstrap values, but these values should not be regarded as directly comparable. Bayesian analyses do not provide better resolved phylogenies than parsimony in our study. The difference between producing a gene-tree (or even a phylogram based on several concatenated genes) and producing a phylogeny should be stressed (e.g. Rieseberg *et al.* 1996; Knowles & Maddison 2002). The true phylogeny will never be more than a hypothesis we infer based on the gene trees. In addition, support values will never be more than a test of consistency within the sampled dataset (Felsenstein 1985; Bremer 1994; Farris *et al.* 1996). Neither parsimony nor Bayesian analysis will be able to sort the homoplasy from the characters defining a branch in a saturated dataset.

Different approaches to analyzing AFLP fragments were tested in paper VI as the amount of samples was much higher than the number of characters; which is not optimal

for parsimony reconstructions (Rokas *et al.* 2003). Three different approaches to improve this ratio were explored: selecting randomly one individual from each population, calculating the populations mean allele frequency of each marker and selecting the most "representative" individual, and last using the population mean frequency of each marker as a continuous character. All these approaches produced congruent trees even though they have different assumptions. To my knowledge, no-one has previously used AFLP allele frequencies as continuous characters for treebuilding. This procedure assumes that the populations evolve as units and that selection or drift will affect the frequencies of the assumed shared ancestral populations more than gene flow between different populations. In paper VI, we consider these assumptions to be met, as the PCO and ANOVA suggest a leading-edge mode of colonization, and there seems to be little or no dispersal or gene flow into already established areas.

Combining different marker systems enhances the possibilities to make reliable hypotheses on the evolution and diversification at short timescales. In paper I, SSRs were used to delimit species where sequences did not provide enough characters. The different marker systems (SSRs, plastid sequences and sequences from two different nuclear regions) provided different supported clusters and clades that in combination could be used to infer the phylogeny and biogeographic history at much greater detail than one marker system alone. In paper VI, by sequencing and making a small phylogeny for related taxa, an outgroup and direction was found for the AFLP analyses, greatly enhancing their explanatory power.

Allopolyploidy is an important evolutionary mechanism, particularly in the Arctic, but provides a challenge to parsimony and Bayesian treebuilding methods (e.g. Brysting *et al.* 2007). Unless concerted evolution of nuclear regions has favored one of the homologues, double (and conflicting) signals are expected in the dataset. Comparing analyses of nuclear and plastid datasets, or cloning of nuclear regions could provide an indication whether allopolyploidy has taken place.

For PCO analyses of microsatellite datasets, polyploidy may in general provide analytical problems. Thus, the microsatellites were scored as dominant markers in this thesis (allele present = 1, allele absent = 0). The information from the codominant properties of microsatellites was lost, but a more trustworthy dataset was gained. As polyploids generally have more bands than diploids, a grouping of polyploids is expected based on the similarity algorithms used. Both Dice and simple matching has been performed on datasets in this thesis, but only the analyses using Dice analyses were shown.

Interestingly, in paper III, grouping of polyploids were not detected in the PCO plots. This was interpreted as support for reoccurring taxonomic autopolyploidy taking place.

Dispersal

Dispersal of plants is often discussed with a short time scale in mind, with only obvious dispersal agents such as fruits and pollen being considered. Improbable dispersal events are disregarded on the basis of being unlikely on a short time scale. However, stochasticity and improbabilities constitute the backbone of evolution, and there is no reason to disregard improbable dispersal events when thinking in an evolutionary or geologic timescale.

In paper II, the phylogenetic trees suggested several long distance dispersal events in *Cardamine* as the species are found on all continents except Antarctica, and in many cases the species from one continent did not represent close relatives. We found a clade of closely related species from Beringia, Australia and South America. This result indicates high dispersal ability across vast distances even though most seeds disperse no more than about one meter (Kimata 1983).

In paper VI, hypotheses based on ecological knowledge and educated guesses on dispersal and establishment are accounted for. Two subspecies of *Cassiope tetragona* that display high similarity in apparent dispersal ability of seeds and in habitat preferences show a remarkable difference in postglacial dispersal and colonization. We believe in paper IV, that a probable explanation in this species is the difference in mycotrophic abilities between the two subspecies. My general statement is that all plants are mycorrhizal, having ectomycorrhiza, arbuscular mycorrhiza, or mycorrhizal root-endophytes. Based on this, it is likely that plants having ability to exploit serveral mutualistic relationships will be more successful in an initial colonizing phase, and studying dispersal abilities of the symbionts may provide additional information to the contrasting dispersal histories seen in the plants.

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PAPER I

Combined data from microsatellites and DNA sequences resolves the evolutionary history of Beringian *Smelowskia* (Brassicaceae)

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Running head: Carlsen et al. - Arctic *Smelowskia*

We used the genus *Smelowskia*, which is distributed in Asia and North America and comprises both diploids and polyploids, as a model to study phylogeny, biogeography and polyploidization in the recently formed arctic biome with particular reference to the Beringian area. To infer the evolutionary history and species delimitation, we combined data from high-resolution nuclear markers (seven SSR loci) with sequences from two nuclear regions (the low copy *RPA*2 intron 23 and the multicopy nrITS region) and five plastid regions (*trn*L^{UAA} and *rp*S16 introns, *trn*H^{GUG}-*psb*A, *trn*L^{UAA}-*trn*F^{GAA}, and 5'*rp*S12-*rp*L20 spacers). The combined use of these markers made it possible to separate species and construct a resolved phylogeny. The different nuclear markers showed a congruent pattern that fits well with that observed in morphology and geography, while the plastid data showed incongruence, suggesting horizontal transfer of the plastid genome. The data supported merging of *S. porsildii, S. spathulatifolia,* and *S. jurtzevii* into a single species (*S. porsildii*). An Asian, in contrast to the previously suggested American, origin was inferred for the study group, with two separate lineages of American-Beringian or American taxa. The SSR data confirmed polyploidy in several species, adding to the evidence showing the major role of this process in the evolution of the arctic flora.

Keywords: Beringia; *Smelowskia*; Phylogeny; Polyploidization

The present-day Arctic is a young biome generated by a climatic shift in the late Tertiary (Lafontaine and Wood, 1988; Bennike and Böcher, 1990; Matthews and Ovenden, 1990; Murray, 1995; Lear et al., 2000; Jahren, 2007). Murray (1995) suggested that the arctic flora of today is composed of a mixture of survivors from the arctic Tertiary forest, Pleistocene immigrants from various mountain areas, and in-situ evolved Pleistocene taxa. Many arctic species are probably of Pleistocene origin, as shown e.g. in *Cerastium* L. (Scheen et al., 2004; Brysting et al., 2007) and *Draba* (Grundt et al., 2004; Grundt et al., 2006).

The region called Beringia, encompassing the region from the Lena River in Northeast Russia to the Mackenzie River in Canada, has probably played a key role in the evolution of the arctic flora and has served as a major refugium during the Pleistocene (Abbott et al., 2000; Weider and Hobæk, 2000; Abbott and Brochmann, 2003; Hewitt, 2004; Alsos et al., 2005; Geml et al., 2006). However, in spite of the importance of this region, detailed reconstructions of the history of Beringian plants and their ancestral lineages are still scarce. Here we selected the genus *Smelowskia* C.A. Mey., which in its widest sense (Al-Shehbaz and Warwick, 2006) comprises several Beringian taxa as well as taxa confined to Central Asia/Himalaya and non-Beringian North America (Fig. 1; Table 1) as a model to study evolution and origin of Beringian taxa. *Smelowskia* is a taxonomically complex genus with both diploid and polyploid species (Al-Shehbaz and Warwick, 2006). It is disjunctly distributed in mountains and arctic areas in northern and central Asia to western Himalaya, northeastern Asia, northwestern North America, and Cordilleran North America south to California (Berkutenko, 1988; Ovchinnikova, 2004; Elven et al., 2006; Fig. 1). The scattered distribution of *Smelowskia* south of the Arctic can be attributed to its preference for base-rich substrates and high mountain scree slopes, rock crevices, and unturfed rubble (Ovchinnikova, 2004).

The phylogenetic position of *Smelowskia* within Brassicaceae is still uncertain. It was assigned to tribe Descurainieae by Schulz (1924; 1936), but transferred to the monotypic tribe

Smelowskieae by Al-Shehbaz et al. (2006). In a molecular study of the family based on ten nuclear and plastid loci, *Smelowskia* was resolved as sister to *Lepidium* L. in tribe Lepidiae (Bailey et al., 2006). However, based on *ndh*F, and with a larger sample of genera, *Smelowskia* was resolved as sister to *Descurainia* Webb & Berthel. in Descurainieae and *Lepidium* as being more distantly related (Beilstein et al., 2006), in agreement with Schulz' original classification.

In a phylogeny inferred from ITS and *trn*L intron sequences (Warwick et al., 2004), *Smelowskia* formed a monophyletic group together with eight other small genera: *Ermania* Cham. ex. Botch., *Gorodkovia* Botch. & Karav., *Hedinia* Ostenf., *Hediniopsis* Botch. & V.V. Petrovsky, *Melanidion* Greene, *Redowskia* Cham. & Schltdl., *Sinosophiopsis* Al-Shehbaz, and *Sophiopsis* O.E. Schulz. This was followed up in the revision by Al-Shehbaz and Warwick (2006), who included all nine genera into a widely circumscribed *Smelowskia*, expanding the number of species from 8-10 to 25. *Redowskia* is the oldest published name, but it is a rare Siberian endemic and not well known. The name *Smelowskia* was thus conserved at the Botanical Congress in Vienna 2005 (Al-Shehbaz, 2003; Brummitt, 2005). This has avoided many nomenclatural changes and kept the traditional naming in horticulture.

Within *Smelowskia* s. lat., five of the nine formerly recognized genera formed a supported monophyletic group with little internal structure (Warwick et al., 2004), including *Smelowskia* s. str., *Ermania, Gorodkovia, Melanidion,* and *Redowskia*. This group (henceforth named the '*Smelowskia* clade') is distributed in South and East Siberia, Russian Far East, Beringia, and Cordilleran North America (Fig. 1). The remaining four among the formerly recognized genera occur in the Central Asian mountains of Tian-Shan, Pamir, and western Himalaya (*Hedinia, Sophiopsis,* and *Sinosophiopsis*) and in eastern Beringia (Chukotka; *Hediniopsis*), and formed a paraphyletic group relative to the *Smelowskia* clade. In this study, we attempt to resolve the relationships within the *Smelowskia* clade.

There has, however, also been extensive disagreement as to the delimitation of species within the *Smelowskia* clade. *Smelowskia calycina* (Stephan) C.A.Mey. was treated by Drury and Rollins (1952) as a widespread, polymorphic species with five varieties (var. *americana*, var. *media*, var. *calycina*, var. *porsildii*, and var. *integrifolia*). This has been the most common treatment in North American floras, as opposed to Russian authors considering *S. calycina* as Central Asian and absent from North America (Velichkin, 1979; Ovchinnikova, 2004). Rydberg (1902) and Velichkin (1979) treated these five taxa as separate species. The variety *integrifolia* was given the new name *S. spathulatifolia* (Velichkin, 1974). Warwick et al. (2004) treated *S. spathulatifolia* as a synonym for *S. americana* (Regel & Herder) Rydb., with a note that the taxon in Velichkin's delimitation includes material that belongs to *S. porsildii* W.H. Drury & Rollins) Jurtsev. However, Al-Shehbaz and Warwick (2006) suggested that *S. spathulatifolia* should be merged with *S. porsildii* into one highly variable species, but they emphasized a need for further studies. Velichkin (1979) also described the new species *S. jurtzevii* and noted its close relationship to *S. spathulatifolia* and *S. porsildii*. This taxon was treated as conspecific with *S. porsildii* by Czerepanov (1995) and Al-Shehbaz and Warwick (2006). *Smelowskia* (*Melanidion*) *borealis* (Greene) W.H. Drury & Rollins was treated as a species with four varieties in Drury and Rollins (1952). According to Warwick et al.(2004), one of these, *S. borealis* var. *jordalii*, is identical to *S. johnsonii*, described as a new species by Mulligan (2001).

As an initial framework for this study, we used the treatment of Al-Shehbaz and Warwick (2006), except that we treated *S. johnsonii* as *S. borealis* var. *jordalii*, and recognized thirteen species within the *Smelowskia* clade (Fig. 1, Table 1). *Smelowskia alba* (Pall.) Regel, *S. bifurcata* (Ledeb.) Botsch and *S. calycina* occur in a belt from Lake Balkash through the Altai mountains to Lake Bajkal, and *S. alba* is also found northwards along the Lena River to its delta. *Smelowskia* (*Melanidion*?) *inopinata* (Kom.) Kom. also has a disjunct

distribution and is found in the Khabarovsk and Okhotsk regions in the Russian Far East. The genus *Melanidion* har partly been recognized in North America, but this species-group probably also includes *S. inopinata*. *Smelowskia* (*Gorodkovia*) *jacutica* (Botsch. & Karav.) Al-Shehbaz & S.I. Warwick is found in the Okhotsk region and in the Verkhoyansk Mountains along the Lena River. *Smelowskia* (*Ermania*) *parryoides* (Cham.)Polunin and *S. porsildii* (including *S. jurtzevii* and *S. spathulatifolia*) are found in the Okhotsk region and on the Kamchatka and Chukchi peninsulas, with *S. porsildii* extending into Alaska. Two of the *Melanidion* species, *S. pyriformis* W.H. Drury & Rollins and *S. ovalis* M.E. Jones have very restricted distributions: *Smelowskia* (*Melanidion*) *pyriformis* is only found in the central mountains of Alaska, while *S.* (*Melanidion*) *ovalis* occurs in Washington, Oregon and the southernmost part of British Columbia. *Smelowskia* (*Melanidion*) *borealis* is found in Alaska and in the Canadian districts of Yukon and the Northwest Territories as far as the Mackenzie River, whilst *S. media* (W.H. Drury & Rollins) Vielchkin is found further east in Alaska, Yukon and the Northwest Territories. All these species are restricted to the unglaciated Beringian regions. *Smelowskia americana* is the most widespread North American species found in the non-Beringian Rocky Mountains and in the Cascade Mountains of Canada and the United States, i.e., south of the Cordilleran and Laurentide glaciations.

Drury and Rollins (1952) assumed the present-day distribution of *Smelowskia* s. str. and *Melanidion* to be a fragmented pattern of an earlier continuous distribution throughout Siberia and North America. They also stated that the most probable place of origin is in North America with a later expansion into Siberia and Altai mountains. Their assumption was based on the present distribution of the genus where no representatives are found west of the Ural Mountains, and the assumption that more species are located in North America than in Siberia. Thus, they assumed that an Asian origin and a subsequent eastwards spread into and speciation into North America to be unlikely.

Polyploidization events are frequent and play a major role in the evolution of the arctic flora (Scheen et al., 2002; Brochmann et al., 2004; Brysting et al., 2004; Popp et al., 2005; Jørgensen et al., 2006; Brysting et al., 2007). The *Smelowskia* clade conforms to this trend, which may provide an explanation for the difficulties in species delimitation in this group. The basic chromosome number in *Smelowskia* is x = 6, with exclusively diploid counts of 2*n* = 12 in *S. alba*, *S. bifurcata*, *S. calycina,* and *S. media* (*Smelowskia* s. str.) and in *S.* (*Melanidion*) *borealis* and *S.* (*Melanidion*) *pyriformis* (Drury and Rollins, 1952; Yurtsev and Zhukova, 1972; Krogluevich, 1976; Dawe and Murray, 1979; Zhukova and Petrovsky, 1980, 1984). Multiple cytotypes including diploids, pointing to frequent and recurrent polyploidizations, have been reported for four species: *S. americana* 2*n* = 12, 22 (Drury and Rollins, 1952; Packer, 1968); *S.* (*Ermania*) *parryoides* 2*n* = 12, 24 (Yurtsev and Zhukova, 1972; Zhukova and Petrovsky, 1977; Zhukova, 1980; Zhukova and Petrovsky, 1980, 1984); *S. porsildii* 2*n* = 12, 18, 22, 24, 32 (Johnson and Packer, 1968; Yurtsev and Zhukova, 1972; Yurtsev et al., 1975; Dawe and Murray, 1979; Zhukova and Petrovsky, 1984; Murray and Kelso, 1997); and *S.* (*Gorodkovia*) *jacutica* 2*n* = 12, 36 (Yurtsev and Zhukova, 1972, 1982).

As typically found in arctic plant groups, many of which may have evolved as late as during the Pleistocene (e.g. Grundt et al., 2004; e.g. Scheen et al., 2004), few phylogenetically informative characters were obtained from DNA sequences of *Smelowskia* in the study of Warwick et al. (2004). There is clearly a need for more high-resolution markers to address species delimitation and history of such groups. DNA fingerprinting such as RAPDs or AFLPs has been successfully used in combination with sequencing in some plant groups (e.g. Grundt et al., 2004; Eidesen et al., 2007a) , but in order to obtain high-quality profiles, such markers requires access to freshly collected material. This is often difficult to obtain for arctic-alpine groups occurring in widely separated, inaccessible areas, such as *Smelowskia*. Microsatellites represent an alternative type of high-resolution markers which can be obtained

from herbarium material, and can be used to infer evolutionary relationships among closely related species where sequence variation is difficult to obtain (Goldstein and Pollock, 1997; Schlötterer, 2001).

In this study, we address species delimitation and historical relationships in the arctic-alpine *Smelowskia* clade using nuclear microsatellites in combination with plastid and low- and multicopy nuclear sequences from several regions (*trn*L^{UAA} intron, *trn*L^{UAA}-*trn*F^{GAA} intergenic spacer, *rp*S16 intron, *trn*H^{GUG}-*psb*A intergenic spacer, 5'*rp*S12-*rp*L20 intergenic spacer, 23rd intron of *RPA*2, and ITS). In particular, we infer the biogeographic history of this North American/Beringian/Asian group, and address the usefulness of microsatellite markers for this kind of studies. Furthermore, we test the validity of separating *S. spathulatifolia, S. porsildii,* and *S. jurtzevii* into distinct species.

MATERIALS AND METHODS

Leaf material was sampled from herbarium specimens held at ALA, CAN, DAO, LE, MHA, and O (Appendix 1). In addition, some fresh leaf material was sampled and dried in silica gel in the field with vouchers deposited in the herbarium at the Natural History Museum, University of Oslo (O).

DNA was extracted using the DNeasy™ Plant Mini Kit or DNeasy™ Plant 96 Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. PCR amplification of nrITS was done with the primers ITS 4 and 5 (White et al., 1990). The *trn*L^{UAA} intron and the *trn*L^{UAA}-*trn*F^{GAA} intergenic spacer region were amplified with the primers c and f (Taberlet et al., 1991), the *rp*S16 intron with the primers of Oxelman et al. (1997) as modified by Shaw et al. (2005), the *trn*H^{GUG}-*psb*A intergenic spacer with the primers of Sang et al. (1997), and the 5'*rp*S12-*rp*L20 intergenic spacer with the primers of Hamilton (1999). PCR reactions were performed with 30 cycles of 30 s at 94 ºC (first cycle 5 min), 30 s at 55 ºC, and 90 s at 72 ºC (last cycle 10 min). PCR products were purified with 10x diluted ExoSAP-IT® (USB Corporation, Cleveland, Ohio, USA) before cycle sequencing with 10x diluted BigDye (Applied Biosystems, Foster city, California, USA) in 25 cycles of 10 s at 96 ºC, 5 s at 50 ºC, and 240 s at 60 ºC and visualized on an ABI 3100 Sequencer (Applied Biosystems).

The 23rd intron of *RPA*2 from four species was amplified with first degenerated RNAP primers and then a nested PCR with subunit specific primers, as described by Popp and Oxelman (2004). The PCR products were cloned using the kit TOPO TA Cloning Kit (Invitrogen) following the manufacturer's protocol except for using only half the reaction volumes. Individual colonies were subjected to PCR and sequenced. Taxon and subunit specific primers were designed and used for direct PCR and sequencing as described above in

subsequent analyses. Smelowskia RPA2F: 5'-ATTGCCATGCTTTTGGAATC-3', Smelowskia RPA2R: 5'-TCTCACACTGCAGCTCTACTCC-3'.

SSR loci were amplified with seven primers (Table 2). All primers were developed for *Arabidopsis thaliana*, but also amplify variable fragments within *Smelowskia* (Skrede et al.). The seven loci are distributed on all five chromosomes of *A. thaliana* (Table "primers"). All forward primers were given a tail with the M13 sequence (5'-CACGACGTTGTAAAACGAC-3'), and used in combination with reverse primers and a third M13-primer dyed with FAM (MWG Biotech AG), VIC (Applied Biosystems), NED (Applied Biosystems), or PET (Applied Biosystems). For the full protocol, see (Skrede et al.). The reactions were run for 5 min at 95ºC, 35 cycles of the three steps 30 s at 94ºC, 30 s at 51ºC, and 45 s at 72ºC, and a final hold of 20 min at 72ºC. The PCR products were multiplexed, and 1 µL of product mixture (FAM:NED:PET:VIC = 2:3:3:2) was added 8.8 µL HiDi (formamide) and 0.2 µL GeneScan Liz 500 size standard (Applied Biosystems). The products were denatured for 5 min at 95ºC and visualized on an ABI 3100 Sequencer (Applied Biosystems).

Sequences were edited in Sequencher 4.1.4 (Gene Codes, Ann Arbor, Michigan, USA), and ambiguous positions were coded according to IUPAC standards. Similar sequences from GenBank were imported and included in the matrices. The sequences were subsequently aligned manually in BioEdit (Hall, 1999) and imported into TNT (Goloboff et al., 2003) through Genetool 2.0 (Biotools, Edmonton, Alberta, Canada).

Parsimony analyses of the sequence data were performed in TNT (Goloboff et al., 2003). Heuristic searches were performed with 1000 random addition sequences and TBR branch swapping, saving ten trees per replication. The resulting trees were swapped on with TBR saving up to 100 000 trees altogether. Collapsing rule was set to minimum length = 0. Random seed was set to "time". Goodness of fit was calculated using CI, RI, RC according to

Kluge and Farris (1969) and Farris (1989). Bremer support (Bremer, 1994) was calculated by producing 60 000 trees that were up to 6 steps longer, starting with saving 10 000 trees one step longer, and successively saving 10 000 tress of up to one step longer in 5 steps. Jackknife (Farris et al., 1996) and traditional bootstrap (Felsenstein, 1985) resampling studies were performed with 1000 replicates (10 random entry orders and 10 trees saved each repetition). Jackknifing was performed with 36% deletion. Both bootstrap and jackknife were performed with cut-off value of 50% and absolute frequencies as output.

Bayesian analyses of the ITS region were performed in MrBayes 3.1.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) with the model SYM+gamma selected by hLRT, and the model GTR+gamma selected by AIC in MrModeltest 2.2 (Nylander, 2004). The analysis was run for 6 000 000 generations in four chains sampling trees every 10 000th generation. Burn-in was set to 25%.

Preliminary phylogenetic analyses were performed to test if *Smelowskia* (*Hedinia*) *altaica* (Pobed.) Botsch. and *Smelowskia* (*Hediniopsis*) *czukotica* (Botsch. & V.V. Petrovsky) Al-Shehbaz & S.I. Warwick could be used as outgroups. This was done by importing ITS sequences of *Arabidopsis* and *Descurainia* to the matrix. In these preliminary analyses *S. altaica* and *S. czukotica* was the closest relatives to, and also distinct from the target group.

Microsatellite profiles were sized and scored using GeneMapper vs. 3.7 (Applied Biosystems). Due to polyploidy, the markers were treated as dominant (see below), and peaks were scored as present (1) or absent (0). The variation in the microsatellite dataset was visualized using principal coordinate analysis (PCO) in NTSYSpc version 2.02 (Rohlf, 1990) based on the similarity measure of Dice (1945). Variation in ploidy level is problematic when scoring microsatellite loci. Different methods have been suggested in order to score and estimate distances among heterozygotes, but all make assumptions about the uncertain pattern of microsatellite evolution (Harr et al., 1998; Bruvo et al., 2004). Thus, we scored the

microsatellites as dominant markers in this paper (allele present = 1, allele absent = 0). We hereby lose the information from the codominant properties of microsatellites, but gain a more trustworthy dataset. Due to high variation, the data matrix was successively split into smaller matrices according to the grouping seen in previous PCO analyses. A Bayesian approach using STRUCTURE version 2 (Pritchard et al., 2000) calculated a logarithmic probability for the data given a number of clusters and assigned the specimens to these clusters probabilistically. The method may be applied to dominant markers under a no-admixture model, assuming no linkage between the loci (Pritchard et al., 2000). Ten replicates of each value of K (= the number of groups) were run for different selections of samples with a burn-in period of 100 000 and 1 000 000 iterations.

RESULTS

The matrix of ITS sequences had 595 characters of which 44 were potentially parsimony informative. None of the ambiguous positions coded according to IUPAC standards were potentially parsimony informative. We found 16 most parsimonious trees (MPTs) of length 84 from one island in the tree space. A Bayesian phylogram combining the results from the parsimony and Bayesian analyses of the nrITS sequences is presented in Fig. 2. Goodness of fit values were CI = 0.833, RI = 0.926, and RC = 0.771. *Smelowskia* (*Redowskia*) *sophiifolia* (Cham. & Schltdl.) Al-Shehbaz & S.I. Warwick was sister to the ingroup with a posterior probability (PP) of 1.0/1.0 (SYMγ/GTRγ), Bremer support (BR) of 4, Jackknife (JK) 97%, and Bootstrap (BS) 96%. *Smelowskia* (*Ermania*) *parryoides* was sister to all other taxa in the ingroup, but this sistergroup relationship only had a posterior probability of 0.81/0.80, BR of 1 and JK and BS below 50%. The tree was further divided into three (by parsimony analyses) or four (by Bayesian analyses) groups, with unresolved relationships. The first group consisted of only one species in both parsimony and Bayesian analyses: *Smelowskia* (*Gorodkovia*) *jacutica* (PP 1.0/1.0, BR 3, JK 94%, BS 93%). The second group consisted of all the *Melanidion* species: *S. borealis, S. ovalis,* and *S. pyriformis* in the Bayesian analysis (PP 0.72/0.68, BR 1). The third group consisted of only one species: *Smelowskia* (*Melanidion*?) *inopinata* in the Bayesian analysis (PP 1.0/1.0, BR 5, JK 97%, BS 99%). The second and third group belonged to the same clade in the parsimony analysis. The fourth group comprised only species of *Smelowskia* s. str.: *S. alba, S. calycina, S. bifurcata, S. porsildii, S. americana*, and *S. media* (PP 1.0/1.0, BR 2, JK 55%).

The matrix of the 23rd intron of *RPA*2 sequences was 309 characters long, of which 15 were potentially parsimony informative. None of the ambiguous positions coded according to IUPAC standards were potentially parsimony informative. The *RPA*2 parsimony analysis (Fig. 3) resulted in 8 MPTs of length 23 from one island in the tree-space. Goodness of fit

values were CI = 1.00, RI = 1.00 and RC = 1.00. One well supported clade (JK = 99%, BS = 99%) was inferred, containing all taxa of *Smelowskia* s. str.

The combined matrix of plastid sequences had 2898 characters of which ~50 were potentially parsimony informative. The parsimony analysis of the plastid matrix (Fig. 4) resulted in 1365 MPTs of length 96 from one island in the tree-space. Goodness of fit values were CI = 0.83, RI = 0.93, and RC = 0.78. The tree had low resolution with one branch separating *S. alba*, *S.* (*Gorodkovia*) *jacutica,* and *S.* (*Ermania*) *parryoides* from the rest of the ingroup (JK 60% and BS 60%). The other supported branches were only for different accessions of individual species (*S. ovalis, S.* (*Ermania*) *parryoides, S.* (*Melanidion*?) *inopinata,* and *S. americana*).

The microsatellite analysis provided a final matrix of 107 individuals with 37 variable markers. The accessions of the outgroup taxa *S.* (*Hediniopsis*) *czukotica* and *S.* (*Hedinia*) *altaica* were excluded from the final analysis as they only yielded private markers for most primers. In the PCO plot, axis 1 and 2 explained 22.2% and 17.9% of the variation, respectively (Fig. 5). The STRUCTURE analysis with K = 3 separated the accessions into three groups, which also could be recognized in the PCO plot. One group comprised the species formerly assigned to the genera *Ermania, Gorodkovia* and *Melanidion*. The remaining two groups comprised the species of *Smelowskia* s. str., one with the Asian (non-Beringian) species (*S. alba, S. bifurcata*, and *S. calycina*) and one with the American/Beringian species (*S. americana, S. media*, and *S. porsildii*). In a separate PCO analysis of the latter group (Fig. 6), *S. americana, S. media* and *S. porsildii* could be separated along the first two axes. The accessions initially referred to *S. jurtzevii* and *S. spathulatifolia* grouped together with *S. porsildii* in this plot, and could neither be separated on any of the next eight axes in the PCO analysis nor in STRUCTURE.

As expected, some accessions of the species reported as polyploid had microsatellite profiles incompatible with diploidy at some loci (AthCTRI, ATTS0191, NGA1145, and IS-17). These profiles contained three or four different alleles (indicated in Appendix 1). All accessions but one of *S. americana* and eleven out of 39 accessions of *S. porsildii* showed a polyploid profile at one or more of these four loci. Most accessions of the species reported as diploid had microsatellite profiles compatible with diploidy at all seven loci. However, six out of 17 accessions of *S. bifurcata*, which previously only has been reported as diploid, showed a polyploid profile at the locus ATHGAPAb.

DISCUSSION

This study has shown that microsatellite analysis in combination with sequencing of several nuclear regions provide a powerful approach to resolve relationships in recently evolved genera, which are frequently encountered in the arctic flora. This is particularly important for groups occurring inaccessible areas from which fresh material for DNA fingerprinting are difficult to obtain.

Mountain systems are frequently characterized as island systems (Billings, 1974; Ribesell, 1982), and one could expect many of the same evolutionary mechanisms taking place. In a study of Canary Island species of the genus *Descurainia*, plastid regions provided many characters for resolved phylogenetic trees (Goodson et al., 2006). However, the ITS region had no informative characters at all. This genus is supposedly closely related to *Smelowskia*, where we found the opposite pattern i.e. good resolution from nuclear data and low resulution from plastid data. Other arctic genera show a pattern similar to that observed in *Smelowskia*; plastid regions in *Draba* and *Cardamine* did not provide resolved phylogenies (Carlsen et al.; Koch and Al-Shehbaz, 2002). This indicates that, at least for these genera, there are different evolutionary mechanisms driving the divergence of closely related taxa. There were no signs that our direct PCR approach amplified different homeologs of nuclear sequences as seen in some other studies of recently diverged and/or polyploid lineages, indicating allopolyploidy or incomplete lineage sorting (Kovarik et al., 2005; Brysting et al., 2007; Eidesen et al., 2007b).

The *Smelowskia* clade - one or several genera?

Our molecular analyses of the *Smelowskia* clade shows a structure corresponding to the former subdivision of this clade into several genera. The analyses based on ITS, *RPA*2 and SSRs all identify *Smelowskia* s. str. as a group separate from *Melanidion* and *Ermania*. The

results of the two nuclear regions and the SSR are congruent and separate *Smelowskia* s. str. from *Melanidion* as sisters. With an assignment of *S. inopinata* to *Melanidion*, the old genus subdivision thus seems to be justified. The sequences also suggest *Ermania* as sister to the *Melanidion*/*Smelowskia* clade, and *Redowskia* as sister to this group.

Within *Smelowskia* s. str. there is a geographic separation of the Old World and New World (including Beringian) accessions in the SSRs, which is not contradicted by the sequence-based phylogenies. Based on the chloroplast phylogeny, however, it would seem that American acessions of *Smelowskia* s. str. and *Melanidion* constitute a separate group originating from Asian ancestors. This does not fit well with morphological and nuclear molecular data, a more likely scenario is horizontal transfer of the plastid genome, which has been more and more frequently reported in plants (Rieseberg and Soltis, 1991; Rieseberg et al., 1996; Petit et al., 2004).

There are two equally justifiable solutions to the problem of genus delimitation. One solution is to lump all species together into one large polymorphic genus, as suggested by Al-Shehbaz and Warwick (2006). The other is to maintain *Smelowskia* s. str. and the satellite genera *Ermania, Melanidion, Gorodkovia*, and *Redowskia.* This solution also implies exclusion from *Smelowskia* of the additional sattelite genera not analyzed in this study (*Hedinia, Hediniopsis, Sophiopsis,* and *Sinosophiopsis*), and that *S. inopinata* is assigned to *Melanidion*. However, as our plastid data suggest gene transfer between the lineages, we will recommend retaining one large genus until supplementary studies are performed.

Number and delimitation of species

Most of the species we tentatively recognized in the initial framework for this study seem to be adequately separable by molecular markers. However, we note that *S. bifurcata* and *S. calycina*, easily separable based on calyx and siliqula characters (Ovchinnikova, 2004) are

barely separable by the markers studied here. Based on their clear morphological differences, we suggest to keep these as two separate species. We also found that accessions of *S. alba* from the Lena River delta differ somewhat from those from the Altai Mountains. This molecular difference may be ascribed to divergence between two disjunct distribution areas wich has not yet resulted in morphological divergence with taxonomic implications. Although the accessions of *S. media* and *S. porsildii* were inadequately separated in all the sequence analyses, they were fairly distinct based on SSRs. Their distinction is also supported by their different leaf shape; *S. media* has pinnatly lobed basal and cauline leaves, whereas *S. porsildii* has leaves that are entire or with shallow apical teeth (Drury and Rollins, 1952).

The SSR data provided further evidence of the importance of frequent polyploidization in this arctic-alpine group. *Smelowskia bifurcata* was reported as diploid with $2n = 12$ by Krogluevich (1976), but we found more than two alleles per individual at the locus ATHGAPAb. This may be due to a single-region duplication, but it is more likely that there are multiple cytotypes also within this species. Another possible explanation is misidentification of the specimens that are counted. Krogluevich (1976) counted specimens from the Putoran Plateu (Lake Bogatyr), which is outside of the commonly recognized distribution area of *S. bifurcata*, but within the range of the morphologically similar *S. alba*, which, however, also has been reported as diploid (Ovchinnikova, 2004). More chromosome counts are clearly necessary for these as well as other species of *Smelowskia*.

Our results suggest that *S. porsildii* should be recognized as one variable species, including *S. spathulatifolia* and *S. jurtzevii*. We found no molecular differences between the accessions referred to them, and there are apparently neither any good morphological characters distinguishing among them (Warwick et al., 2004; Al-Shehbaz and Warwick, 2006). The name *Smelowskia porsildii* (W.H. Drury & Rollins) Jurtzev has priority (Jurtzev, 1970), and he following names should be treated as synonyms; *Smelowskia spathulatifolia*

Velichkin and *Smelowskia jurtzevii* Velichkin. For a full list of synonyms see Velichkin (1974; 1979).

The Beringian species *S. porsildii* does not show a close relationship with the Asian (non-Beringian) taxa, but rather with the American *S. media* and *S. americana*. These three species are allopatric, occurring in three separate areas left unglaciated during the Wisconsian maximum (Fig. 1; Dyke, 2004). It is possible that they result from divergent speciation in different glacial refugia. Alternatively, supported by the varying ploidy level and large morphological variation within *S. porsildii,* this species may result from recurrent hybridization and allopolyploidization events between Asian and North American lineages. Muliple polyploid origins have been reported for many other arctic taxa (Brochmann et al., 2004; Brysting et al., 2004).

Origin and spread of the *Smelowskia* clade

Based on its sister group relationships, it is most likely that the *Smelowskia* clade originated in Central Asia. Except for *S. czukotica*, all species outside what we have called the *Smelowskia* clade are Central Asian. The American and Beringian representatives within the clade are restricted to two phylogenetic lineages, whereas the Asian representatives are spread throughout the tree. If the clade originated in Asia, only two dispersal events from Asia to North America have to be inferred. Alternatively, a North American origin would necessitate at least four dispersal events. Thus, our results are not consistent with a North American origin of the group (from the *S. media* lineage) as suggested by Drury and Rollins (1952) . They only studied the North American representatives, but looking at this region separately, there is still no indication from our study that *S. media* has any key role in the origin of the North American taxa.

FIGURE LEGENDS

Fig. 1.

Sampling sites and geographic distribution of the members of the *Smelowskia* clade.

Fig. 2.

ITS phylogram from the Bayesian analysis. Posterior probability values from two different models (SYMγ/GTRγ) are shown above branches. Jackknife and bootstrap support values from the parsimony analysis are shown below branches (JK/BS). Bremer support values are shown in bold below branches.

Fig.3.

Strict consensus tree of 45 MPTs from the analysis of the RPA2 sequences. Numbers above branches are jackknife/bootstrap support values.

Fig. 4.

Strict consensus tree of 1365 MPTs from the analysis of the combined plastid sequences. Numbers above branches are jackknife/bootstrap support values. Numbers below branches are Bremer support values.

Fig. 5.

PCO plot based on the microsatellite dataset. The colours identify the different genetic groups recognized in the STRUCTURE analysis: black - Beringian and American species of *Smelowskia* s. str., green - Asian (non-Beringian) species of *Smelowskia* s. str., red - species formerly referred to *Melanidion*, *Ermania*, and *Gorodkovia*.

Fig 6.

Separate PCO plot of the Beringian and American species of *Smelowskia* s. str. based on the microsatellite dataset.

Table 1. Taxa included in this study, with their former (pre Al-Shehbaz and Warwick, 2006) genus name, chromosome numbers[1] and approximate distribution area.

Taxon	Former genus name	2*n* =	Area
Ingroup taxa:			
Smelowskia alba (Pall.) Regel	*Smelowskia*	12	Central North Asia
Smelowskia americana (Regel & Herder) Rydb.	*Smelowskia*	12, 22	Western America
Smelowskia bifurcata (Ledeb.) Botsch.	*Smelowskia*	12	Central Asia
Smelowskia borealis (Greene) W.H. Drury & Rollins	*Melanidion*	12	American Beringia
Smelowskia calycina (Stephan) C.A. Mey	*Smelowskia*	12	Central Asia
Smelowskia inopinata (Kom.) Kom.	*Melanidion*?[2]		Eastern Asia
Smelowskia jacutica (Botsch. & Karav.) Al-Shehbaz & S.I. Warwick	*Gorodkovia*	12, 36	North north-eastern Asia
Smelowskia media (W.H. Drury & Rollins) Vielchkin	*Smelowskia*	12	American Beringia
Smelowskia ovalis M.E. Jones	*Melanidion*		Western America
Smelowskia parryoides (Cham.) Polunin	*Ermania*	12, 24	Asian Beringia
Smelowskia porsildii (W.H. Drury & Rollins) Jurtsev	*Smelowskia*	12, 18, 22, 24, 32	Amphi-Beringia
Smelowskia pyriformis W.H. Drury & Rollins	*Melanidion*	12	American Beringia
Smelowskia sophiifolia (Cham. & Schltdl.) Al-Shehbaz & S.I. Warwick	*Redowskia*		Central Asia
Outgroup taxa:			
Smelowskia altaica (Pobed.) Botsch.	*Hedinia*		Central Asia
Smelowskia czukotika (Botsch. & V.V. Petrovsky) Al-Shehbaz & S.I. Warwick	*Hediniopsis*	24	Asian Beringia

1 From (Drury and Rollins, 1952; Johnson and Packer, 1968; Packer, 1968; Yurtsev and Zhukova, 1972; Krogluevich, 1976; Zhukova and Petrovsky, 1977; Dawe and Murray, 1979; Zhukova, 1980; Zhukova and Petrovsky, 1980, 1984; Murray and Kelso, 1997)

2 The genus *Melanidion* har partly been recognized in North America, but this species-group probably also includes *S. inopinata*

Table 2 Microsatellite primers used in this study. The chromosome where they are situated on *Arabidopsis thaliana* is given for each locus.

Name	Chromo-some number	Forward primer 5'-3'	Reverse primer 5'-3'
AthCTRI	5	TATCAACAGAAACGCACCGAG	CCACTTGTTTCTCTCTCTAG
SSL2	1	CATGTACTGGGATTCAGTGTCC	CGTCCTTTGTGTGGTTACACG
ATHGAPAb	3	CACCATGGCTTCGGTTACTT	TCCTGAGAATTCAGTGAAACCC
IS-17	4	TTTGTTGCATCATCCTTTGC	GGCCTGCAATTTGAGACCTA
AthS0392	1	TTGGAGTTAGACACGGATCTG	GTTGATCGCAGCTTGATAAGC
nga129	5	TCAGGAGGAACTAAAGTGAGGG	CACACTGAAGATGGTCTTGAGG
nga1145	2	CCTTCACATCCAAAACCCAC	GCACATACCCACAACCAGAA

1 Appendix 1. Specimens analyzed

Species	PopID	Country	Year	Collector	Y_ Coordinate	X_ Coordinate	Alleles[1]	Herb
S. alba	TC03-044	RUS	1980	B. Khanminchun, M. Danilov & N. Zolobina	52.083	92.750	α	LE
S. alba	TC03-045	RUS	1970	E. Velichkin	54.000	109.000	α	LE
S. alba	TC03-046	RUS	1970	E. Velichkin	53.617	109.617	α	LE
S. alba	TC06-003	RUS	1982	B. Yurtsev	49.633	88.917	α	LE
S. alba	TC06-001	RUS	1979	I. Krasnoborov	53.317	107.650	α	MHA
S. alba	SUP-4013	RUS	2004	L. Kuznetsova	71.925	127.318	α	O
S. alba	773	RUS	2004	L. Kuznetsova	71.925	127.318	α	O
S. altaica	TC03-61	RUS	1931	B. Shishkin, L. Chilikina & G. Sumnevich			na	LE
S. americana	TC03-204	USA	1990	L. Vierling 86	39.131	-106.182	ε	ALA
S. americana	TC03-255	USA	1997	A. R. Batten 97-7	40.717	-110.300	na	ALA
S. americana	TC03-400	CAN	1964	J. A. Calder	50.594	-114.962	ε	DAO
S. americana	TC03-401	USA	1965	C. L Hitchcock	44.968	-109.467	α	DAO
S. americana	TC03-405	USA	1972	G. W. & G. G. Douglas	48.679	-119.909	na	DAO
S. americana	TC03-406	CAN	1969	W. Blais & J. Nagy	49.150	-113.883	εβ	CAN
S. americana	ALA6	USA	1984	Init. Kirkpatrick	43.866	-109.373	ε	ALA
S. bifurcata	SUP03-300-1	RUS	2002	A. Tribsch	50.331	87.736	α	O
S. bifurcata	SUP03-300-2	RUS	2002	A. Tribsch	50.331	87.736	β	O
S. bifurcata	SUP03-300-3	RUS	2002	A. Tribsch	50.331	87.736	β	O
S. bifurcata	SUP03-300-4	RUS	2002	A. Tribsch	50.331	87.736	β	O
S. bifurcata	SUP03-317-1	RUS	2002	A. Tribsch	49.658	88.175	α	O
S. bifurcata	SUP03-317-2	RUS	2002	A. Tribsch	49.658	88.175	α	O
S. bifurcata	SUP03-317-3	RUS	2002	A. Tribsch	49.658	88.175	α	O
S. bifurcata	SUP03-317-4	RUS	2002	A. Tribsch	49.658	88.175	β	O
S. bifurcata	SUP03-317-5	RUS	2002	A. Tribsch	49.658	88.175	β	O
S. bifurcata	SUP03-326-1	RUS	2002	A. Tribsch	49.422	88.035	α	O
S. bifurcata	SUP03-326-2	RUS	2002	A. Tribsch	49.422	88.035	α	O
S. bifurcata	SUP03-326-3	RUS	2002	A. Tribsch	49.422	88.035	α	O
S. bifurcata	SUP03-326-4	RUS	2002	A. Tribsch	49.422	88.035	α	O
S. bifurcata	SUP03-326-5	RUS	2002	A. Tribsch	49.422	88.035	β	O
S. bifurcata	TC03-047	RUS	1966	I. Krasnoborov & B. Luzhecky	52.750	96.000	na	LE
S. bifurcata	TC03-049	RUS	1963	Alenskaya, Kozhevnikova & Surova	52.283	104.267	na	LE
S. bifurcata	TC03-050	RUS	1970	E. Velichkin	52.500	83.000	α	LE
S. bifurcata	TC03-051	RUS	1947	K. A. Sobolevskaya & A. Bezsonov	50.550	89.800	α	LE
S. bifurcata	TC06-005	RUS	1966	I. Krasnoborov & B. Luzhecky	53.125	93.558	α	LE

S. bifurcata	TC06-006	RUS	1966	M. Ivanova	55.484	110.101		LE
S. borealis	SUP03-127-1	CAN	2003	R. Elven & H. Solstad	65.506	-138.239	α	O
S. borealis	SUP03-127-2	CAN	2003	R. Elven & H. Solstad	65.506	-138.239	α	O
S. borealis	SUP03-127-4	CAN	2003	R. Elven & H. Solstad	65.506	-138.239	α	O
S. borealis	SUP03-127-5	CAN	2003	R. Elven & H. Solstad	65.506	-138.239	α	O
S. borealis	SUP03-146-1	CAN	2003	H. Solstad & R. Elven	64.969	-138.325	α	O
S. borealis	SUP03-146-2	CAN	2003	H. Solstad & R. Elven	64.969	-138.325	α	O
S. borealis	SUP03-146-3	CAN	2003	H. Solstad & R. Elven	64.969	-138.325	α	O
S. borealis	SUP03-146-4	CAN	2003	H. Solstad & R. Elven	64.969	-138.325	α	O
S. borealis	SUP03-146-5	CAN	2003	H. Solstad & R. Elven	64.969	-138.325	α	O
S. borealis	TC03-407	USA	1962	L. A. Viereck & K. Jones	63.883	-147.333	na	CAN
S. calycina	TC03-052	RUS	1970	E. Velichkin	49.714	87.392	α	LE
S. calycina	TC03-053	RUS	1970	E. Velichkin	49.714	87.392	α	LE
S. calycina	TC03-247	RUS	1987	D. Murray, W. A. Weber & I. Krasnoborov	49.924	86.444	α	ALA
S. calycina	TC06-004	RUS	1976	I. Krasnoborov & V. Khanminchun	na	na	α	MHA
S. czukotika	TC03-60	RUS	1985	V. Petrovsky & T. Plieva	na	na	na	LE
S. inopinata	TC03-054	RUS	1935	V. Vasilyev	56.440	138.214	α	LE
S. inopinata	TC03-055	RUS	1924	V. Vasilyev	56.440	138.214	α	LE
S. inopinata	TC06-002	RUS	1978	T. Buch & V. Yakubov	48.398	135.295	α	MHA
S. inopinata	TC06-007	RUS	1965	V. N. Voroshilov	48.398	135.295	α	MHA
S. jacutica	3080	RUS	1957	B. Yurtzev	71.633	128.867	α	O
S. jacutica	TC07-1	RUS					α	
S. jacutica	TC07-2	RUS					α	
S. media	TC03-254	USA	1978	D. F. Murray	69.583	-145.917	na	ALA
S. media	TC03-296	USA	1982	D. A. Walker	69.833	-149.500	α	ALA
S. media	RE03-0455-1	CAN	2003	R. Elven & H. Solstad	67.050	-136.250	α	O
S. media	RE03-0455-2	CAN	2003	R. Elven & H. Solstad	67.050	-136.250	α	O
S. media	RE03-0455-3	CAN	2003	R. Elven & H. Solstad	67.050	-136.250	α	O
S. media	RE03-0455-4	CAN	2003	R. Elven & H. Solstad	67.050	-136.250	α	O
S. media	RE03-0455-5	CAN	2003	R. Elven & H. Solstad	67.050	-136.250	α	O
S. ovalis	TC03-404	USA	1938	W. C. Maccalla & R. C. Rollins	46.883	-121.885	α	DAO
S. ovalis	TC03-409	USA	1953	G. W. Gillett	40.488	-121.505	α	CAN
S. parryoides	05/0224-1	RUS	2005	H. Solstad & R. Elven	64.830	-173.087	α	O
S. parryoides	05/0224-2	RUS	2005	H. Solstad & R. Elven	64.830	-173.087	α	O
S. parryoides	05/0042-1	RUS	2005	H. Solstad & R. Elven	64.500	172.833	α	O
S. parryoides	05/0042-2	RUS	2005	H. Solstad & R. Elven	64.500	172.833	α	O
S. parryoides	05/0042-3	RUS	2005	H. Solstad & R. Elven	64.500	172.833	α	O

S. parryoides	05/0042-4	RUS	2005	H. Solstad & R. Elven	64.500	172.833	α	O
S. parryoides	05/0042-5	RUS	2005	H. Solstad & R. Elven	64.500	172.833	α	O
S. porsildii	TC03-291	USA	1972	M. Lenarz	65.450	-167.150	γ	ALA
S. porsildii	TC06-108	USA	1997	D. F. Murray & R. Lipkin	65.367	-166.450	γδ	ALA
S. porsildii	TC03-057	RUS	1971	N. Sekretareva, A. Sytin & B. Yurtsev	66.556	-171.085	ε	LE
S. porsildii	TC03-058	RUS	1972	B. Yurtsev	65.069	-172.960	α	LE
S. porsildii	TC03-059	RUS	1967	A. Korobkov & B. Yurtsev	67.026	-178.917	β ε	LE
S. porsildii	TC03-062	RUS	1967	A. Korobkov & B. Yurtsev			na	LE
S. porsildii	ALA93349	RUS	1972	P. Zukova & B. Yurtsev	66.333	-171.583	ε	ALA
S. porsildii	ALA83158	RUS	1979	P. Zmylev et al	67.500	-178.667	γ	ALA
S. porsildii	AK-904-5	USA	2003	C. L. Parker, R. Elven & H. Solstad	67.967	-161.617	β ε	O
S. porsildii	AK-904-6	USA	2003	C. L. Parker, R. Elven & H. Solstad	67.967	-161.617	α	O
S. porsildii	AK-904-7	USA	2003	C. L. Parker, R. Elven & H. Solstad	67.967	-161.617	β ε	O
S. porsildii	SUP02-272-1	USA	2002	R. Elven	68.182	-152.734	α	O
S. porsildii	SUP02-272-2	USA	2002	R. Elven	68.182	-152.734	ε	O
S. porsildii	SUP02-272-3	USA	2002	R. Elven	68.182	-152.734	ε	O
S. porsildii	SUP02-272-4	USA	2002	R. Elven	68.182	-152.734	ε	O
S. porsildii	SUP02-272-5	USA	2002	R. Elven	68.182	-152.734	ε	O
S. porsildii	TC03-224	USA	2001	C. L. Parker, C. R. Meyers & N. Eagleson	66.967	-159.683	δ	ALA
S. porsildii	TC03-276	USA	2001	C. L. Parker	68.383	-158.850	na	ALA
S. porsildii	SUP02-137-1	USA	2002	R. Elven, T. M. Gabrielsen & M. H. Jørgensen	64.695	-164.182	α	O
S. porsildii	SUP02-137-2	USA	2002	R. Elven, T. M. Gabrielsen & M. H. Jørgensen	64.695	-164.182	α	O
S. porsildii	SUP02-137-3	USA	2002	R. Elven, T. M. Gabrielsen & M. H. Jørgensen	64.695	-164.182	α	O
S. porsildii	SUP02-137-4	USA	2002	R. Elven, T. M. Gabrielsen & M. H. Jørgensen	64.695	-164.182	α	O
S. porsildii	SUP02-137-5	USA	2002	R. Elven, T. M. Gabrielsen & M. H. Jørgensen	64.695	-164.182	α	O
S. porsildii	SUP02-148-2	USA	2002	R. Elven, T. M. Gabrielsen & M. H. Jørgensen	64.907	-166.180	α	O
S. porsildii	SUP02-148-3	USA	2002	R. Elven, T. M. Gabrielsen & M. H. Jørgensen	64.907	-166.180	α	O
S. porsildii	SUP02-148-4	USA	2002	R. Elven, T. M. Gabrielsen & M. H. Jørgensen	64.907	-166.180	α	O
S. porsildii	SUP02-148-5	USA	2002	R. Elven, T. M. Gabrielsen & M. H. Jørgensen	64.907	-166.180	α	O
S. porsildii	SUP02-176-1	USA	2002	R. Elven, T. M. Gabrielsen & M. H. Jørgensen	64.933	-164.998	α	O
S. porsildii	SUP02-176-2	USA	2002	R. Elven, T. M. Gabrielsen & M. H. Jørgensen	64.933	-164.998	α	O
S. porsildii	SUP02-176-3	USA	2002	R. Elven, T. M. Gabrielsen & M. H. Jørgensen	64.933	-164.998	α	O
S. porsildii	SUP02-176-4	USA	2002	R. Elven, T. M. Gabrielsen & M. H. Jørgensen	64.933	-164.998	α	O
S. porsildii	SUP02-176-5	USA	2002	R. Elven, T. M. Gabrielsen & M. H. Jørgensen	64.933	-164.998	α	O
S. porsildii	TC03-210	USA	1984	T. Kelso	65.167	-162.117	na	ALA
S. porsildii	TC03-213	USA	1971	A. Springer	64.650	-165.717	na	ALA
S. porsildii	05/1239-1	USA	2005	C. L. Parker & H. Solstad	64.648	-164.347	α	O

S. porsildii	05/1239-2	USA	2005	C. L. Parker & H. Solstad	64.648	-164.347	α	O
S. porsildii	05/1239-3	USA	2005	C. L. Parker & H. Solstad	64.648	-164.347	α	O
S. porsildii	05/1239-4	USA	2005	C. L. Parker & H. Solstad	64.648	-164.347	α	O
S. porsildii	05/1239-5	USA	2005	C. L. Parker & H. Solstad	64.648	-164.347	α	O
S. porsildii	05/1300-1	USA	2005	C. L. Parker & H. Solstad	64.657	-165.725	α	O
S. porsildii	05/1300-2	USA	2005	C. L. Parker & H. Solstad	64.657	-165.725	α	O
S. porsildii	05/1300-3	USA	2005	C. L. Parker & H. Solstad	64.657	-165.725	α	O
S. porsildii	05/1300-4	USA	2005	C. L. Parker & H. Solstad	64.657	-165.725	α	O
S. porsildii	05/1300-5	USA	2005	C. L. Parker & H. Solstad	64.657	-165.725	α	O
S. pyriformis	Ala128297	USA	1999	C. L.Parker & Blank	61.783	-155.550	α	ALA
S. pyriformis	Ala150307	USA	2004	C. L. Parker	59.211	-161.658	α	ALA

1 α diploid profile at all loci, β tetraploid at AthCTR1, γ tetraploid at IS-17, ε tetraploid at nga1145, δ tetraploid at SSL2.

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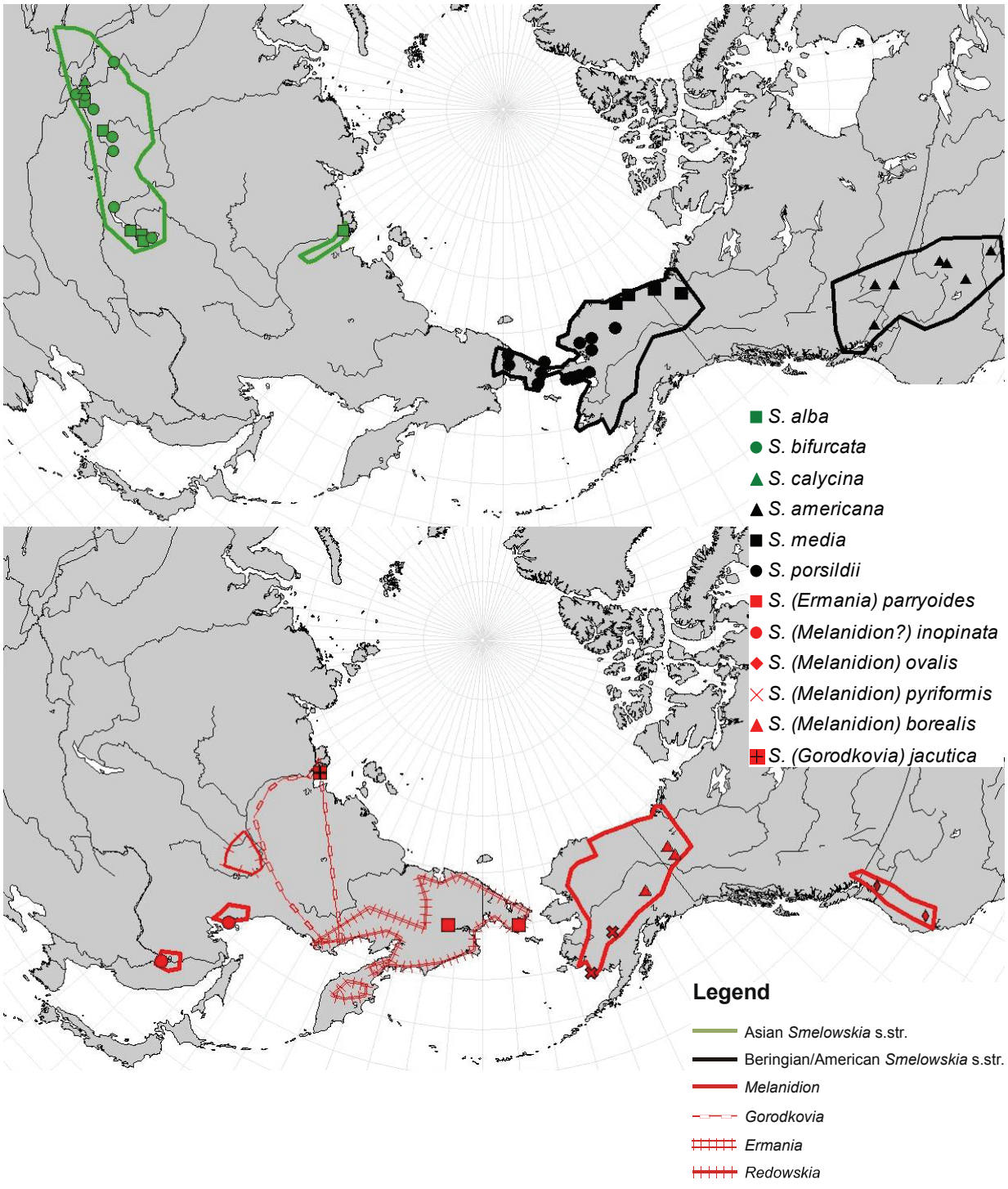
Fig. 1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S. (Melanidion) ovalis
S. (Melanidion) pyriformis
S. (Melanidion) borealis
S. (Gorodkovia) jacutica
Legend
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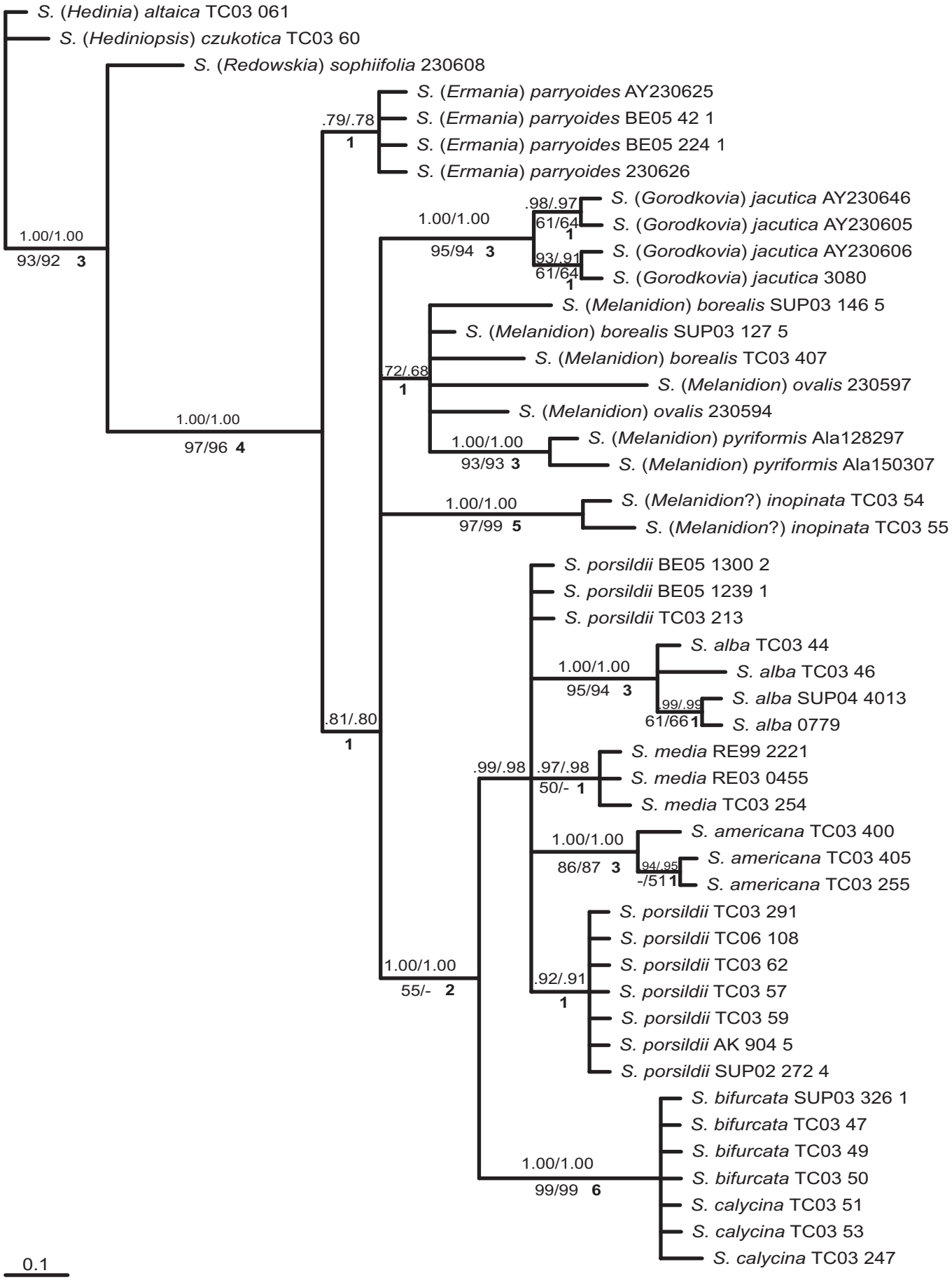
Beringian/American Smelowskia s.str.
Melanidion
Gorodkovia
Ermania
Redowskia

Fig. 2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S. (Melanidion) borealis SUP03 127 5
S. (Melanidion) borealis TC03 407
S. (Melanidion) ovalis 230597
S. (Melanidion) ovalis 230594
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S. (Melanidion) pyriformis Ala150307
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S. (Melanidion?) inopinata TC03 55
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S. porsildii BE05 1239 1
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S. americana TC03 255
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S. porsildii TC03 62
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1
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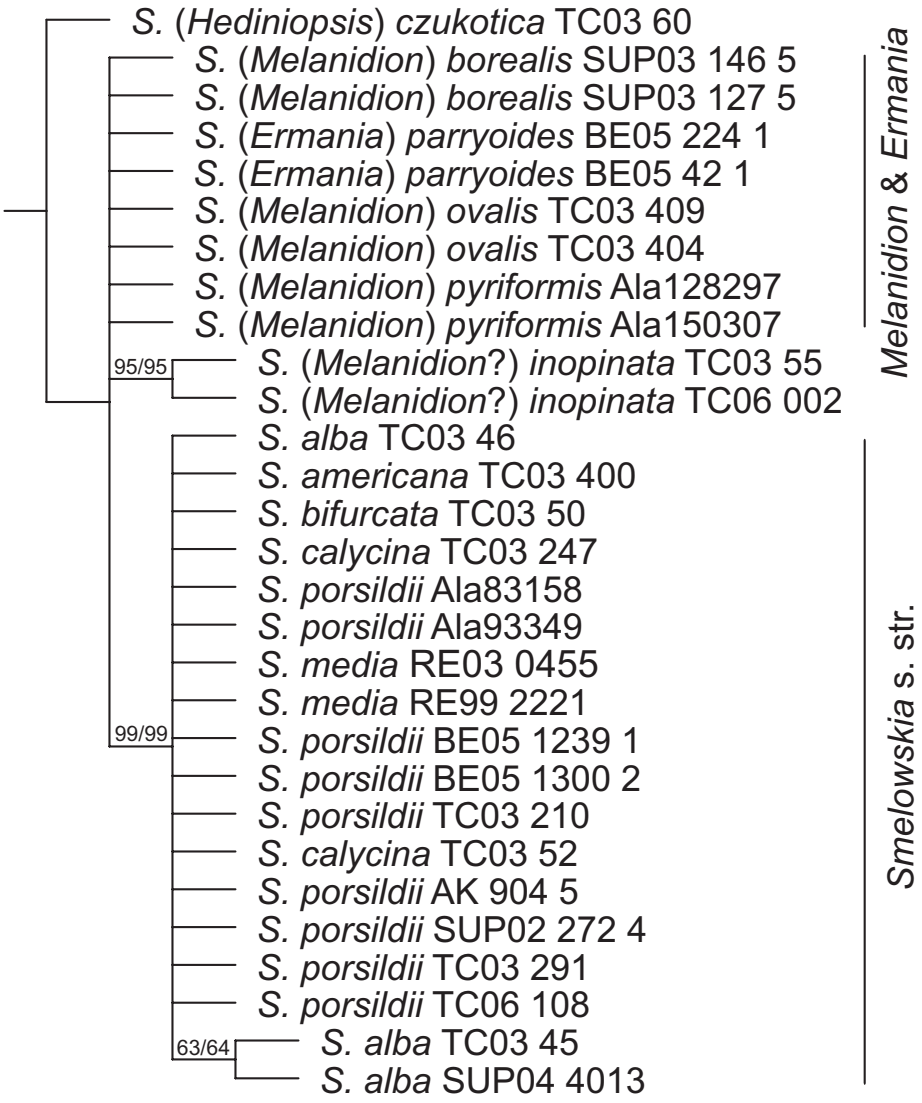
Fig. 3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95/95
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S. (Melanidion?) inopinata TC06 002
S. alba TC03 46
S. americana TC03 400
S. bifurcata TC03 50
S. calycina TC03 247
S. porsildii Ala83158
S. porsildii Ala93349
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S. media RE99 2221
99/99
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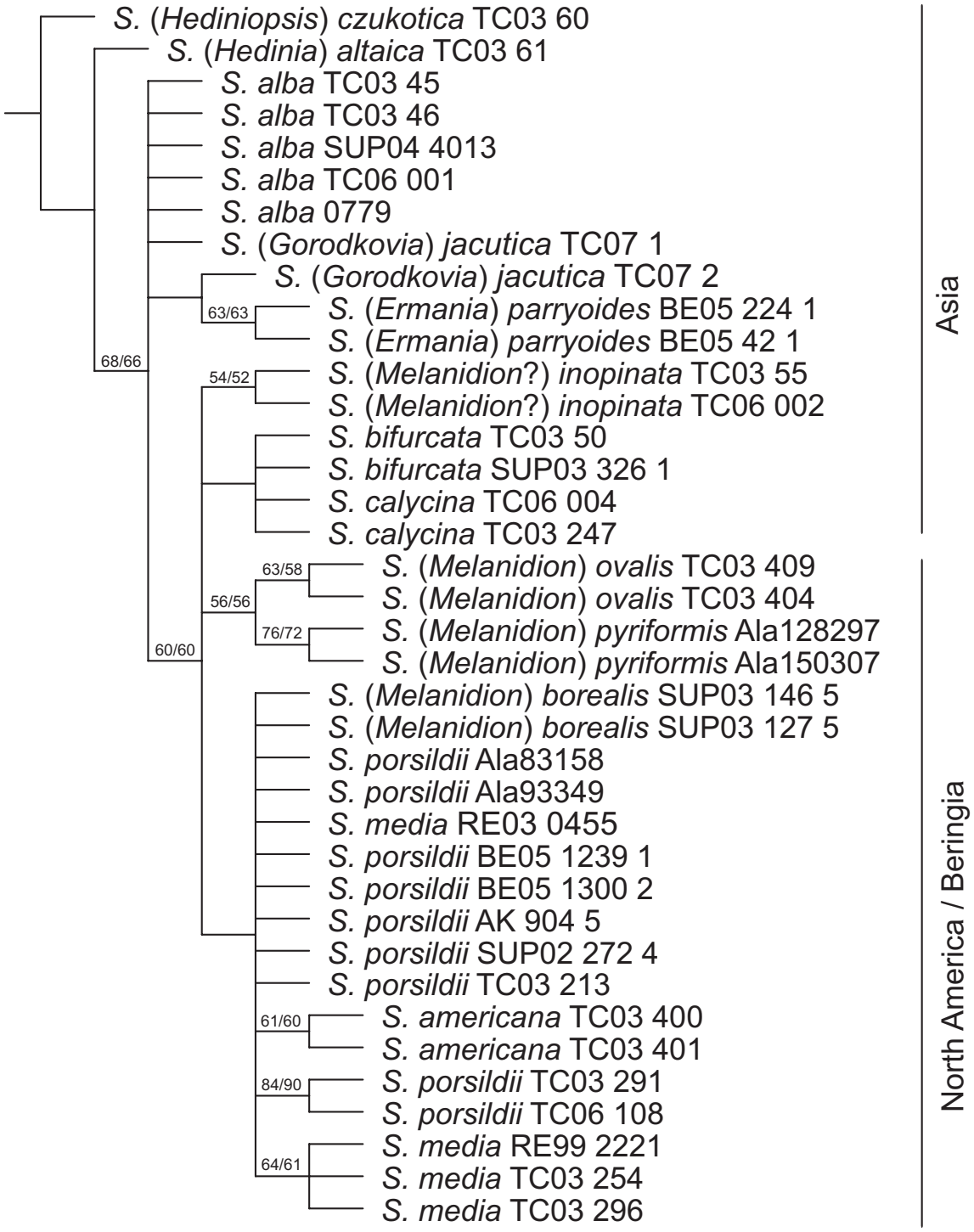
Smelowskia s. str.

Fig. 4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S. calycina TC03 247
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S. (Melanidion) pyriformis Ala128297
S. (Melanidion) pyriformis Ala150307
S. (Melanidion) borealis SUP03 146 5
S. (Melanidion) borealis SUP03 127 5
S. porsildii Ala83158
S. porsildii Ala93349
S. media RE03 0455
S. porsildii BE05 1239 1
S. porsildii BE05 1300 2
S. porsildii AK 904 5
S. porsildii SUP02 272 4
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S. americana TC03 401
S. porsildii TC03 291
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S. media TC03 296
63/63
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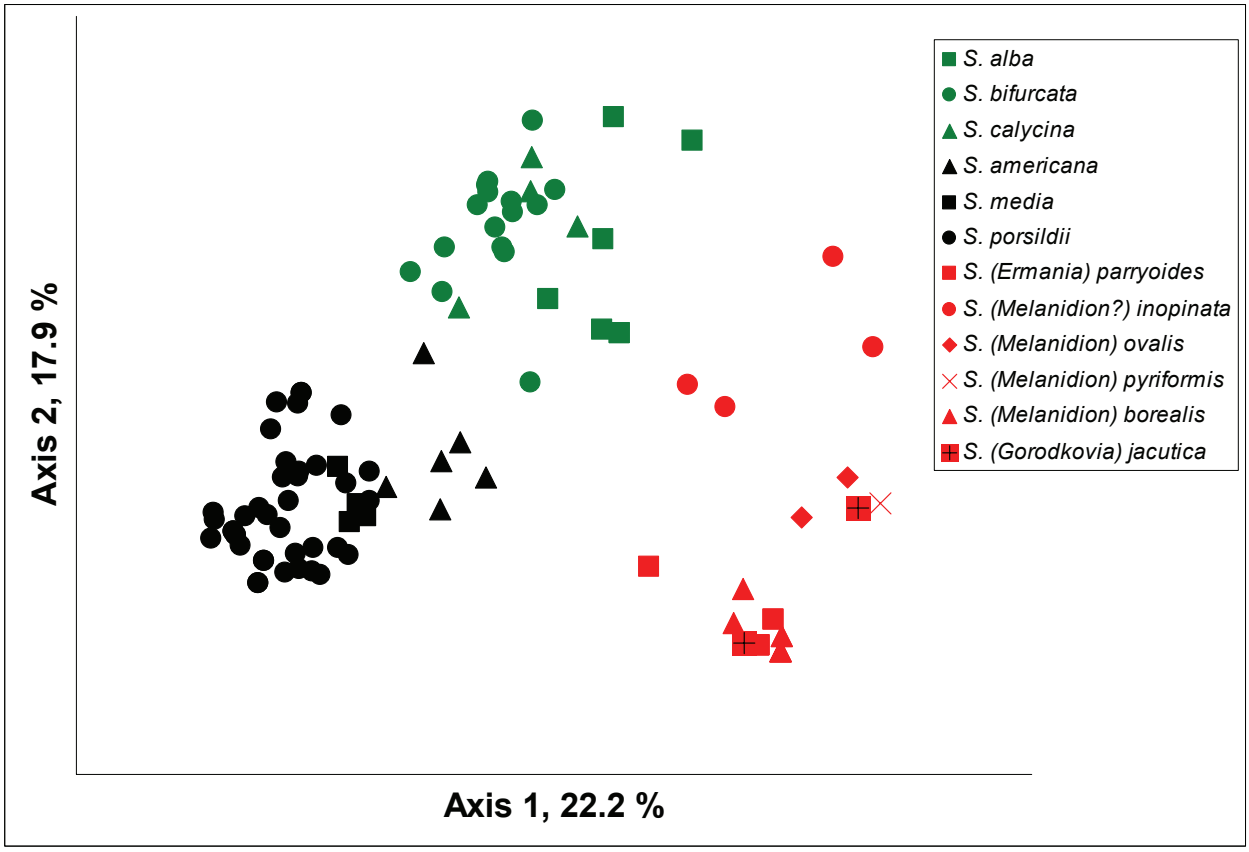
Asia
North America / Beringia

Fig. 5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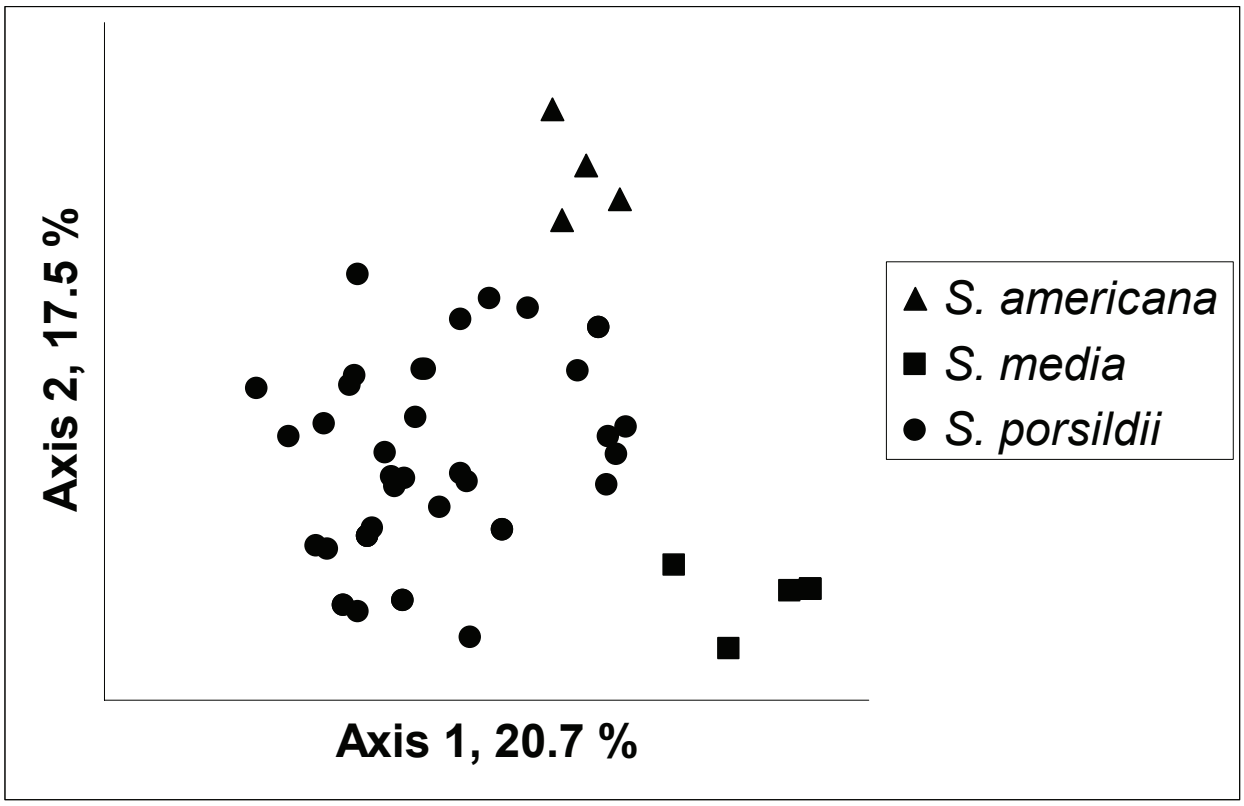
S. (Melanidion?) inopinata
S. (Melanidion) ovalis
S. (Melanidion) pyriformis
S. (Melanidion) borealis
S. (Gorodkovia) jacutica
Axis 2, 17.9 %
Axis 1, 22.2 %

Fig. 6



PAPER II

Biogeography and phylogeny of *Cardamine* (Brassicaceae) [1]

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The biogeography and phylogeny of *Cardamine* were inferred based on sequences of the nuclear ribosomal ITS regions and the plastid *trn*L intron and *trn*L-F spacer regions. This genus is one of the largest and polyploid-rich genera of the Brassicaceae, and has its centre of diversity in Eurasia. Species from all continents, representing all sections except two monotypic ones, were included. The results support a hypothesis of recent and rapid speciation in the genus. The traditional sectional classification was not supported. We found evidence for several extremely long-distance dispersal events. Colonization of the southern hemisphere and the Arctic has occurred repeatedly; we identified at least three phylogenetically distinct arctic lineages, two distinct Oceanian lineages, and four distinct South American lineages. Polyploidization has occurred independently many times during the evolution of *Cardamine*. Recent divergence combined with widespread polyploidization offer an explanation for the complex taxonomy of the genus.

Keywords: Arctic; Biogeography; *Cardamine*; Phylogeny; Polyploidization

Cardamine L. is a taxonomically complex, cosmopolitan genus with at least 160--200 arctic, alpine, and boreal species, and is thus one of the most species-rich genera of the Brassicaceae (Sjöstedt, 1975; Hewson, 1982; Al-Shehbaz, 1988; Webb et al., 1988; Al-Shehbaz et al., 2006). The number of species accepted varies considerably among different authors, illustrating the notorious taxonomic complexity of this genus. The centre of diversity is clearly situated in Eurasia. According to conservative estimates (mainly based on Al-Shehbaz, 1988), approximately 95 species are Eurasian (~48 in China and ~25 in Europe including the Caucasus). There are also many species in North and Central America (~40), and at least nine species extend into arctic areas. Some species are invasive cosmopolitan weeds, such as *C. hirsuta, C. impatiens, C. flexuosa,* and *C. parviflora*. There are much fewer native species in the southern hemisphere: ten in Australia and New Zealand, five in South America (likely underestimated), three in Africa, and four in New Guinea.

In Schulz' (1903) monograph of the genus, 116 species were accepted and classified into 12 sections. Schulz (1936) later extended his account to include ~130 species in 13 sections (Table 1). Of special interest in a biogeographic context are the three largest sections: *Cardamine* (Schulz' *Eucardamine*), *Dentaria* and *Cardaminella*. In Schulz' treatments, section *Cardamine* includes ~74 species, has a global distribution and encompasses a wide range of morphological variation. His section *Dentaria* contains 16 perennial species from North America and Europe, characterized by fleshy creeping rhizomes and typically large showy flowers. Schulz' section *Cardaminella* has a highly disjunct distribution, including four species from the Arctic (including Beringia), three species from alpine areas in Europe, one species from Japan, and three species from Oceania, all of them small, cold-adapted plants. Also noteworthy are six monotypic sections (Table 1).

Many new species have been described since Schulz' (1903; 1936) revisions, but species delimitation is difficult and the total number of species in *Cardamine* remains

controversial. The sectional partitioning of Schulz has been criticized by several authors for over-emphasizing a few morphological characters (Al-Shehbaz, 1988; Rashid and Ohba, 1993). Previous molecular studies in *Cardamine* have shown that some of his sections (*Cardamine, Dentaria, Macrocarpus, Macrophyllum, and Papyrophyllum)* are not monophyletic (Rashid and Ohba, 1993; Franzke et al., 1998; Sweeney and Price, 2000; Bleeker et al., 2002). Several species groups in *Cardamine* have been studied quite extensively based on molecular as well as cytological and morphological data. The *C. pratensis* complex, for example, has a history of recurrent polyploidization events and dispersals over relatively long distances (Marhold and Ancev, 1999; Franzke and Hurka, 2000; Marhold et al., 2002; Lihova et al., 2004; Marhold et al., 2004; Marhold and Lihova, 2006). However, no genus-wide molecular analysis of *Cardamine* has been performed so far.

Cardamine is probably a fairly young genus. Molecular data indicate that a clade comprising the genera *Barbarea*, *Armoracia* and *Rorippa* is sister to a *Cardamine* – *Nasturtium* clade (Franzke et al., 1998; Yang et al., 1999; Koch et al., 2001). *Rorippa* pollen is first found in sediments from the Pliocene (2.5--5 MYA; (Mai, 1995). Koch et al. (2000) used this time span to estimate that the lineages that gave rise to *Cardamine* and *Barbarea* diverged 6.0 MYA. This was suggested to be an underestimate by (Heads, 2005). However, based on the nuclear dataset of Koch et al. (2000), Haubold and Wiehe (2001) performed a more thorough study under various evolutionary rate assumptions, all resulting in a divergence time of 6.2 MYA.

Most species of *Cardamine* are polyploid, and up to five basic chromosome numbers have been suggested (Al-Shehbaz, 1988). The most probable basic number for the majority of species is $x = 8$ (Kucera et al., 2005). For some species, such as the Beringian taxa in section *Cardaminella*, the most probable basic number is $x = 7$ (Elven et. al., 2006). Diploids are only

known with $2n = 16$, and the highest recorded number is $2n = 32x = 256$ (*C. concatenata* and *C. diphylla*; (Kucera et al., 2005).

The seeds of *Cardamine* are shot out by curling of the silique walls, a typical short-distance mode of dispersal (Kimata, 1983). *Cardamine* is nevertheless found on all continents except Antarctica. Under moist conditions the seeds can become mucilaginous and adhere to animals (Al-Shehbaz, 1988). As the majority of *Cardamine* species occur in moist habitats, this may be a common mode of dispersal, also across vast areas via birds. Dispersal between Eurasia and North America may have occurred stepwise via the Tertiary Beringian land bridge that existed until 5.4 -- 5.5 MYA (Marincovich and Gladenkov, 1999, 2001; Gladenkov et al., 2002), but dispersal over longer distances must have occurred between these *Cardamine*-rich continents and Oceania, South America, and Africa.

In this paper, we particularly address the occurrence of such long-distance colonization events, including the colonization of the biogeographically young arctic region. Among the ~9 species of *Cardamine* occurring in the Arctic, two (*C. bellidifolia* and *C. pratensis* s. lat.) have complete circumpolar distributions, and seven are restricted to the Beringian region (*C. blaisdellii*, *C. digitata*, *C. purpurea*, *C. pedata*, *C. microphylla*, *C. victoris*, and *C. sphenophylla*). The current arctic tundra replaced a more or less continuous forest following the climatic shift in the late Tertiary (Lafontaine and Wood, 1988; Bennike and Böcher, 1990; Matthews and Ovenden, 1990; Murray, 1995; Lear et al., 2000). Murray (1995) suggested that the arctic flora of today is composed of a mixture of survivors from the arctic Tertiary forest, Pleistocene immigrants from various mountain areas, and in-situ evolved Pleistocene taxa.

Here we attempt to reconstruct the phylogeny of *Cardamine* based on extensive, genus-wide species sampling and sequencing of several DNA regions (using the nuclear ribosomal ITS regions and the plastid *trn*L intron and *trn*L-F spacer regions in the final

analysis). In particular, we address the infrageneric classification of *Cardamine*, especially that of (Schulz, 1903; Schulz, 1936; Franzke et al., 1998; Sweeney and Price, 2000; Bleeker et al., 2002). We also examine biogeographic patterns in this widespread genus, and particularly address dispersals and source areas for colonization of the Arctic and the southern hemisphere.

MATERIALS AND METHODS

Fresh leaf material was sampled and dried in silica gel in the field. Vouchers are deposited in the herbaria at the Natural History Museum, University of Oslo (O), and the University of Osnabrück (OSBU). Leaf material was also sampled from herbarium specimens in ALA, CAN, CANB, DAO, HBG, LE, O, OSBU, OSC, S, UPS, and WU (Appendix 1). Species of *Cardamine* representing all continents and 11 of the 13 sections were included. The exceptions are the monotypic sections *Girardiella* and *Lygophyllum* (Table 1).

DNA was extracted using the DNeasy™ Plant Mini Kit or DNeasy™ Plant 96 Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. We initially tested several DNA regions for a subset of species. The mitochondrial *nad*6 gene, the nuclear 5S non-transcribed spacer region, and the plastid regions *trn*T-*trn*L spacer, *psb*A-*trn*H spacer, *trn*S-*trn*G spacer, and *ndh*F gene were tested but either found not variable enough (*nad*6), too variable and difficult to align (the 5S non-transcribed spacer and *psb*A-*trn*H), or difficult to sequence (*ndh*F, *trn*S-*trn*G, and *trn*T-*trn*L). The only useful regions were found to be ITS and the plastid *trn*L intron and *trn*L-F spacer regions.

PCR amplification of ITS was done with the primers ITS 4 and 5 (White et al., 1990) using 30 cycles of 45 s at 94 ºC (first cycle 5 min), 45 s at 55 ºC, and 90 s at 72 ºC (last cycle 10 min). The *trn*L intron was amplified with the primers c and d, and the *trn*L-*trn*F intergenic spacer region with the primers e and f (Taberlet et al., 1991), using 30 cycles of 30 s at 94 ºC (first cycle 5 min), 30 s at 55 ºC, and 90 s at 72 ºC (last cycle 10 min). PCR products were purified with ExoSAP-IT® (USB Corporation, Cleveland, Ohio, USA) before cycle sequencing with BigDye (Applied Biosystems, Foster City, California, USA) using 25 cycles of 10 s at 96 ºC, 5 s at 50 ºC, and 240 s at 60 ºC.

Sequences were edited in Sequencher 4.1.4 (Gene Codes, Ann Arbour, Michigan, USA), and ambiguous positions were coded according to IUPAC standards. Sequences were translated to RNA and analyzed in RNAfold (Hofacker et al., 1994) and MARNA (Siebert and Backofen, 2005) to detect secondary structure and to ensure that conserved stem (helix) regions were aligned correctly. The sequences were subsequently aligned manually in BioEdit (Hall, 1999). The three regions corresponding to the hairpin loop in helix III in ITS 2 and to loops in helices III and IV in ITS 1 could not be unambiguously aligned, and these regions were therefore excluded from the final matrix. For the *trn*L-F spacer region, non-homologous pseudogene replications were excluded from the matrix prior to all analyses (Koch et al., 2005). In addition, plastid and nuclear sequences of *Cardamine* and related genera from GenBank were imported into the matrices and aligned manually, resulting in a dataset including a total number of 111 species of *Cardamine* (Appendix 1). Several genera were tested as outgroup, but *Rorippa* was chosen in the end as the most suitable alternative.

Parsimony analyses were performed in TNT (Goloboff et al., 2003) with potential parsimony informative gaps coded as present/absent (Simmons and Ochoterena, 2000). Heuristic searches were performed with 1000 random addition sequences and TBR branch swapping, saving ten trees per replication. The resulting trees were swapped on with TBR saving up to 100 000 trees. Collapsing rule was set to min. length = 0. Random seed was set to "time". Goodness of fit was calculated using CI, RI, and RC according to (Kluge and Farris, 1969; Farris, 1989). Bremer support (Bremer, 1994) was calculated by producing 120 000 trees that were up to 12 steps longer, starting with saving 10 000 trees one step longer, and successively saving 10 000 tress of up to one step longer in 11 steps. Jackknife (Farris et al., 1996) and bootstrap (Felsenstein, 1985) resampling were performed with 1000 replicates (10 random entry orders and 10 trees saved in each repetition) and collapsing rule = TBR. Jackknifing was performed with 36% deletion. Bootstrap and jackknife were performed with

a cut-off value of 50% and absolute frequencies as output. Implied weighing (Goloboff, 1993) was done with K = 1, 3, 6, 8, 20, and 50 In addition to the analysis of all taxa, separate analyses were done on diploid taxa, tetraploid taxa, and diploid and tetraploid taxa together.

A Bayesian analysis was performed on the ITS dataset in MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) with the model GTR+gamma provided by MrAIC (Nylander, 2004). The analysis was run with the default settings in MrBayes, random starting trees and run for 3 000 000 generations with sampling of Markov chains for each 100^{th} generation. The first 25% of the trees were discarded as "burn-in" samples.

RESULTS

The final aligned ITS matrix included 629 characters of which 188 were parsimony informative (186 when excluding the outgroup). There were four potential parsimony informative coded gaps in the data matrix (2 gaps of length 1 bp and 2 gaps of length 2 bp). The most parsimonious trees (MPTs) inferred from the ITS dataset were 749 steps long with CI = 0.530, RI = 0.692, and RC = 0.367; one of them, as well as the strict consensus tree, is presented in Fig. 1. The separate parsimony analyses of diploids, tetraploids, and diploids and tetraploids together did not give better resolution or conflicting topologies (result not shown). Using implied weighing did not give better resolution or conflicting topologies (result not shown). The Bayesian analysis of the ITS dataset is presented in Fig. 2.

The aligned *trn*L-F matrix included 765 characters of which 77 characters were parsimony informative (71 when excluding the outgroup). There were eleven potential parsimony informative coded gaps in the data matrix with lengths spanning from 1 bp to 11 bp. The MPTs resulting from the *trn*L-F analysis were 258 steps long with CI = 0.694, RI = 0.807, and RC = 0.560; one of them, as well as the strict consensus tree, is presented in Fig. 3. In terms of initial similarity retained as synapomorphy (RI), the plastid characters were more self-congruent than the ITS characters. The analysis of the plastid data set (Fig. 3) resulted in a poorly resolved strict consensus tree, but several interesting groups were recovered in the analyses. The European diploid *Cardaminella* taxa (*C. bellidifolia*, *C. alpina*, and *C. resedifolia*) constituted a monophyletic group with 91% jackknife (JK) support, 88% bootstrap (BS) support, and a Bremer (BR) support of 2 with *C. glauca* as sister (JK 67%, BS 55%, and BR = 1). There was also support (BR = 1) for monophyly of the Oceanian *Cardaminella* species (*C. corymbosa*, *C. debilis*, and *C. lilacina*) with the inclusion of the Beringian *C. victoris* and *C. umbellata*, the North American *C. cordifolia* and the South

American *C. glacialis*. The four European species previously shown to be related to the *C. pratensis* complex formed a group supported by 81% in both JK and BS, and 3 in BR. The North American high polyploids (*C. angustata*, *C. concatenata*, *C. diphylla*, and *C. dissecta*) also formed a clade (supported 82% JK, 76% BS, and BR = 1).

The following presentation focuses on the ITS trees (Figs. 1 and 2). We partitioned the ITS trees into nine operational groups to simplify presentation (marked a--j in Figs.1 and 2).

Group "a" was supported by a posterior probablity (PP) of 0.96 and BR = 1. This group included only diploid ($2n = 16$) European species (with *C. bellidifolia* extending into the circumpolar area), four of them belonging to section *Cardaminella* (*C. alpina, C. bellidifolia, C. plumieri,* and *C. resedifolia*), and one (*C. carnosa*) to section *Pteroneurum*.

Group "b" was supported by BR = 1 and comprised the East Asian *C. tenuifolia* of the monotypic section *Sphaerotorrhiza* and the African *C. trichocarpa* of section *Cardamine*.

Group "c" was supported by BR = 1 and included Eurasian and North American taxa. North American high-polyploids ($2n = 12x = 96$ to $2n = 32x = 256$) of section *Dentaria* (*C. angustata, C. concatenata, C. dissecta, and C. diphylla*) formed a clade supported by JK 93%, BS 88%, BR = 5, and PP 1.0. The European species of section *Dentaria* (*C. bipinnata, C. bulbifera, C. glanduligera, C. abchasica,* and *C. quinquefolia*) together with the Asian diploid *C. leucantha* of section *Macrophyllum* formed a clade supported by JK 91%, BS 87%, BR = 4, and PP 1.0.

Group "d" was supported by BR = 1 and included the East Asian polyploids *C. macrophylla* and *C. tangutorum* and the European diploid *C. trifolia*, belonging to three different sections (*Macrophyllum, Dentaria*, and *Coriophyllum*, respectively).

Group "e" was supported by BR = 1 and PP 1.0. This group included the European *C. waldsteinii* of section *Dentaria* in addition to two well-supported clades with East Asian and North American taxa, respectively. The East Asian clade (JK 98%, BS 97%, BR = 5, and PP

1.0) included members of sections *Cardaminella* (*C. nipponica*) and *Cardamine* (*C. microzyga*). The North American clade (JK 100%, BS 99%, BR = 12, and PP 1.0) also included the cosmopolitan weed *C. hirsuta* of section *Cardamine*.

Group "f" was supported by BR = 1 and PP 1.0. This group included species of section *Cardamine* from South America, East Asia, and Africa.

Group "g" was supported by BR = 1 and PP 0.98. This group included Eurasian, African and South American taxa. Most Asian species grouped together, containing members of both sections *Cardamine* and *Macrophyllum*. Notably, the accessions of *C. scutata* from Japan and Taiwan did not group together. The South American *C. ovata* grouped with African and South American *C. africana*, both belonging to section *Papyrophyllum*. The chromosome numbers in this group spanned from $2n = 16$ (diploid) to $2n = 56$.

Group "h" was supported by JK 65%, BS 50%, BR = 4 and PP 1.0. This group included a specimen from New Guinea referred to as *C. africana*; it did not group with the other *C. africana* accessions (group g) and most likely represent a different taxon. All four species known from New Guinea belonged to this group, which also included European species of section *Dentaria* (*C. heptaphylla, C. kitaibelii*, and *C. pentaphyllos*).

Group "i" was supported by BR = 1 and PP 0.98. This group included the *C. pratensis* species group and its closely related European species, which also formed a clade in the plastid analysis (Fig. 3; JK 81%, BS 81%, BR = 3). As this group has been extensively studied earlier (Franzke et al., 1998; Franzke and Hurka, 2000; Lihova and Marhold, 2003) and our analyses supported their findings without adding new information, we pruned out several taxa of this complex from our final analyses and retained only four species related to *C. pratensis* to simplify this presentation (*C. acris, C. flaccida, C. pratensis,* and *C. tenera*).

Group "j" had no support and was only present in the MPTs and a combinable components consensus tree, but included several supported subgroups. This group included

most of the Beringian taxa, all of the Australian and New Zealand taxa, and many North American taxa in addition to one species from South America and East Asia, respectively. The Australian and New Zealand taxa, *C. debilis, C. lacustris, C. lilacina,* and *C. paucijuga*, constituted a monophyletic group (JK 55%, BS 57%, BR = 1 and PP 0.98) with the inclusion of the South American *C. glacialis* and the amphi-Beringian/Pacific *C. umbellata*. The ITS dataset was inconclusive about the monophyly of the remaining Beringian species (*C. blaisdellii*, *C. digitata*, *C. purpurea*, *C. pedata*, *C. microphylla*, *C. victoris*, and *C. sphenophylla*). However, most of the most parsimonious trees supported the Beringian species as a monophyletic group with North American taxa as sister groups. The remaining trees supported the Beringian species as two separate groups, but both of them with North American species as sister groups. This led to collapse of these branches in the strict consensus tree and the resampling analyses.

The South American species appeared scattered in the tree. *Cardamine glacialis* was most closely related to the taxa from Oceania in group "j", *C. bonariensis* and *C. flaccida* close to or nested within the *C. pratensis* group in group "i", and *C. ecuadorensis* and *C. rhizomata* were resolved as a sister group to *C. griffithii* in group "f".

The three African species did not form a monophyletic group. *Cardamine trichocarpa* was found in group "b" whilst *C. obliqua* was sister to *C. lihengiana* in group "f" with BR = 2 and PP 0.96. A specimen from South America referred to *C. africana* was more closely related to South American *C. ovata* than to a *C. africana* specimen from Kilimanjaro.

The Oceanian taxa occurred in two distinct clades. In group "h" (JK 65%, BS 50%, BR = 4 and PP 1.0), three European species were nested among four species from New Guinea (*C. "africana", C. altigena, C. keysseri,* and *C. papuana*). In group "j", four species from Australia and New Zealand (*C. paucijuga, C. lilacina, C. lacustris,* and *C. debilis*) were

most closely related (JK 54%, BS 57%, and PP 0.98) to the arctic *C. umbellata* and the South American *C. glacialis*.

The Beringian/circumpolar taxa occurred in three different groups. *Cardamine pratensis* (group “i”) and *C. bellidifolia* (group “a”) had their closest relatives in Europe. In group ”j”, there were seven Beringian species (*C. blaisdellii*, *C. digitata*, *C. purpurea*, *C. pedata*, *C. microphylla*, *C. victoris*, and *C. sphenophylla*) possibly having their closest relatives in Oceania and North America.

DISCUSSION

Rapid diversification and widespread polyploidization—Although 29% of the characters in the ITS data set were phylogenetically informative, only a few of them were useful for resolving deeper relationships in *Cardamine*. Even though the overall RI for the ITS trees might suggest that there is substantial homoplasy in the dataset, with 31% of all characters being retained as such, implied weighing did not affect the topology or improve the resolution of the deeper relationships as one might expect when reducing the effect of homoplastic characters. The most likely explanation for the lack of resolution is therefore that the initial diversification in *Cardamine* occurred rapidly.

Polyploidization, a common mode of evolution in *Cardamine* as well as in the Brassicaceae in general (Al-Shehbaz, 1988; Kucera et al., 2005; Beilstein et al., 2006; Marhold and Lihova, 2006), could also have affected the resolution of the ITS phylogeny. Our results imply that polyploidization has happened independently in many lineages in *Cardamine*. We found that eight of nine groups contain diploid species, that only one group is exclusively diploid, and that most groups contain species with very high chromosome numbers. However, our separate analyses of the ITS region for diploids and tetraploids did not provide better resolution nor conflicting topologies, neither did the plastid tree show better resolution. These results support our hypothesis that the lack of resolution is due to rapid speciation rather than breakdown of phylogenetic signal from frequent allopolyploidization.

It is difficult and requires a huge sequencing effort to untangle the hierarchical structure among rapidly diverging lineages (Fishbein et al., 2001). We did an extensive survey of different regions in the preliminary analyses for this study, and a search for more phylogenetically informative molecular markers in *Cardamine* may prove fruitless.

Our results corroborate those of Koch et al. (2000) and Haubold and Wiehe (2001), who used molecular dating to demonstrate that *Cardamine* is a relatively young genus. After the split between *Cardamine* and *Barbarea* as late as 6.2 MYA (2--8 MYA), *Cardamine* rapidly diversified into one of the most species-rich genera in the Brassicaceae. This is consistent with the pattern observed in other large genera of this family, such as *Lepidium* (2.1--4.2 MYA, ~175 species; Mummenhoff et al., 2001) and *Draba* (4.5 to 9 MYA, ~350 species; Koch and Al-Shehbaz, 2002).

Several recent papers have addressed the importance and previous underestimation of long-distance dispersals to explain biogeographic patterns (Donoghue and Smith, 2004; Givnish and Renner, 2004; Thorne, 2004; Cook and Crisp, 2005; McGlone, 2005; Queiroz, 2005). In *Cardamine*, continental drift causing vicariant speciation can be ruled out since the present constellation of continents was established millions of years before the origin of the genus. Based on the estimate of 2--8 MYA, it is likely that the large late Tertiary forest in the northern hemisphere (Lafontaine and Wood, 1988; Bennike and Böcher, 1990; Matthews and Ovenden, 1990; Murray, 1995) provided the first habitat for establishment, divergence and spread of *Cardamine*. It is possible that the later submerging of the Bering Land Bridge (Gladenkov et al., 2002) and the successive cooling of the Holarctic region (Zachos et al., 2001) has caused vicariant speciation in the genus. Neverteless, our phylogeny provides, in spite of its low overall resolution, evidence for several extensive long-distance dispersal events (further discussed below).

Sectional partitioning—We found no support for monophyly of any of Schulz' (1903, 1936) large sections (Figs 1--3). Section *Cardaminella* was not monophyletic; the species of this section occurred in different supported clades intermingled with species of other sections (compare clades in groups "a", "e" and "j"; Fig. 1 and 2). Our results also reject the

monophyly of section *Dentaria*, in agreement with (Franzke et al., 1998; Sweeney and Price, 2000). Furthermore, the species included in the largest section, *Cardamine*, are spread among different supported groups and intermingled with species of other sections (e.g. groups ”g” and ”j”). This section has apparently served to include species that did not fit morphologically into any of the other 12 sections, as suggested by (Sweeney and Price, 2000). We also have shown that *Macrophyllum* and *Papyrophyllum* are not monophyletic, in agreement with Bleeker (2002) and Sweeny and Price (2000; cf. Figs 1--3).

Rapid colonization and subsequent dispersals — Because of its poor resolution, our phylogeny was not suitable for biogeographic analyses such as DIVA to reconstruct ancestral areas (Ronquist, 1997). However, because of the considerably higher diversity of species and, in particular, diploid ones, Eurasia is certainly the most likely area of origin of *Cardamine*. In spite of its typically short-distance main mode of dispersal, we hypothesize that the genus rapidly colonized the entire northern hemisphere and later spread across vast distances to the southern hemisphere as several distinct lineages (cf. also Bleeker et al. 2002). Some distinct colonization episodes can be inferred based on supported groupings in our phylogeny and are shown in Fig. 4.

Oceania— One example of very long-distant colonization (arrow f in Fig. 4) followed by rapid speciation is provided by the Australian and New Zealand taxa, which form a monophyletic group together with one Beringian (*C. umbellata*) as well as one South American species (*C. glacialis*; subclade in ”j”, Figs. 1 and 2). Notably, these oceanian species are morphologically diverse and comprise lowland as well as alpine taxa, but appear genetically similar. The four oceanian species from New Guinea (*C.* sp. *“africana”, C. altigena, C. keysseri,* and *C. papuana*), on the other hand, belonged to another distinct clade,

which also comprised northern hemisphere taxa (group "h"; Figs 1 and 2). Thus, Oceania appears to have been colonized at least twice from the Northern Hemisphere.

South America—We can discern at least four different dispersals into South America, two of which might originate from other Southern Hemisphere regions. We have shown that the accessions of *C. africana* are monophyletic only by the inclusion of the South American *C. ovata* (which earlier have been suggested to be conspecific with *C. africana*, (Sjöstedt, 1975); see subclade in group "g"). This must represent an independent dispersal event into South America, either from Africa or from the northern hemisphere with later dispersal to Africa (arrow d in Fig. 4).

As noted above, the South American *C. glacialis* is most closely related to the species from Australia, Tasmania and New Zealand in both the *trn*L-F (JK 52%, BR = 1) and ITS (JK 55%, BS 57%, BR = 1, PP 0.98) tree, and must represent a separate dispersal event into South America (arrow g in Fig. 4; subclade in group "j").

The South American *C. flaccida* and *C. bonariensis*, suggested by Sjöstedt (1975) to be conspecific, form a clade with the European *C. pratensis* complex and thus provide an example of yet another dispersal event (arrow c in Fig. 4; group "i" in Figs 1--2). From the parsimony ITS tree the direction is impossible to determine as both ways are equally parsimonious (Cook and Crisp, 2005). We can infer either two dispersal events into South America or one old dispersal event to South America with a subsequent speciation event and then dispersal back to Europe.

Evidence for a fourth dispersal event into South America is provided by the sister group relationship (PP 0.86, BR = 1) between the Asian *C. griffithii* and the South American *C. ecuadorensis* and *C. rhizomata*, which form a separate clade within group "f" and may

have originated from Eurasia (not indicated in Fig. 4). Our results reject Sjöstedt's (1975) hypothesis that *C. ecuadorensis* and *C. rhizomata* are conspecific with *C. africana*.

Africa—The African species did not form a monophyletic group in our analyses. The sister group of the African *C. obliqua* differs between the ITS tree (group "f") and the plastid tree, as also found by Bleeker (2002), but it is possible that it has a Eurasian origin (arrow e in Fig. 4). The African *C. trichocarpa* (see group "b") is highly divergent with its 15 ITS and 17 *trn*L-F autapomorphies and has no unambiguous sister group. It is not possible to infer its origin except that it is not sister to the other African taxa. Finally, *C. africana*, which occurs both in Africa and South America (cf. above), is member of yet another clade, nested with European and Asian taxa (group "g"; PP 0.98 and BR = 1).

The Arctic—We can infer at least three major colonization episodes into the Beringian/circumpolar region. Many species of *Cardamine* reach the Arctic (e.g. *C. macrophylla, C. conferta, C. tenuifolia, C. prorepens, C. scutata,* and *C. amara*), but here we focus on the nine taxa having the major part of their distribution in this region (*C. bellidifolia*, *C. blaisdellii*, *C. digitata*, *C. pratensis* ssp. *angustifolia, C. purpurea*, *C. pedata*, *C. microphylla*, *C. victoris*, and *C. sphenophylla*).

Both the chloroplast and nuclear data demonstrate that the broadly circumpolar diploid *C. bellidifolia* in group "a" is sister (JK 77%, BS 70%, PP 1.0, and BR = 3 in ITS and JK 91%, BS 88%, and BR = 2 in *trn*L-F) to European diploids, specifically in the Alps and the Pyrenees (arrow b in Fig. 4). This clade is sister to other European Mediterranean-Alpine diploids such as *C. glauca* in the *trn*L-F tree (JK 67%, BS 55%, and BR = 1) and *C. carnosa* and *C. plumieri* in the ITS tree (PP 0.96, and BR = 1).

Another example of European origin is provided by the arctic circumpolar *C. pratensis* ssp. *angustifolia* (group ”i”), by some authors regarded as a separate species, *C. nymanii* (Franzke and Mummenhoff, 1999). *Cardamine pratensis* is a common boreal polyploid and belongs to a complicated species complex with many described diploids and low-polyploids distributed throughout Europe. Our results are consistent with those of (Franzke and Hurka, 2000), who concluded that *C. pratensis* ssp. *angustifolia* colonized the Arctic from southern Europe during the Holocene (arrow h in Fig. 4).

The remaining seven arctic species (group ”j”), which are restricted to the amphi-Beringian region, may have originated from one or two colonization events from North America (arrow a in Fig. 4), as these taxa are nested with North American species. However, this is only inferred from the MPTs and the combinable components tree. As we use collapsing rules that do not allow zero length branches, we did not get support for this hypothesis in the resampling analyses.

Thus, there are two distinct examples of European origin of arctic *Cardamine*, including two different species which have become broadly distributed in the Arctic without further diversification. In addition, there is one example of a probable North American origin followed by diversification into many species in Beringia, but without further expansion into the circumarctic.

Table 1. Sectional classification, geographic distribution and number of species according to Schulz (1923)

Section		Abbreviation (cf. Fig. 1)	No. of spp	Geographic distribution
I.	*Dentaria* (L.) O. E. Schulz	Dent	16	Eurasia & Atlantic North America
II.	*Eutreptophyllum* O. E. Schulz	Eutr	2	Pacific North America
III.	*Sphaerotorrhiza* O. E. Schulz	Sphae	1	Siberia
IV.	*Coriophyllum* O. E. Schulz	Corio	1	Middle Europe
V.	*Girardiella* O. E. Schulz	Girar	1	China
VI.	*Macrophyllum* O. E. Schulz	Mac-ph	7	Asia & North America
VII.	*Lygophyllum* O. E. Schulz	Lygo	1	Himalaya
VII.	*Papyrophyllum* O. E. Schulz	Papyro	8	Tropical mountains
IX.	*Eucardamine* Gren. et. Godr.	Card	~74	Cosmopolitan
X.	*Cardaminella* Prantl.	C-nella	12	Cold areas all over the world
XI.	*Pteroneurum* (DC.) Nyman	Ptero	5	East Mediterranean region
XII.	*Spirobolus* O. E. Schulz	Spiro	1	Mediterranean region
XIII.	*Macrocarpus* O. E. Schulz	Mac-ca	1	South America

Appendix 1. Specimens included in this study. Chromosome counts in parentheses refer to rare reports. Note: GenBank accession numbers will be added when the manuscript is accepted.

Species	Section	2*n* =	Country/Territory (Herbarium) Collector/Determinator[a]	ITS	*trn*L intron	*trn*L-F spacer
Rorippa palustris (L.) Bess.			(OSBU)			
Rorippa sylvestris (L.) Bess.			(OSBU)			
abchasica Govaerts.	*Dentaria*		Russia (MW)			
acris Griseb.	*Eucardamine*	16	Montenegro, Marhold et al. 2004	AY245977 AY246007		
acris Griseb.	*Eucardamine*	16	Greece, Marhold et al. 2004	AY246002 AY246032		
"*africana* L." (sp.)[b]	*Papyrophyllum*	16	Papua New-Guinea, Franzke et al. 1998	AF078009 AF078010	AF079342	AY047650
africana L.	*Papyrophyllum*	16	Tanzania, Bleeker et al. 2002	AY047612 AY047623	AY047639	AY047655
africana L.	*Papyrophyllum*	16	Equador, Bleeker et al. 2002	AY047611 AY047622	AY047642	AY047658
alpina Willd.	*Cardaminella*	16	Italy (OSBU), Hurka			
altigena Schltr. ex O. E. Shulz			Papua New-Guinea, Franzke et al. 1998	AF078011 AF078504	AF079343	
amara L. subsp. amara	*Eucardamine*	16	Slovakia, Marhold et al. 2004	AY245985 AY246015		
amara L. subsp. amara	*Eucardamine*	16	Italy, (Lihova et al., 2004)	AY260579		
amara L. subsp. pyrenaea	*Eucardamine*	16	Spain, Franzke and Hurka 2000		AF266633	
amara L. subsp. pyrenaea	*Eucardamine*	16	Spain, (Lihova et al., 2004)	AY260580		
amara L.	*Eucardamine*	16	Norway (O) Wesenberg			
amporitana Sennen & Pau	*Eucardamine*	32	Spain (SAV), Lihova et al. 2004	AY260585		
amporitana Sennen & Pau	*Eucardamine*	32	Italy, (SAV) Lihova et al. 2004	AY260608		
angulata Hook.	*Macrophyllum*	40	USA (MO) Thysell			
angulata Hook.	*Macrophyllum*	40	USA (S) Calder, Savile & Taylor			
angustata O.E.Schulz	*Dentaria*	128	USA (MO) Kral			
angustata O.E.Schulz	*Dentaria*	128	USA (GA) P. Sweeney & Price 2000		AF198121	
appendiculata Franchet et Savatier	*Macrophyllum*		Japan (O) Hiroshi Ogura			
arisanensis Hayata			Taiwan (S) C. Beu			
asarifolia L.	*Eucardamine*	48	Italy, (Lihova et al., 2004)	AY260620		
barbaraeoides Halacsy	*Eucardamine*	32	Greece, Lihova et al. 2004	AY260614		
bellidifolia L.	*Cardaminella*	16	Russia (MW)			
bellidifolia L.	*Cardaminella*	16	Russia (MW)			
bellidifolia L.	*Cardaminella*	16	Norway, (Sweeney and Price, 2000)		AF198122	
bellidifolia L.	*Cardaminella*	16	Spitsbergen (O) Elven			
bellidifolia L.	*Cardaminella*	16	Russia (O) Maksimova			
bipinnata (C.A.Meyer)O.E.Schulz	*Dentaria*		Russia (WU)			
blaisdellii Eastw.	*Cardaminella*	28,42	USA (O) R. Elven			
blaisdellii Eastw.	*Cardaminella*	28,42	USA (O) R. Elven			
blaisdellii Eastw.	*Cardaminella*	42 *[c]	Russia (LE) P. Zhukova			
blaisdellii Eastw.	*Cardaminella*	28 *	Russia (LE) P. Zhukova			
bonariensis Pers.	*Eucardamine*		Equador (S) Ishan A. Al-Shehbaz			
bonariensis Pers.	*Eucardamine*		Peru (MO) Duncan 2663			
bradei O.E.Schulz			Costa Rica (S) R. L. Liesner			
breweri Watson	*Eucardamine*	84-96	USA (S) C. L. Porter & Marjorie W. Porter			
breweri Watson	*Eucardamine*	84-96	USA (GA) R. Price 1359		AF198123	
bulbifera (L.)Crantz	*Dentaria*	96	(OSBU)			
bulbosa (Schreb. ex Muhl.) Britton		32-64	USA (MO) Kral			
bulbosa (Schreb. ex Muhl.) Britton		32-64	Canada (S) Gilles Lemieux			

bulbosa (Schreb. ex Muhl.) Britton		32-64	USA (O) B. O. Wolden			
bulbosa (Schreb. ex Muhl.) Britton		32-64	USA (Sweeney and Price, 2000)		AF198124	
californica (Nutt.) Greene	*Eutreptophyllum*	32	USA (OSBU) Hurka			
carnosa Waldstein et Kitaibel	*Pteroneurum*		Greece (S) K. H. Rechinger			
castellana Lihova & Marhold		16	Spain, Lihova et al. 2004	AY260578		
chelidonia L.	*Spirobolus*	64	Italy (O) J. Poelt			
clematitis Shuttleworth	*Eucardamine*		USA (S) S. W. Leonard & A. E. Radford 1431			
concatenata (Michaux)Schwarz	*Dentaria*	256	Canada (S) Dorothy E. Swales			
concatenata (Michaux)Schwarz	*Dentaria*	256	Canada (WU)			
conferta Jurtz.		48	Russia (LE) L. Fokina			
constancei Detling			USA (S) R. C. Rollins			
cordifolia A. Gray	*Eucardamine*	24	USA (S) Holmgren, Reveal & LaFrance			
cordifolia A. Gray	*Eucardamine*	24	USA (O) E. Dahl			
corymbosa Hook.	*Cardaminella*	48	Australia, Bleeker et al. 2002	AF078003 AF078004	AF079339	AY047645
corymbosa Hook.	*Cardaminella*	48	New Zealand, Bleeker et al. 2002	AY047613 AY047624	AY047633	AY047646
crassifolia Pourr.		16	Spain, Lihova et al. 2004	AY260605		
debilis Banks ex DC.	*Eucardamine*	48	New Zealand, Bleeker et al. 2002	AY047614 AY047625	AY047643	AY047660
delavayi Franch.			China (MO) 14549			
densiflora Gontsch.			Tadschikistan (MW)			
dentipetala Matsum			Japan (S) M. Mizushima			
digitata Richardson	*Cardaminella*	28,42	USA (O) R. Elven			
digitata Richardson	*Cardaminella*	28,42	USA (O) R. Elven			
digitata Richardson	*Cardaminella*	28,42	Canada (O) ver. by Elven			
digitata Richardson	*Cardaminella*	28,42	Russia (MW)			
diphylla (Michaux)Wood	*Dentaria*	96-256	Canada (S) Marcel Blondeau			
diphylla (Michaux)Wood	*Dentaria*	96-256	USA (MO) Kral			
dissecta (Leavenw.) Al-Shehbaz	*Dentaria*		USA (WU)			
douglassii Britton		28-72	USA (S) Hainault			
douglassii Britton		28-72	Canada (O) Hainault			
ecuadorensis Hieronymus	*Eucardamine*		Equador (S) O. Brekke			
enneaphyllos (L.)Cranz	*Dentaria*	80	Austria (OSBU) Hurka			
flaccida Cham. et Schlechtend.	*Eucardamine*	16	Chile (O) Skottsberg			
flexuosa Withering	*Eucardamine*	32	Germany, Franzke et al. 1998	AF077999 AF077800	AF079337	AY047644
fragariifolia O.E.Schulz	*Eucardamine*		China (MO) 317			
franchetiana Diels			China (MO) 20021			
gallaecica (M. Laínz) Rivas Mart. & Izco		(32)48	Spain, Lihova et al. 2004	AY260613		
glacialis (Forster)DC.	*Eucardamine*	72	Chile, Bleeker et al. 2002	AY047615 AY047626	AY047634	AY047648
glacialis (Forster)DC.	*Eucardamine*	72	Argentinia, Bleeker et al. 2002	AY047616 AY047627	AY047635	AY047649
glanduligera O.Schwarz	*Dentaria*	42	Regensburg Botanical Garden			
glanduligera O.Schwarz	*Dentaria*		Russia (MW)			
glauca Sprengel	*Pteroneurum*	16	Italia (S) Ivar Segelberg			
graeca L.	*Pteroneurum*	16-18	Romania (O) Al. Borza & Gh. Bujorean			
griffithii Hooker fil. et Thomson	*Eucardamine*		China (MO) 496			
heptaphylla (Villars) O. E. Shulz	*Dentaria*	48	Italy (OSBU) Bernhardt			
hirsuta L.	*Eucardamine*	16	Germany, Franzke et al. 1998	AF077997 AF077998		
impatiens L.	*Eucardamine*	16	Germany, Franzke et al. 1998	AF078015 AF078016		
impatiens L.	*Eucardamine*	16	Norway (O) Lye			
keysseri O.E.Schulz			New Guinea, Franzke et al. 1998	AF078013 AF078014	AF079344	AY047651

kitaibelii Becherer	*Dentaria*		Bosnia-Herzegowina (WU)			
kitaibelii Becherer	*Dentaria*		Switzerland (S) C. Simon			
laciniata (Muhlenb.)Wood	*Dentaria*		Canada (S) Dorothy E. Swales			
lacustris (Garn.-Jones & P.N.Johnson) Heenan		48	New Zealand (Mitchell and Heenan, 2000)	AF100683		
leucantha O.E.Schulz	*Macrophyllum*	16	Japan (S) Masami Mizushima			
leucantha O.E.Schulz	*Macrophyllum*	16	Japan (S) Miyoshi Furuse			
lihengiana Al-Shehbaz			China (MO) 1207			
lilacina Hook.		48	Australia, Franzke et al. 1998	AF078007 AF078008	AF079341	AY047659
lilacina Hook.		48	Australia, Franzke et al. 1998	AF078005 AF078006	AF079340	AY047647
longii Fernald			USA (S) M. L. Fernald & Bayard Long			
lyallii S. Watson	*Eucardamine*		USA (S) R. C. Collins			
macrophylla Willd.	*Macrophyllum*	64-96	Japan (MO) Al-Shehbaz 9342			
matthioli Moretti	*Eucardamine*	16	Slovenia (Lihova et al., 2004)	AY260606		
matthioli Moretti	*Eucardamine*	16	Bulgaria, Franzke and Hurka 2000		AF266642	
matthioli Moretti	*Eucardamine*	16	Slovakia, Franzke et al. 1998		AF079330	Af266597
microphylla Adams	*Cardaminella*	28-64	Russia (LE) A. J. Tolmachev & T. G. Polozova			
microphylla Adams	*Cardaminella*	28 *	Russia (LE) P. Zhukova			
microphylla Adams	*Cardaminella*	28-64	Russia (LE) A. Tolmachev & B. Yurtsev			
microphylla Adams	*Cardaminella*	28-64	Russia (O) Plyeva			
microzyga O. E. Schulz	*Eucardamine*		China (S) J. F. Rock			
nipponica Franchet Savatier	*Cardaminella*		Japan (S) M. Tamura 9141			
nuttallii Greene	*Eutreptophyllum*		USA (MO) Taylor			
obliqua Hochst.	*Eucardamine*	36-72	Kenya, Bleeker et al. 2002	AY047617 AY047628	AY047636	AY047652
obliqua Hochst.	*Eucardamine*	36-72	Ethiopia, Bleeker et al. 2002	AY047618 AY047629	AY047638	AY047654
occidentalis (Watson) O. E. Shulz	*Eucardamine*	64	USA (OSC) 335917			
occidentalis (Watson) O. E. Shulz	*Eucardamine*	64	USA (OSC) 357322			
ovata Bentham	*Papyrophyllum*		Equador (S) Ishan A. Al-Shehbaz			
ovata Bentham	*Papyrophyllum*		Equador (S) Ishan A. Al-Shehbaz			
ovata Bentham	*Papyrophyllum*		Equador (MO) Ishan A. Al-Shehbaz			
papuana (Lauterb.) O. E. Shulz	*Papyrophyllum*		Papua New-Guinea (CANB)			
parviflora L.	*Eucardamine*	16	Slovakia (O) Fr. Cernoch			
pattersonii Henderson			USA (OSC) 245394			
paucijuga Turcz.			Australia, Bleeker et al. 2002	AY047619 AY047630	AY047640	AY047656
pedata Regel et. Tiling	*Cardaminella*	30 *	Russia (LE) P. Zhukova, B. Yurtsev, V. Petrovsky			
pensylvanica Muhl. ex Willd.		32,64	USA (ALA)M. Duffy 93-807			
pensylvanica Muhl. ex Willd.		32,64	USA (ALA) J. De Lapp & M. Duffy 93-539			
pentaphyllos (L.) Crantz	*Dentaria*	48	V7030-2003			
pentaphyllos (L.) Crantz	*Dentaria*	48	Switzerland (OSBU)			
penzesii Anchev et Marhold		16	Bulgaria, Franzke and Hurka 2000	AF265182 AF265202	AF266643	AF266600
penzesii Anchev et Marhold		16	Bulgaria, Franzke and Hurka 2000	AF265675 AF265681		
plumeri Villars	*Cardaminella*	16	Italy (GAT)			
pratensis L.	*Eucardamine*	16-118	Portugal, Marhold et al. 2004	AY245995 AY246025		
prorepens Fisch. ex DC.	*Eucardamine*	18,20	Russia (LE) A. P. Arkhangelskya			

prorepens Fisch. ex DC.	*Eucardamine*	18,20	Russia (MW)			
purpurea Cham et Schlechtend	*Cardaminella*	96 *	Russia (LE) V. Petrovsky			
purpurea Cham et Schlechtend	*Cardaminella*	96 *	Russia (LE) V. Petrovsky			
purpurea Cham et Schlechtend	*Cardaminella*	80	USA (O) R. Elven			
purpurea Cham et Schlechtend	*Cardaminella*	80	USA (O) R. Elven			
purpurea Cham et Schlechtend	*Cardaminella*	80	USA (O) Murray, Yurtzev & Kelso			
purpurea Cham et Schlechtend	*Cardaminella*	80	USA (O) Elven & Grundt			
purpurea Cham et Schlechtend	*Cardaminella*	80	Russia (MW)			
quinquefolia (M.B.)Schmalhausen	*Dentaria*		Russia (MW)			
resedifolia L.	*Cardaminella*	16	Austria (S) Erik Emanuelsson 1292			
resedifolia L.	*Cardaminella*	16	Italy (OSBU)			
rhizomata Rollins			Equador (S) Ishan A. Al-Shehbaz			
rivularis Schur		16	Romania, Franzke et al. 1998	AF077981 AF077982	AF079328	AF266595
rivularis Schur		16		AF265201 AF265181		
rockii O.E.Schulz	*Eucardamine*		China (S) J. F. Rock			
rotundifolia Michaux	*Eucardamine*		USA (S) F. A. Gilbert			
rupicola (Schulz) Hitch.			USA (S) Arthur Cronquist			
scutata Thunberg		32	Japan (MO) 18034			
scutata Thunberg		32	Taiwan (S) C. Hsu			
scutata Thunberg var. *formosana* (Hayata) T.S.Liu & S.S.Ying		32	Taiwan (Yang et al., 1999)	AF128107		
seidliziana Albov	*Eucardamine*		Russia (Marhold et al., 2004)	AY245979 AY246009		
sphenophylla Jurtz. (Isotypus)	*Cardaminella*	28 *	Russia (LE) Yu. Kozhevnikov & B. Yurtsev			
tanakae Franchet et Savatier	*Eucardamine*		Japan (S) Miyoshi Furuse			
tanakae Franchet et Savatier	*Eucardamine*		Japan (S) Miyoshi Furuse			
tangutorum O.E.Schulz	*Dentaria*	42	China (S) O. E. Schulz			
tenella (Pursh) O. E. Schultz	*Eutreptophyllum*		USA (S) Cronquist			
tenera C. A. Mey	*Eucardamine*	16	Russia (Marhold et al., 2004)	AY245980 AY246010		
tenuifolia (Ledeb.) Turcz.	*Sphaerotorrhiza*	32	Russia (S) Petrovsky & Plieva			
torrentis Nakai		56	Japan (S) M. Mizushima			
trichocarpa Hochst.	*Eucardamine*	16,32	Ethiopia, Bleeker et al. 2002	AY047620 AY047631	AY047641	AY047657
trichocarpa Hochst.	*Eucardamine*	16,32	Tanzania, Bleeker et al. 2002	AY047621 AY047632	AY047637	AY047653
trifolia L.	*Coriophyllum*	16	Austria, Bernhardt			
uliginosa M. Bieb.	*Eucardamine*	16	Georgia, Marhold et al. 2004	AY245981 AY260707 AY246011		
umbellata Greene	*Cardaminella*	32,48	USA (O) R. Elven			
umbellata Greene	*Cardaminella*	32,48	USA (O) R. Elven			
umbellata Greene	*Cardaminella*	32,48	USA (O) R. Elven			
victoris N.Busch	*Cardaminella*	28 *	Russia (LE) Yu. Kozhevnikov & B. Yurtsev			
victoris N.Busch	*Cardaminella*	28 *	Russia (LE) B. Yurtsev & P. Zhukova			
victoris N.Busch	*Cardaminella*	28	Russia (LE) A. Koropkov			
victoris N.Busch	*Cardaminella*	28	Russia (O) ver. by R. Elven			
victoris N.Busch	*Cardaminella*	28 *	Russia (LE) A. Korobkov			
waldsteinii Dyer.	*Dentaria*		Bosnia-Herzegovina (WU)			
waldsteinii Dyer.	*Dentaria*		(Sweeney and Price, 2000)			
waldsteinii Dyer.	*Dentaria*		Austria (S) H. Teppner			
waldsteinii Dyer.	*Dentaria*		Austria (S) Dr. Korb			

yezoensis Maxim.	*Macrophyllum*	32	Russia (MW)
yunnanensis Franchet	*Eucardamine*		China (MO) 12360

a Where sequences have been retrieved from GenBank, only country/territory and reference is given.

b This specimen was identified as *C. africana*, but results from our study indicate that this specimen represents a separate taxon.

c * indicates that this specimen has been chromosome-counted.

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1 FIGURE LEGENDS

2 Fig 1. One of the most parsimonious trees based on the ITS dataset showing branches kept in
3 the strict consensus tree. Letters a-j designate groups commented upon in the text. To simplify
4 presentation, multiple accessions are given as a single open circle for one terminal.
5 Chromosome numbers are given after the species name based on previously published data
6 (Appendix 1(Kucera et al., 2005), followed by section in brackets (cf. Table 1).
7
8 Fig. 2. Bayesian phylogram based on the ITS dataset. Letters a-j designates groups
9 commented upon in the text and used in Fig. 1.
10
11 Fig. 3. One of the most parsimonious trees based on the *trn*L-F dataset showing branches kept
12 in the strict consensus tree. Numbers above branches are Bremer support values. Numbers
13 below branches are jackknife/bootstrap support values.
14
15 Fig 4. Map defining areas as used in the text and in Figs. 1 and 2. Arrows indicate inferred
16 major dispersal events discussed in the text (marked a-h).
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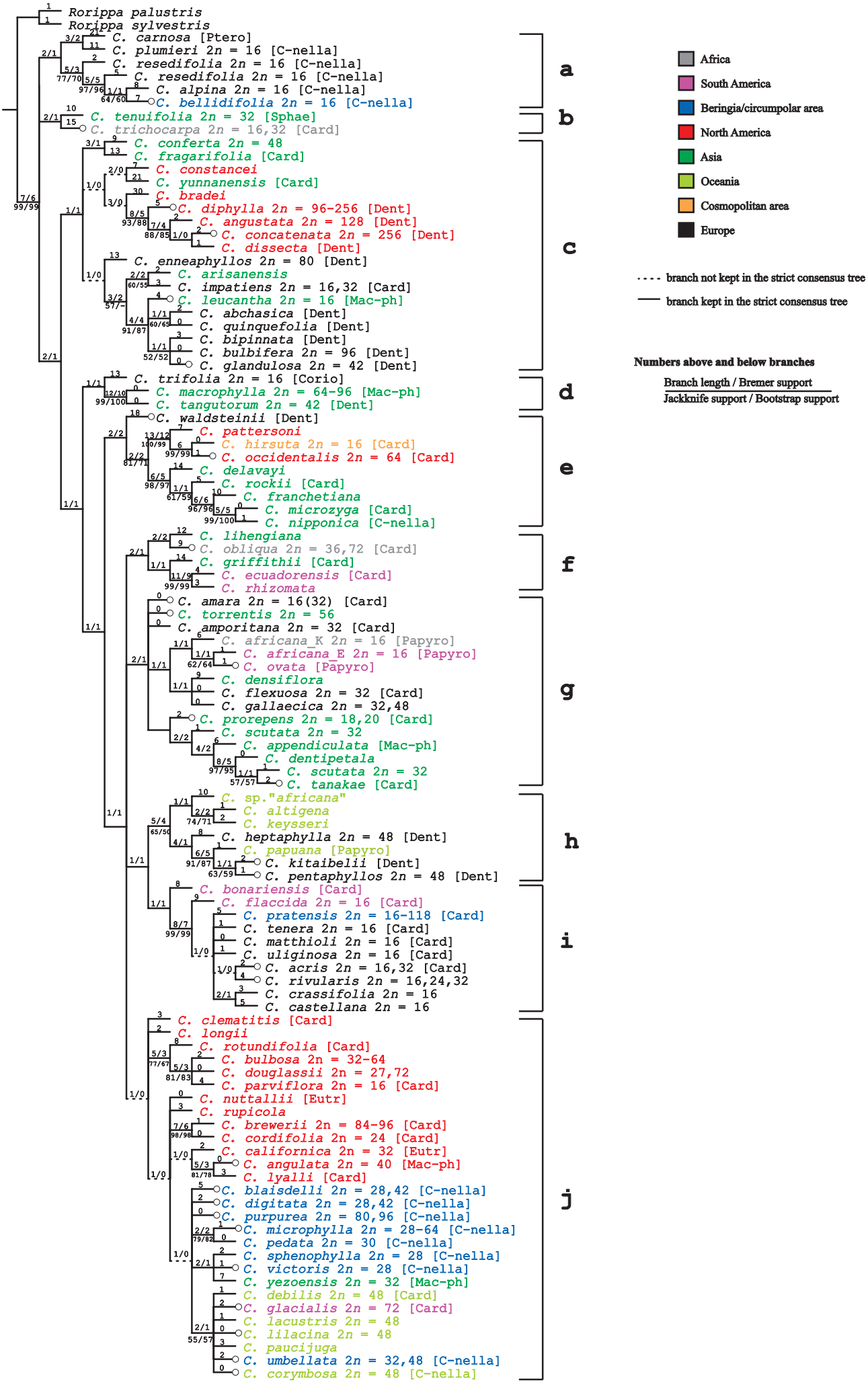
Fig. 1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Rorippa sylvestris
C. carnosa [Ptero]
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C. resedifolia 2n = 16 [C-nella]
C. resedifolia 2n = 16 [C-nella]
C. alpina 2n = 16 [C-nella]
C. bellidifolia 2n = 16 [C-nella]
C. tenuifolia 2n = 32 [Sphae]
C. trichocarpa 2n = 16,32 [Card]
C. conferta 2n = 48
C. fragarifolia [Card]
C. constancei
C. yunnanensis [Card]
C. bradei
C. diphylla 2n = 96-256 [Dent]
C. angustata 2n = 128 [Dent]
C. concatenata 2n = 256 [Dent]
C. dissecta [Dent]
C. enneaphyllos 2n = 80 [Dent]
C. arisanensis
C. impatiens 2n = 16,32 [Card]
C. leucantha 2n = 16 [Mac-ph]
C. abchasica [Dent]
C. quinquefolia [Dent]
C. bipinnata [Dent]
C. bulbifera 2n = 96 [Dent]
C. glandulosa 2n = 42 [Dent]
C. trifolia 2n = 16 [Corio]
C. macrophylla 2n = 64-96 [Mac-ph]
C. tangutorum 2n = 42 [Dent]
C. waldsteinii [Dent]
C. pattersoni
C. hirsuta 2n = 16 [Card]
C. occidentalis 2n = 64 [Card]
C. delavayi
C. rockii [Card]
C. franchetiana
C. microzyga [Card]
C. nipponica [C-nella]
C. lihengiana
C. obliqua 2n = 36,72 [Card]
C. griffithii [Card]
C. ecuadorensis [Card]
C. rhizomata
C. amara 2n = 16(32) [Card]
C. torrentis 2n = 56
C. amporitana 2n = 32 [Card]
C. africana_K 2n = 16 [Papyro]
C. africana_E 2n = 16 [Papyro]
C. ovata [Papyro]
C. densiflora
C. flexuosa 2n = 32 [Card]
C. gallaecica 2n = 32,48
C. prorepens 2n = 18,20 [Card]
C. scutata 2n = 32
C. appendiculata [Mac-ph]
C. dentipetala
C. scutata 2n = 32
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C. acris 2n = 16,32 [Card]
C. rivularis 2n = 16,24,32
C. crassifolia 2n = 16
C. castellana 2n = 16
C. clematitis [Card]
C. longii
C. rotundifolia [Card]
C. bulbosa 2n = 32-64
C. douglassii 2n = 27,72
C. parviflora 2n = 16 [Card]
C. nuttallii [Eutr]
C. rupicola
C. brewerii 2n = 84-96 [Card]
C. cordifolia 2n = 24 [Card]
C. californica 2n = 32 [Eutr]
C. angulata 2n = 40 [Mac-ph]
C. lyalli [Card]
C. blaisdelli 2n = 28,42 [C-nella]
C. digitata 2n = 28,42 [C-nella]
C. purpurea 2n = 80,96 [C-nella]
C. microphylla 2n = 28-64 [C-nella]
C. pedata 2n = 30 [C-nella]
C. sphenophylla 2n = 28 [C-nella]
C. victoris 2n = 28 [C-nella]
C. yezoensis 2n = 32 [Mac-ph]
C. debilis 2n = 48 [Card]
C. glacialis 2n = 72 [Card]
C. lacustris 2n = 48
C. lilacina 2n = 48
C. paucijuga
C. umbellata 2n = 32,48 [C-nella]
C. corymbosa 2n = 48 [C-nella]
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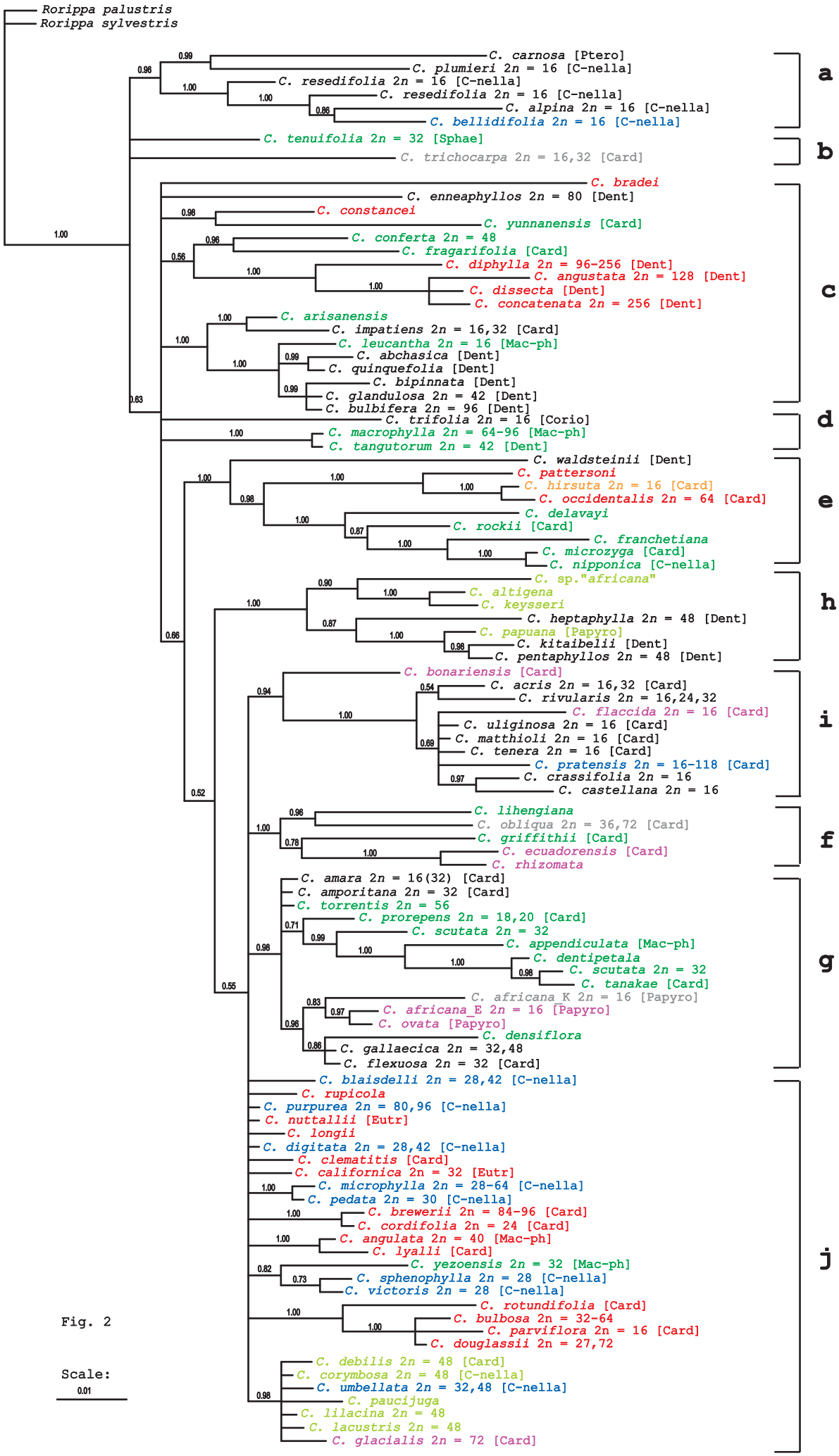
Numbers above and below branches
Branch length / Bremer support
Jackknife support / Bootstrap support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C. yunnanensis [Card]
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C. rockii [Card]
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C. microzyga [Card]
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C. angulata 2n = 40 [Mac-ph]
C. lyalli [Card]
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C. sphenophylla 2n = 28 [C-nella]
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C. paucijuga
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Fig. 2
Scale:
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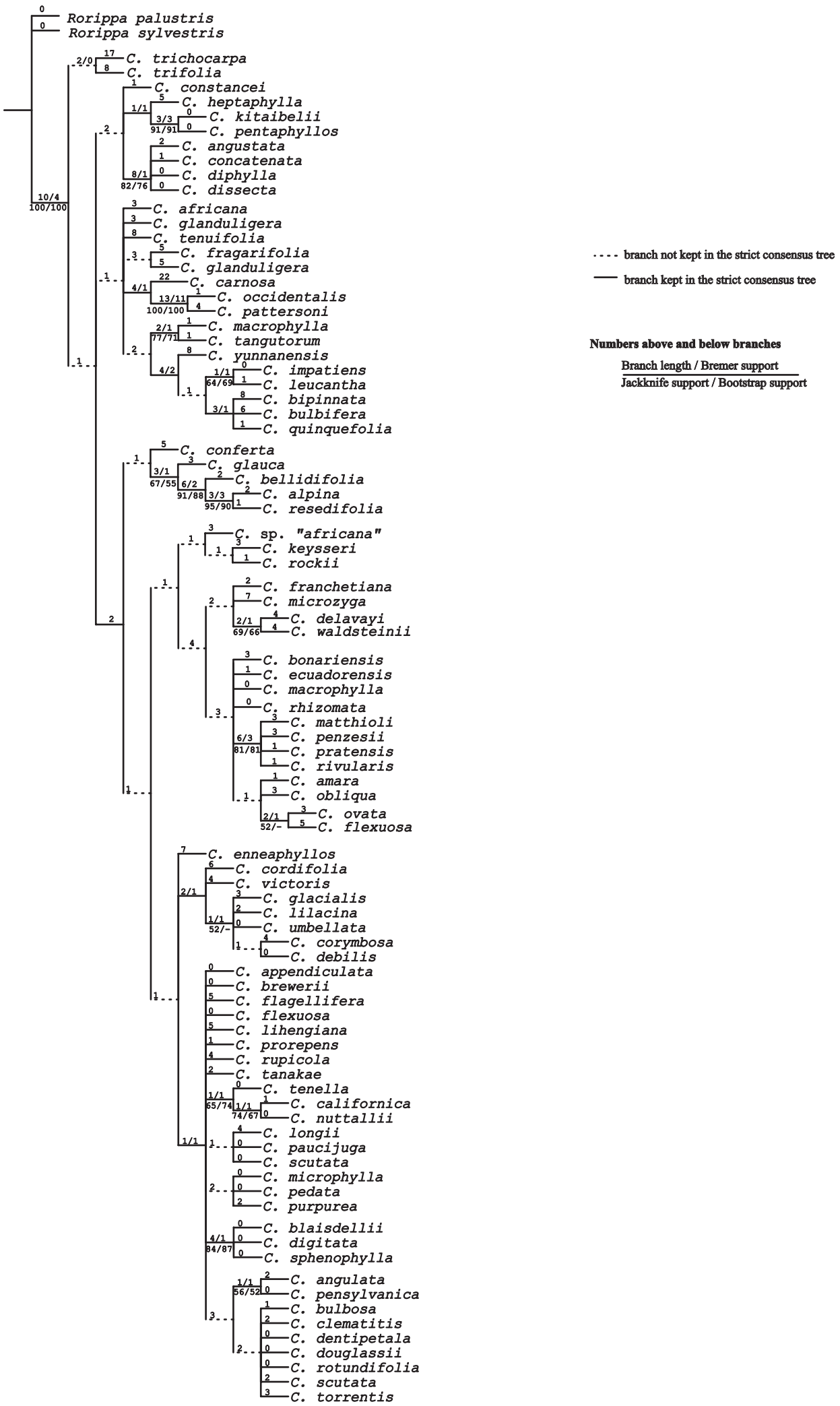
Fig. 3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C. angustata
C. concatenata
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C. dentipetala
C. douglassii
C. rotundifolia
C. scutata
C. torrentis
10/4
100/100
2/0
1/1
3/3
91/91
8/1
82/76
4/1
13/11
100/100
2/1
77/71
1/1
64/69
3/1
3/1
67/55
6/2
91/88
3/3
95/90
2/1
69/66
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81/81
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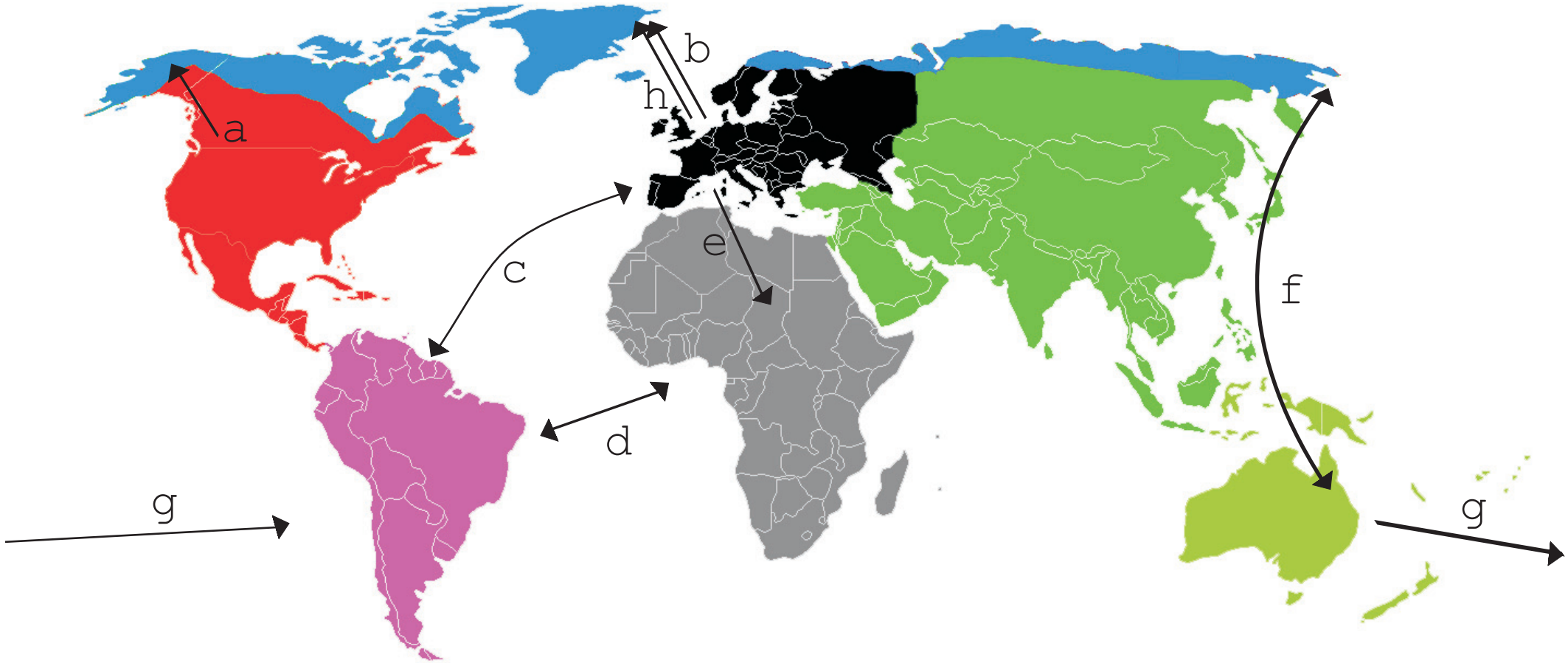
branch kept in the strict consensus tree
Numbers above and below branches
Branch length / Bremer support
Jackknife support / Bootstrap support

Fig. 4



PAPER III

Microsatellites resolve the taxonomy of the polyploid *Cardamine digitata* aggregate (Brassicaceae)

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Polyploidy, probably the single most important mode of sympatric speciation in plants, tends to result in complicated evolutionary patterns. The *Cardamine digitata* aggregate is a species complex where polyploidy has resulted in taxonomic and nomenclatural controversies. Two basic chromosome numbers are found ($x = 7$ and $x = 8$), and all plants studied so far are tetra- to dodecaploids. We used six microsatellite loci originally developed for the *Arabidopsis* genome to identify evolutionary and taxonomic units within the *C. digitata* aggregate, obtaining 102 polymorphic markers. Using different analysis methods (PCO, CVA, STRUCTURE, and parsimony), we recognised four approximately equidistant units corresponding in morphology with the four species; *C. blaisdellii*, *C. digitata*, *C. microphylla*, and *C. purpurea*. All taxa include at least two ploidal levels; thus recurrent taxonomic autopolyploidy is indicated.

Keywords: *Cardamine*, Arctic, polyploidy, taxonomy, nomenclature, microsatellites

INTRODUCTION

Polyploidy may be the single most important mode of sympatric speciation in the plant kingdom (Otto & Whitton 2000). With consequences such as rapid genomic rearrangements, genomic downsizing, movement of genetic elements across genomes, and movement of foreign genetic material into the polyploid genome (reviewed by Soltis & al. 2003), polyploidy is an evolutionary trigger. Evidence of these genomic prosesses induced by polyploidy is mostly collected from studies on crop plants, and the direct consequences of polyploidisation in natural populations are still essentially unknown (Soltis & al. 2003). Identifying polyploid units and their origins is a necessary prerequisite before studies of these consequences can begin.

Cardamine L. is a nearly cosmopolitan genus with 160--200 species (Sjöstedt 1975, Hewson 1982, Al-Shehbaz 1988, Webb & al. 1988, Al-Shehbaz & al. 2006, Lihová & Marhold 2006). The taxonomy of many species complexes is unexplored and remains controversial. An exception is the *C. pratensis* group and many of its close relatives, which has been studied extensively using nuclear and plastid sequences and fingerprinting methods such as amplified fragment length polymorphism (AFLP; Franzke & Hurka 2000, Marhold & al. 2002, Lihová & al. 2004, Marhold & al. 2004, Marhold & Lihová 2006).

Schulz (1903, 1936) considered section *Cardaminella* Prantl to be one of the main sections in the genus *Cardamine*. However, a recent study of the phylogeny of *Cardamine* has found support for the long-time suspicion that section *Cardaminella* is polyphyletic (Carlsen & al. submitted). The circumpolar and alpine *C. bellidifolia* L. and some of its European alpine relatives constitute a separate and distinct branch, whereas other *Cardaminella* species appear in several parts of the tree. There is, however, a consistent and monophyletic Pacific--Beringian branch. In this branch, we find the *C. digitata* Richardson aggregate and the Asian Beringian *C. victoris* N. Busch and *C. sphenophylla* Jurtsev, see Petrovsky in Tolmachev

(1975). From morphological evidence *C. sphenophylla* and *C. victoris* appear as two distinct species but are probably more closely related to each other than to their next closest relative, the *C. digitata* aggregate. The nomenclature and circumscription of some of the species in the *C. digitata* aggregate have been disputed. See discussion for further details on previous disagreements.

Most species of *Cardamine* are polyploid, and up to five basic chromosome numbers have been suggested (Al-Shehbaz 1988). The most probable basic number for the majority of species is $x = 8$ (Kučera & al. 2005). For some species, such as the Beringian taxa in section *Cardaminella*, the most probable basic number is $x = 7$ (Elven & al. 2006). Diploids are known only with $2n = 16$, and the highest recorded number is $2n = 32x = 256$ (*C. concatenata* O. Schwarz and *C. diphylla* Wood; Kučera & al. 2005). The reliable reports for *Cardamine microphylla* Adams are of tetraploid and hexaploid ($2n = 28, 42$) plants from S Chukotka (Zhukova 1980). There are other reports, but all are dubious and must be checked against vouchers before acceptance: Zhukova & Tikhonova (1973), Krogulevich (1976), and Mulligan (1965). Reports for *C. digitata* are considered reliable; the species is tetraploid and hexaploid ($2n = 28, 42$). Tetraploids are reported from Alaska (Rollins 1966, Johnson & Packer 1968) and Chukotka (Zhukova 1969, Zhukova & Petrovsky 1971, 1972, Zhukova & al. 1973, Zhukova & Petrovsky 1984), while hexaploids are reported from Chukotka (Zhukova 1965 also one count as $2n = 40$, 1966, Zhukova & Petrovsky 1972, Zhukova & al. 1973, Petrovsky & Zhukova 1981). The identity of some of the plants counted as *C. blaisdellii* Eastw. is problematic, but reports show the taxon to be tetraploid and hexaploid. Tetraploids are reported from Alaska (Murray & Kelso 1997) and from Chukotka (Zhukova & al. 1973 as *C. hyperborea* O.E. Schulz, Zhukova & Tikhonova 1973 as *C. hyperborea*, Zhukova & Petrovsky 1975, 1976, 1977 all as *C. hyperborea*, 1980, 1984, 1987), while hexaploids are only reported from Chukotka (Zhukova 1966, 1969, Zhukova & al. 1973,

Zhukova & Petrovsky 1984). Reports of ploidy level for *C. purpurea* Cham. & Schtdl. are considered reliable. The single American report, from NW Alaska, is of a decaploid number ($2n$ = c. 80, x = 8?, Johnson & Packer 1968) whereas several counts of a dodecaploid number are reported from Wrangel Island ($2n$ = 96, x = 8, Zhukova & Petrovsky 1972, Petrovsky & Zhukova 1981).

Microsatellites are widely used molecular markers for population genetic studies and have also been used to infer evolutionary relationships among closely related species (Harr & al. 1998, Petren & al. 1999, Alvarez & al. 2001, Chirhart & al. 2005). The evolutionary rate of microsatellites has been suggested to be too fast for phylogenetic studies. However, for several studies of closely related species, where sequence variation is difficult to obtain, microsatellites have been a useful marker system (Goldstein & Pollock 1997, Schlötterer 2001). *Cardamine* is a young genus with little sequence variation (Carlsen & al. submitted; Koch & al. 2000, Haubold & Wiehe 2001), microsatellites were therefore used in order to obtain enough variation to delimit species in the genus.

We tested the number and the circumscription of taxa in the *Cardamine digitata* aggregate using microsatellites. We conclude that there are four distinct taxonomic units at equal rank, preferrably as species: *C. blaisdellii* Eastw., *C. digitata* Richardson, *C. microphylla* Adams, and *C. purpurea* Cham. & Schtdl.. Furthermore, there might be an additional unit we informally indicate as *C. 'hyperborealis'*. We use these names as a framework.

MATERIAL AND METHODS

Material. --- We included 131 specimens from 54 populations, a sampling covering most of the known ranges (Fig. 1; Appendix). Both herbarium (denoted TC) and silica-dried (denoted BE or SUP) specimens were included (Appendix). The herbarium specimens are

deposited at the V. L. Komarov Botanical Institute, Russian Academy of Sciences, St. Petersburg (LE; denoted TC03) and the University of Alaska Museum of the North Herbarium, Fairbanks (ALA; denoted TC06; Appendix). Vouchers for the remaining populations are deposited at the Natural History Museum, University of Oslo, Oslo (O).

Microsatellite analysis. --- DNA was extracted using the DNeasy™ Plant Mini Kit or DNeasy™ Plant 96 Kit (Qiagen) following the manufacturer's protocol. Microsatellites were amplified using marker specific primers (Table 1; MWG Biotech AG) and M13-primers (5'-CACGACGTTGTAAAACGAC-3') dyed with FAM (MWG Biotech AG), VIC (Applied Biosystems), and NED (Applied Biosystems). A PCR reaction volume of 10 µL contained 0.1 mM dNTP, 1.5--3.0 mM $MgCl_2$ (Table 1; Applied Biosystems), 0.01% BSA (Roche), 10 µM TMA (Sigma), 0.2 µM reverse primer (Table 1), 0.06 µM forward primer (Table 1), 0.2 µM M13 labeled primer (Table 1), 0.15 U AmpliTaq Gold (Applied Biosystems), 1x PCR Gold Buffer (Applied Biosystems), mqH_2O, and 1.0 µL diluted DNA template. The reactions were run with GeneAmp PCR system 9700 (Applied Biosystems) at the Natural History Museum, University of Oslo, using one of three different PCR programs (depending on marker; Table 1). The PCR products of different dyes were coloaded, and 1 µL of product mixture (FAM:NED:VIC = 2:3:2) was added 8.8 µL HiDi (formamide) and 0.2 µL GeneScan Rox 500 size standard (Applied Biosystems). The products were denatured for 5 min at 95ºC and analyzed with an ABI 3100 Sequencer (Applied Biosystems) with 10 s injection time and 3 kV injection voltage, otherwise default conditions.

Numerous microsatellite markers previously developed for *Arabidopsis thaliana* were tested (Skrede & Carlsen submitted), and six markers with an appropriate level of variation were chosen (Table 1). Three replicates were made for every sample included. The resulting profiles were visualized, sized and scored using GeneMapper vs. 3.7 (Applied Biosystems). Due to polyploidy, the markers were treated as dominant, and peaks in the range of 50--500

base pairs (Table 1) were scored as present (1) or absent (0). Variation in ploidy level is problematic when scoring microsatellite loci due to partial heterozygous individuals (e.g., AAAB, ABCC, ABBB, etc.). Studies analysing microsatellites for polyploid species often score dosage differences. As the plants in the present study have a ploidy level from diploid to dodecaploid scoring dosage differences is an impossible task. We therefore decided to score the microsatellites as phenotypes rather than genotypes. The final dataset consisted of 131 individuals and 102 bands.

Data analysis. --- The variation in the dataset was visualized using principal coordinate analysis (PCO) in NTSYSpc version 2.02 (Rohlf 1999) based on the similarity measure of Dice (1945). The first 96 of the resulting 131 eigenvectors explained 100% of the variation in the dataset, and these were analyzed with canonical variate analysis (CVA) and multivariate analysis of variance (MANOVA) in PAST version 1.29 (Hammer & Harper 2004), following the procedure of Anderson & Willis (2003), of a priori dividing the specimens into groups according to the results of other analyses (PCO, STRUCTURE and parsimony).

A Bayesian approach using STRUCTURE version 2 (Pritchard & al. 2000) calculated a logarithmic probability for the data being assigned to a given number of clusters. The method was originally designed for codominant markers but may be applied to dominant markers under a no-admixture model, assuming no linkage among loci (Pritchard & al. 2000). Ten replicates of each value of K (= the number of groups) were run for different selections of samples with a burn-in period of 100,000 and 1,000,000 iterations. Similarity coefficients comparing the resulting assignments were calculated using Structure-Sum (Rosenberg & al. 2002, Ehrich 2006).

Parsimony analyses were performed in TNT (Goloboff & al. 2000) with bands coded as present or absent. Heuristic searches were performed with 10,000 random additional

sequences and TBR branch swapping, saving ten trees per replication. The resulting trees were swapped with TBR saving up to 100,000 trees altogether. Collapsing rule was set to minimum length = 0. Random seed was set to "time". Goodness of fit was calculated using CI, RI, and RC (Kluge & Farris 1969, Farris 1989). Bremer supports (Bremer 1994) were calculated producing 60,000 trees, of which 10,000 were one step longer, 10,000 were two steps longer, etc., up to six steps longer. Jackknife (Farris & al. 1996) and traditional bootstrap (Felsenstein 1985) resampling studies were performed with 1,000 replicates (10 random entry orders and 10 trees saved for each repetition). Jackknifing was performed with 36% deletion. Both bootstrap and jackknife were performed with cut-off value of 50% and absolute frequencies as output.

RESULTS

Scoring. --- The six microsatellite loci provided a matrix of 102 scored variable alleles. AthCTRI was the most conservative locus providing only one or two alleles even for the high-ploid individuals. Atts0239 was the most variable and allele-rich locus providing from two to nine alleles for each individual (Table 2). All isolates had a number of alleles that corresponded well to known or expected ploidy level, except for some accessions of *C. microphylla* from the Sakha Republic (Table 2) that had seven to nine alleles at the locus Atts0239.

Ordination analyses. --- PCO analysis of all samples separated *Cardamine purpurea* and *C. microphylla* from *C. digitata* and *C. blaisdellii* along axis 1 (spanning 17.6% of the variation; Fig. 2A), although a few specimens of *C. digitata* were placed closer to *C. microphylla* than the remaining specimens of *C. digitata*. Axis 2 (10.7%) separated *C. purpurea* from *C. microphylla* and *C. digitata* from *C. blaisdellii*. The *C. 'hyperborealis'*

specimens grouped with *C. digitata* (Fig. 2A). Axis 3 (8.2%) gave no further information (not shown).

In an analysis excluding the *C. purpurea* samples, *C. microphylla* was found at high values along axis 1 (19.4%), *C. blaisdellii* was found at low values, and *C. digitata* was intermediate and partially overlapping with *C. microphylla* (Fig. 2B). The *C. 'hyperborealis'* specimens grouped with *C. digitata* along the first axis, whereas one (TC03-28) grouped with *C. blaisdellii* along the second axis (11.1%; Fig. 2B).

A PCO analysis excluding both *C. purpurea* and *C. microphylla* separated *C. blaisdellii* and *C. digitata* along the first axis (20.8%; Fig. 2C). One of the *C. 'hyperborealis'* plants (TC03-28) grouped with *C. blaisdellii*, and the other two with *C. digitata*. The second axis gave no additional taxonomic information (10.2%; Fig. 2C).

The CVA analysis separated the five a priori defined groups completely and significantly (Wilk's lambda = 3.485E-11, df1 = 384, df2 = 122.8, F = 131.8, p(same) = 5.636E-102; Pillai trace = 3.986, df1 = 384, df2 = 132, F = 94.65, p(same) = 8.102E-100; Fig. 3). The first axis (61.9%) separated *C. microphylla* from the other groups, whereas the second axis (26.2%) separated the remaining groups. *Cardamine 'hyperborealis'* was intermediate between *C. blaisdellii* and *C. digitata* along the second axis (Fig. 3).

STRUCTURE analyses. --- The STRUCTURE analysis including all specimens, separated the plants into two groups; one comprising *C. microphylla* and *C. purpurea*, and one comprising *C. blaisdellii* and *C. digitata*. An increase in number of groups resulted in ambiguous division of the dataset (similarity coefficiants < 1). Further analyses of the two groups separately gave no further division of the first group, while the second was unambiguously divided in two: one comprising *C. blaisdellii*, one specimen of *C. digitata* (SUP02-177-3), and one of *C. 'hyperborealis'* (TC03-28), and one comprising *C. digitata* and the other two *C. 'hyperborealis'* specimens.

Parsimony analyses. --- Heuristic search and subsequent TBR swapping gave 6,336 most parsimonious trees of length 568, from two different "islands" in tree-space. Goodness of fit values were CI 0.180, RI 0.721, and RC 0.130. A strict consensus tree is presented in Fig. 4. Resampling analyses gave support only for internal nodes within species (result not shown). Bremer support value for the branch separating *C. purpurea* from the other species is 2 (Fig. 4). Bremer support value for the branch separating *C. microphylla* from *C. blaisdellii* and *C. digitata* is 1 (Fig. 4; Bremer support for all other branches not shown).

DISCUSSION

The combination of analyses presented here supports the recognition of four evolutionary units in the *Cardamine digitata* aggregate. Although they give partly different results and resolutions, the analyses are not conflicting. The separation of *C. purpurea* and *C. microphylla* from the remaining units is supported in both PCO and parsimony analyses, and the isolation of the groups even got Bremer support of 2 and 1, respectively. *Cardamine digitata* and *C. blaisdellii* were separated by both STRUCTURE and PCO analyses. The resolution in the parsimony analyses gave no support to, but neither contradicted the separation of the two groups. As the four groups correspond to morphologically defined and comparatively distinct units, we suggest to acknowledge the groups as four taxa at the rank of species: *C. blaisdellii, C. digitata, C. microphylla,* and *C. purpurea*.

Murray & Kelso (1997) suggested to reduce the rank of *C. blaisdellii* to subspecific, as *C. microphylla* ssp. *blaisdellii* (Eastw.) D.F. Murray & S. Kelso. We found no transition between *C. blaisdellii* and *C. microphylla* in our study. On the contrary, we found all four units to be approximately equidistant, suggesting similar ranking for all units. The closest relationship of *C. blaisdellii* is to *C. digitata* rather than to *C. microphylla*. Furthermore, *C. blaisdellii* and *C. microphylla* are largely sympatric, and the genetic and morphological

differences observed are therefore probably not resulting from the geographical isolation often defining subspecies.

The three specimens we have named *C. 'hyperborealis'* are morphologically intermediates between *C. digitata* and *C. blaisdellii* (pers. obs.), and they are genetically grouped with both taxa. Deciding whether these represent an additional stabilised allopolyploid taxon requires further studying. Checking the vouchers of these plants revealed that they all had pollen of irregular size, indicating sterility and hybrid origin (pers. obs.). They were all, however, collected at an early developmental stage, making it impossible to examine fruits and seeds.

Chromosome numbers and ploidy levels. --- The basic chromosome number of *C. purpurea* is $x = 8$, but $x = 7$ for the other study species. All numbers reported from the relevant units are polyploid, from tetraploid to dodecaploid. Assignment of chromosome number reports to taxa is made difficult by the different application of names and circumscriptions of species.

Two or more ploidy levels are documented for each of the four species. We have studied the numerous vouchers for the Russian chromosome counts (in LE) and find no evident morphological difference among plants at different ploidy levels in *C. blaisdellii*, *C. digitata* or *C. microphylla*. Taxonomic autopolyploidy is therefore indicated. In *C. purpurea* there is a slight difference in leaf shape between the isolated Wrangel Island plants (counted with $2n = 96$, chromosome number vouchers studied) and the NW Alaskan plants (counted with $2n =$ c. 80, but voucher for the chromosome count not studied by us) but neither of them approach any of the other species morphologically. The several NE Asian chromosome number vouchers annotated as *C. hyperborea* are another matter. The vouchers for tetraploid counts ($2n = 28$) correspond closely morphologically (and also in microsatellite markers) with tetraploid *C. blaisdellii*. The vouchers for hexaploid counts ($2n = 42$) are intermediate in shoot

and leaf morphology between *C. blaisdellii* and *C. digitata*, and we informally name them as *C. 'hyperborealis'*.

Chromosome counts above hexaploid level (Mulligan 1965, Zhukova & Tikhonova 1973, Krogulevich 1976) are dubious in *C. microphylla*, but as we have found up to nine alleles in the locus Atts0239 in plants from the Sakha Republic (cf. Table 2), this could indicate that cytotypes exist at higher ploidy levels.

Cytotypes, not autopolyploid speciation --- The *Cardamine digitata* aggregate can be listed as one of the many plant groups showing that autopolyploids are much more common than traditionally maintained (reviewed by Soltis & al. 2003). Soltis & al. (2003) suggested that recurrent formation of polyploids is the rule, not the exception, and the different levels of polyploidy in at least three of four units in our study support this statement. But even though our knowledge on autopolyploid dynamics and the frequency of formation has dramatically increased during the past decade, the recognition of autopolyploidy as a major mode of speciation has not (Soltis & al. 2007). Soltis & al. (2007) claimed that a failure to name autopolyploids as separate species is caused by the adherence of plant systematists to a strict taxonomic species concept stressing morphological features, resulting in a serious underestimate of the role of polyploidy in plant speciation. The lack of both genetic and morphological distinction among cytotypes within our four units, however, indicates frequent gene flow, a condition considered by most systematists (including Soltis & al. 2007) to characterise conspecificity. Thus, we choose the conservative approach and suggest the cytotypes to be conspecific.

Concluding notes on nomenclature and taxonomy. --- *Cardamine purpurea* Cham. & Schtdl. (Chamisso & Schlechtendal 1826) was described from western Alaska: St. Lawrence Island in the northern Bering Sea. A specimen from "Ins. St. Laurentii", leg. L. K. A. von Chamisso (HAL-85360), has been indicated as the holotype, but an alternative is "St.-

Laurence Isl.", 1816, leg. J. F. G. von Eschscholtz (LE). Lectotypification may be needed. The meaning and application of the name has been unambiguous since its description. The species is mainly Beringian American (Yukon Territory and Alaska, widespread) and the only Asian occurrence reported by Petrovsky in Tolmachev (1975) is on Wrangel Island.

Cardamine microphylla Adams (Adams 1817) was described from the estuary of Lena River in northern Siberia (Sakha Republic): "Promontorio Bykovský Mys, ora fl. Lena", leg. M.F. Adams (MW) lectotype. The name was until fairly recently applied collectively to include all plants with broad leaf lobes and comparatively large, white flowers (e.g., Hultén 1968, also tentatively Petrovsky in Tolmachev1975, Porsild & Cody 1980, Berkutenko 1988), i.e., including *C. blaisdellii* (published 1902) which these authors have considered as a later synonym. With the late recognition of the more narrowly amphi-Beringian *C. blaisdellii*, by Khatri (1990) as a variety of *C. microphylla*, by Murray & Kelso (1997) as a subspecies, the questions arise how to circumscribe *C. microphylla* s.str. and where it occurs. Plants identified morphologically as *C. microphylla* s.str. are found in three separate regions: in a restricted area around the Lena River estuary in N Siberia (Petrovsky in Tolmachev 1975), in a wider area in W and E Chukotka in NE Russian Far East (about half of what Petrovsky in Tolmachev 1975 mapped as *C. microphylla*, the other half is part of *C. blaisdellii*), and in a significant area in NE Alaska, N Yukon Territory, and NW Mackenzie District. The gap between the N Siberian and Chukotkan parts of the range is c. 1,900 km, that between the Chukotkan and NW American parts c. 1,500 km.

Cardamine digitata Richardson (Richardson 1823) was described from NW North America with a type from NW Canada, Mackenzie District: "Barren Grounds from lat. 64° to Arctic Sea, in lat. 69°", leg. N. Richardson (BM) holotype. There has been much confusion concerning the name. Trautvetter (1879) applied Richardson's name to plants with broad leaf lobes and coined the name *C. digitata* var. *oxyphylla* Trautv. for the plants with narrow leaf

lobes (i.e., those of the current-day opinion of *C. digitata* s.str.). In the only global revision of *Cardamine*, Schulz (1903) rejected the name *C. digitata* Richardson as he assumed that it was predated by *C. digitata* Lam. (Lamarck 1786) and thereby a later homonym. He coined *C. hyperborea* O.E.Schulz as a nomen novum for *C. digitata* in the Richardson meaning (Schulz 1903). He also followed Trautvetter (1879) in assuming this to be a plant with broad leaf lobes and made the combination *C. hyperborea* var. *oxyphylla* (Trautv.) O.E.Schulz for the plant with narrow leaf lobes (Schulz 1903). Both propositions were erroneous. Lamarck (1786) described his species as a *Dentaria*, not as a *Cardamine*, and the name therefore does not invalidate Richardson's *C. digitata* which has priority at the rank of species. Richardson's plant is that with narrow leaf lobes (as seen in the type specimen). Only such plants occur in the region from where it was described. Schulz' *C. hyperborea* is therefore a homotypic synonym of Richardson's *C. digitata*, and Trautvetter's and Schulz' var. *oxyphylla* is the true *C. digitata* whereas their application of the names *C. digitata* (excl. var. *oxyphylla*) and *C. hyperborea* (excl. var. *oxyphylla*) refers to something else, probably *C. blaisdellii*. That *C. hyperborea* is synonymic and homotypic with the validly described *C. digitata* and is a superfluous synonym was already pointed out by Shetler (1961) and Rollins (1993) and confirmed by Egorova (pers. comm.): "*Cardamine hyperborea* is a nomen superfluvum illegitimum [for *C. digitata* Richardson] according to Art. 52.1--52.2 of the St. Louis Code". The next step in the confusion of names and applications is the introduction of the name *C. richardsonii* Hultén. Hultén (1945) coined this name as a nomen novum for *C. digitata* Richardson as also he, on the authority of Schulz, erroneously assumed that Richardson's name was predated by Lamarck's name and in addition that the name *C. hyperborea* as applied by Schulz belonged to the plants with broad leaf lobes. Hultén's name is therefore a superfluous, full synonym of *C. digitata*. It has been applied by, e.g., Löve & Löve (1975), but was already rejected by Hultén (1968) in favour of *C. hyperborea*. *Cardamine digitata* is

a widespread northern Beringian and North American plant with a nearly continuous range from W Chukotka eastwards to Hudson Bay (Petrovsky in Tolmachev 1975, Porsild & Cody 1980).

The meaning of the name *C. hyperborea* is now clear, as a homotypic synonym of *C. digitata*. Its application is problematic, especially on the Asian side. Petrovsky in Tolmachev (1975 Russian Arctic) entered *C. digitata* and *C. hyperborea* as two species and only tentatively indicated *C. blaisdellii* as possibly synonymous with the latter. Petrovsky still (pers. comm.) suggests that there may be a third entity present in NE Asia, besides *C. digitata* and *C. blaisdellii* (which he now accepts as a species and as a part of his 1975 concept of *C. hyperborea*). Berkutenko in Charkevicz (1988 Russian Far East) also entered *C. digitata* and *C. hyperborea* but without reference to *C. blaisdellii*. Doronkin in Malyschev & Peschkova (1994 Siberia, i.e., excl. Russian Far East) synonymized *C. hyperborea* in the Schulz meaning (excluding the plants with narrow leaf lobes) with *C. microphylla*.

Cardamine blaisdellii Eastw. (Eastwood 1902) was described from W Alaska with type from Seward Peninsula: Cape Nome, summer 1900, leg. F.E. Blaisdell (CAS) holotype. This name was largely forgotten until Khatri's revision of the *Cardaminella* group (Khatri 1990) and especially a closer study of the Alaskan plants by Murray & Kelso (1997), but see Porsild's (1938) discussion on the taxon and applications of names in the aggregate. Since then *C. blaisdellii* or *C. microphylla* subsp. *blaisdellii* has been accepted as the correct name for at least the NW North American parts of Schulz' *C. hyperborea* with broad leaf lobes, recently also for at least a major part of the NE Asian plants (Petrovsky pers. comm.). As currently understood *C. blaisdellii* is amphi-Beringian with a small part area in E Chukotka and a larger one in Alaska, Yukon Territory, and probably reaching Mackenzie District in the mountains west of Mackenzie River.

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Table 1. Microsatellite markers used in this study. All forward primers also contain a M13-tail (5'-CACGACGTTGTAAAACGAC-3') at the 5' end of the sequence. The PCR programs contained the following steps: 51 (5 min at 95ºC, 35 cycles of the three steps 30 s at 94ºC, 30 s at 51ºC, and 45 s at 72ºC, and a final hold of 20 min at 72ºC), TD50 (5 min at 95ºC, 16 cycles of the three steps 30 s at 94ºC, 30 s at 58-50.5ºC (decreasing 0.5ºC every cycle), and 45 s at 72ºC, 35 cycles of the three steps 30 s at 94ºC, 30 s at 50ºC, and 45 s at 72ºC, and a final hold of 20 min at 72ºC), or TD48 (5 min at 95ºC, 10 cycles of the three steps 30 s at 94ºC, 30 s at 53-48.5ºC (decreasing 0.5ºC every cycle), and 45 s at 72ºC, 28 cycles of the three steps 30 s at 94ºC, 30 s at 48ºC, and 45 s at 72ºC, and a final hold of 20 min at 72ºC).

Marker	Forward	Reverse	Dye	[$MgCl_2$]	PCR program	App. range
ICE14	5'-TCGAGGTGCTTTCTGAGGTT-3'	5'-TACCTCACCCTTTTGACCCA-3'	FAM	2.5 mM	51	220-280
MR187	5'-GAGTTTTGGTTCCACCATTA-3'	5'-CCCTTCAGCCTTTGATAAAT-3'	NED	3.0 mM	51	145-195
Atts0191	5'-GACTGATGTTGATGGAGATGGTCA-3'	5'-CTCCACCAATCATGCAAATG-3'	VIC	1.5 mM	TD48	190-205
Atts0392	5'-GACGTTGATCGCAGCTTGATAAGC-3'	5'-TTGGAGTTAGACACGGATCTG-3'	FAM	2.5 mM	TD50	145-225
nga1145	5'-GACCCTTCACATCCAAAACCCAC-3'	5'-GCACATACCCACAACCAGAA-3'	VIC	2.0 mM	TD50	245-275
AthCTRI	5'-GACTATCAACAGAAACGCACCGAG-3'	5'-CCACTTGTTTCTCTCTCTAG-3'	NED	2.5 mM	TD50	145-160

Table 2: Number of alleles per plant found for each taxon and locus. 2*n* gives chromosome counts of plants included in this study, cf. Appendix.

	2*n*	ICE14	nga1145	Atts0392	MR187	AthCTRI	Atts0191
C. blaisdellii	28	1-2	2-4	2-4	1-4	1	1-2
C. digitata	28, 42	1-3	2-4	3-6	1-4	1-2	2-5
C. 'hyperborealis'	42	2-3	3-4	4-5	3-4	1	2-4
C. microphylla	28, 42, 52	1-5	2-4	2-9	1-5	1-2	1-4
C. purpurea	96	1-4	1-4	2-6	2-7	1-2	1-2

Fig. 1. Sampling of the *Cardamine digitata* aggregate included in this study. The map shows aprroximatly the known ranges of the complex. See Appendix for further details.

Fig. 2. PCO analysis of the *Cardamine digitata* aggregate based on six microsatellite loci and Dice 's similarity. A. Analysis of the total material of 131 plants. B. Analysis of 21 populations of *C. microphylla*, seven populations of *C. blaisdellii*, 12 populations of *C. digitata*, and three populations of *C. 'hyperborealis'*. C. Analysis of seven populations of *C. blaisdellii*, 12 populations of *C. digitata*, and three populations of *C. 'hyperborealis'*.

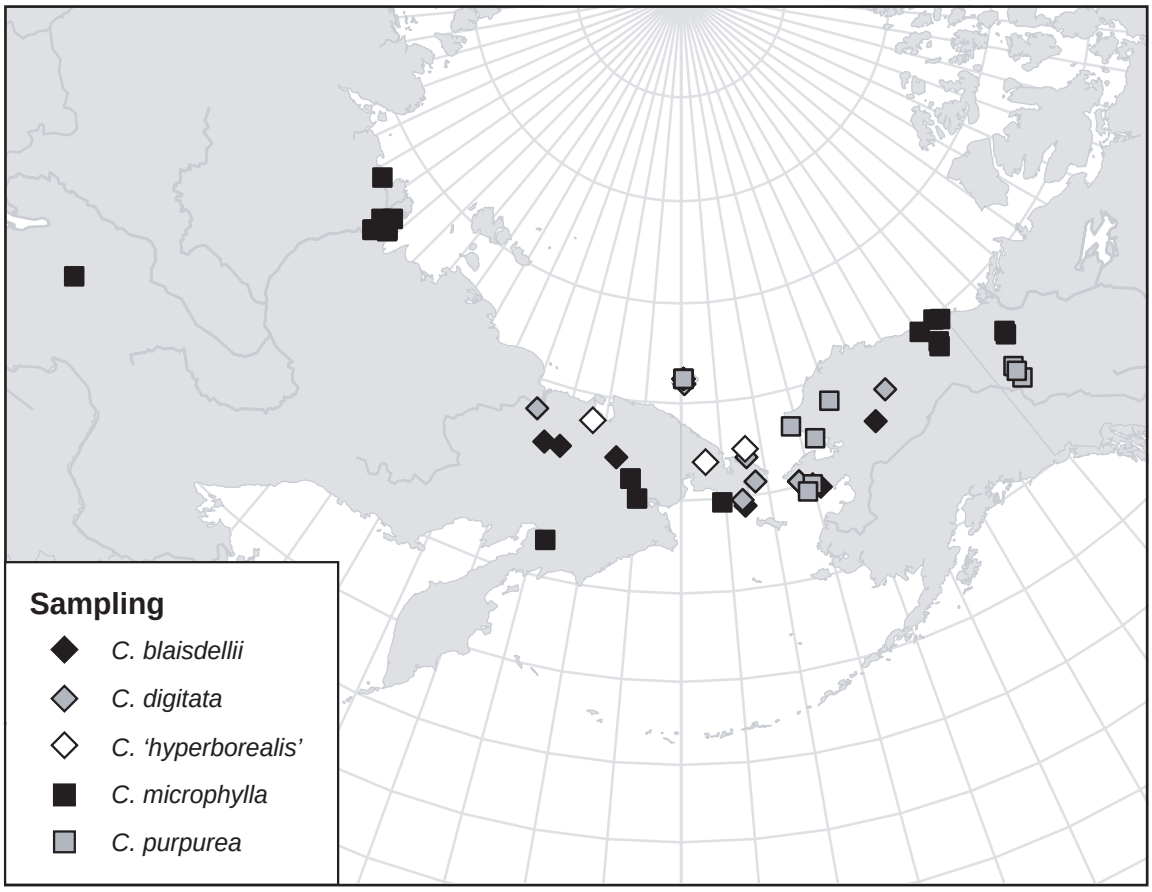
Fig. 3. CVA analysis of 96 eigenvectors from the PCO analysis of the *Cardamine digitata* aggregate, a priori grouped according to taxa. See text for further details.

Fig. 4. Unrooted strict consensus tree of 6,336 most parsimonious trees. Bremer support (BS) values are noted for two branches.

Appendix. Sampled material of the *Cardamine digitata* aggregate. The states are abbreviated as follows: ALA - Alaska, CAN - Canada, DFO - Far Eastern Federal District, and SFO - Siberian Federal District. Positive and negative values for longitudes and latitudes give N/E and S/W, respectively.Chromosome counts give the original collection numbers, chromosome numbers, and publications: [1]Zhukova & Petrovsky (1976), [2]Zhukova & Petrovsky (1980), [3]Zhukova & Petrovsky (1977), [4]Petrovsky & Zhukova (1981), [5]Zhukova & al. (1973), [6]Zhukova & Petrovsky (1972), [7]Zhukova & Petrovsky (1987), [8]Zhukova & Petrovsky (1984), [9]Zhukova (1969), [10]Zhukova & Tikhonova (1973), and [11]Zhukova (1980).

Species: Population/herbarium number (No. of specimens, state, region, locality, latitude, longitude, year, collectors, chromosome counts)
***Cardamine blaisdellii*:** BE05-41 (5, DFO, Chukotka Autonomous Okrug, Chukchi Peninsula, Novo Chaplino, 64.50, -172.83, 2005, Solstad, Elven), SUP02-166 (5, ALA, Nome Census Area, Seward Peninsula, Solomon River East Fork, 64.70, -164.18, 2002, Elven, Gabrielsen, Jørgensen), SUP02-193 (5, ALA, Nome Census Area, Seward Peninsula, Teller, coast/cliffs W of Teller, 65.25, -166.41, 2002, Elven, Gabrielsen, Jørgensen), SUP02-243 (5, ALA, Northwest Arctic Borough, Kobuk River Area, Anayucham Mts, Fritts Mt, 66.92, -155.52, 2002, Elven), TC03-30 (1, DFO, Chukotka Autonomous Okrug, W Chukotka, Bilibino settlement, 67.00, 172.00, 1974, Tikhonova, 74-216 T, $2n = 28$[1]), TC03-31 (1, DFO, Chukotka Autonomous Okrug, W Chukotka, Anyuy Mts, Pogynden River basin, Alarmagtyn River, 67.00, 165.00, 1974, Tikhonova, 74-10 T, $2n = 28$[2]), TC03-32 (1, DFO, Chukotka Autonomous Okrug, W Chukotka, Anyuy Mts, Mainghy-Pauktuvaam River, 67.00, 163.00, 1976, Petrovsky, Koroleva, 76-86, $2n = 28$[3]); ***C. digitata*:** BE05-555 (5, DFO, Chukotka Autonomous Okrug, Chukchi Peninsula, Lavrentiya Bay, bay 19 km NW of Lavrentiya, 65.70, -171.32, 2005, Solstad, Elven), BE05-260 (3, DFO, Chukotka Autonomous Okrug, Chukchi Peninsula, Penkigney Bay, Pestolova River, 64.83, -173.09, 2005, Solstad, Elven), BE05-979 (5, DFO, Chukotka Autonomous Okrug, Wrangel Isl, Somnitelnaya settlement and River, 70.98, -179.55, 2005, Solstad, Elven), SUP02-177 (4, ALA, Nome Census Area, Seward Peninsula, Kigluaik Mts, Mt W of Shaw Creek, 64.93, -164.99, 2002, Elven, Gabrielsen, Jørgensen), SUP02-189 (5, ALA, Nome Census Area, Seward Peninsula, Teller, coast/cliffs W of Teller, 65.25, -166.41, 2002, Elven, Gabrielsen, Jørgensen), SUP02-274 (5, ALA, North Slope Borough, Brooks Range, Endicott Mts, river south of Chandler Lake, 68.20, -152.74, 2002, Elven), TC03-33 (1, DFO, Chukotka Autonomous Okrug, Wrangel Isl, Samnitelnaya harbour, 71.25, -179.67, 1979, Petrovsky, 79-224, $2n = 42$[4]), TC03-34 (1, DFO, Chukotka Autonomous Okrug, Wrangel Isl, Samnitelnaya harbour, 71.25, -179.67, 1971, Petrovsky, Steinberg, 71-47, $2n = 42$[5]), TC03-35 (1, DFO, Chukotka Autonomous Okrug, Wrangel Isl, Mamontoraya River, 71.25, -179.67, 1970, Zhukova, Petrovsky, 70-213, $2n = 42$[6]), TC03-36 (1, DFO, Chukotka Autonomous Okrug, W Chukotka, Anyuisky Mts, by Anyuy River basin, Rybnaya River, 68.50, 160.82, 1982, Plieva, Petrovsky, 82-143/82-144, $2n = 28$[7]), TC03-37 (1, DFO, Chukotka Autonomous Okrug, Wrangel Isl, Gusinaya River, 71.25, -179.67, 1970, Zhukova, Petrovsky, 70-66, $2n = 28$[6]), TC03-38 (1, DFO, Chukotka Autonomous Okrug, E Chukotka, Lavrentiya settlement, 67.00, -172.00, 1972, Zhukova, 72-20 9?, $2n = 28$[8]); ***C. 'hyperborealis'*:** TC03-27 (1, DFO, Chukotka Autonomous Okrug, E Chukotka, Lavrentiya settlement, 67.50, -172.00, 1972, Zhukova, 72-38, $2n = 42$[8]), TC03-28 (1, DFO, Chukotka Autonomous Okrug, W Chukotka, Baranikha settlement, 68.85, 168.25, 1967, Korobkov, 67-18 K, $2n = 42$[9]), TC03-29 (1, DFO, Chukotka Autonomous Okrug, E Chukotka, Amguema R., 115 km road Egvekinot - Iultin, 67.00, -177.00, 1970, Kozlova, Tikhonova, 70-52 T, $2n = 42$[10]); ***C. microphylla*:** SUP-3912 (5, DFO, Sakha Republic, Lena River west bank, Chekurovka village, valley 1-2 km N of settlement, 71.06, 127.51, 2004, Solstad, Elven), SUP-3946 (5, DFO, Sakha Republic, Lena River west bank, plateau mountain 3-5 km W of Chekurovka village, 71.06, 127.47, 2004, Solstad, Elven), SUP-4093 (5, DFO, Sakha Republic, Lena River estuary, Area of Lena-Nordenskiöld Research Station, NE-most Kharaulakh Mts and Lena River delta flat, 72.20, 128.06, 2004, Solstad, Elven), SUP-4128 (5, DFO, Sakha Republic, Tiksi S, valley and small mountain E of town, 71.64, 128.86, 2004, Solstad, Elven), SUP03-372 (5, CAN, Yukon

Territory, Richardson Mts, Wright Pass W side, Dempster hwy, 463-465 km , 67.05, -136.25, 2003, Solstad, Elven), TC03-130 (1, DFO, Chukotka Autonomous Okrug, Beringovsky, W border of Pekulnejskoe Lake, 65.00, 175.00, 1984, Korobkov), TC03-132 (1, DFO, Koryak Autonomous Okrug, North Korjakia, 10 (..), coast of Majnip (Majnik?) Lake, 62.00, 166.00, 1984, Razzhivin), TC03-24 (1, DFO, Chukotka Autonomous Okrug, E Chukotka, Nunligran settlement, 64.80, -175.40, 1970, Korobkov, 70-25 K, $2n = 52^{10}$), TC03-25 (1, DFO, Chukotka Autonomous Okrug, S Chukotka, Pekulney Ridge, Bychya River, 66.00, 174.00, 1977, Zhukova, 77-214, $2n = 28^{11}$), TC03-26 (1, DFO, Chukotka Autonomous Okrug, S Chukotka, Pekulney Ridge, Bychya River, 66.00, 174.00, 1977, Zhukova, 77-162, $2n = 42^{11}$), TC03-3 (1, DFO, Sakha Republic, Lena River, Olenek Gulf, Stannakh-Khocho settlement, 72.95, 121.67, 1956, Tolmachev, Polozova), TC03-4 (1, DFO, Sakha Republic, Lena River, Tas-Azy Isl, 71.75, 127.00, 1956, Norin, Petrovsky, Shtepa), TC03-5 (1, DFO, Sakha Republic, Lena River, Sietachar River mouth, 71.08, 127.50, 1956, Norin, Petrovsky, Shtepa), TC03-6 (1, DFO, Sakha Republic, Tiksi harbour, Kengdey River basin, 71.58, 129.00, 1956, Tolmachev, Yurtsev), TC03-7 (1, SFO, Buryat Republic, Stanovoe Mts, South Muysky Ridge, Kindikan River source, 56.00, 115.00, 1965, Petrochenko), TC06-100 (1, ALA, North Slope Borough, Table Mountain Quad, Ambresvajun Lake, Last Lake, 68.60, -143.75, 1975, Batten, Batten), TC06-101 (1, ALA, North Slope Borough, Demarcation Point Quad, Beaufort Lagoon, Nuvagapak Point, 69.88, -142.30, 1974, Murray, Batten), TC06-102 (1, ALA, North Slope Borough, Demarcation Point Quad, Arctic National Wildlife Range, Pingokraluk Lagoon, Raluk, 69.70, -141.52, 1970, Murray), TC06-103 (1, ALA, North Slope Borough, Mount Michelsen Quad, Marsh Creek, app. 15 mi. inlan, 69.79, -144.82, 1985, Lipkin), TC06-104 (1, ALA, North Slope Borough, Table Mountain Quad, 32 km N of Ambresvajun Lake (Last Lake), Sheenjek River floodplain, 68.83, -143.50, 1975, Batten, Batten), TC06-105 (1, CAN, Yukon Territory, Eagle River Quad, Rock River, 66.87, -136.38, 1978, Russell); ***C. purpurea*:** SUP02-212 (4, ALA, Nome Census Area, Seward Peninsula, Kigluaik Mts, Mt E of Shaw Creek, 64.92, -164.97, 2002, Elven, Gabrielsen, Jørgensen), SUP02-225 (5, ALA, Nome Census Area, Seward Peninsula, Teller Road, Mt W of Penny River, 64.63, -165.68, 2002, Elven, Gabrielsen, Jørgensen), SUP03-129 (5, CAN, Yukon Territory, Ogilvie Mts C, Dempster Hwy, km 91. Seepage on alpine mountain slope, 64.63, -138.37, 2003, Elven, Solstad), SUP03-16 (5, ALA, Northwest Arctic Borough, Noatak Quad, Igichuk Hills, Kaksurok Mt, N side of mountain, 67.21, -163.22, 2003, Parker, Elven, Solstad), SUP03-373 (5, CAN, Yukon Territory, N Ogilvie Mts, Ogilvie River at confluence with Engineer Creek, Dempster hwy 196 km, 65.39, -138.27, 2003, Elven, Solstad), SUP03-382 (2, CAN, Yukon Territory, N Ogilvie Mts, steep limestone mountain S of W end of Windy Pass, Dempster hwy 157 km, 65.07, -138.33, 2003, Solstad, Elven), TC03-12 (1, DFO, Chukotka Autonomous Okrug, Wrangel Isl., Draga harbour, 71.25, -179.67, 1954, Sey), TC03-16 (1, DFO, Chukotka Autonomous Okrug, Wrangel Isl., Draga harbour, 71.25, -179.67, 1970, Petrovsky, 70-300, $2n = 96^{6}$), TC03-17 (1, DFO, Chukotka Autonomous Okrug, Wrangel Isl., Red Flag R., 71.25, -179.67, 1979, Petrovsky, 79-116, $2n = 96^{4}$), TC06-106 (1, ALA, North Slope Borough, Point Hope Quad., Ogotoruk Creek, Headwaters of Snowbank Creek, 68.12, -165.78, 1980, Murray, Johnson), TC06-107 (1, ALA, North Slope Borough, Misheguk Mountain Quad., Noluck Lake, Storm Creek, 68.80, -160.00, 1972, Parker)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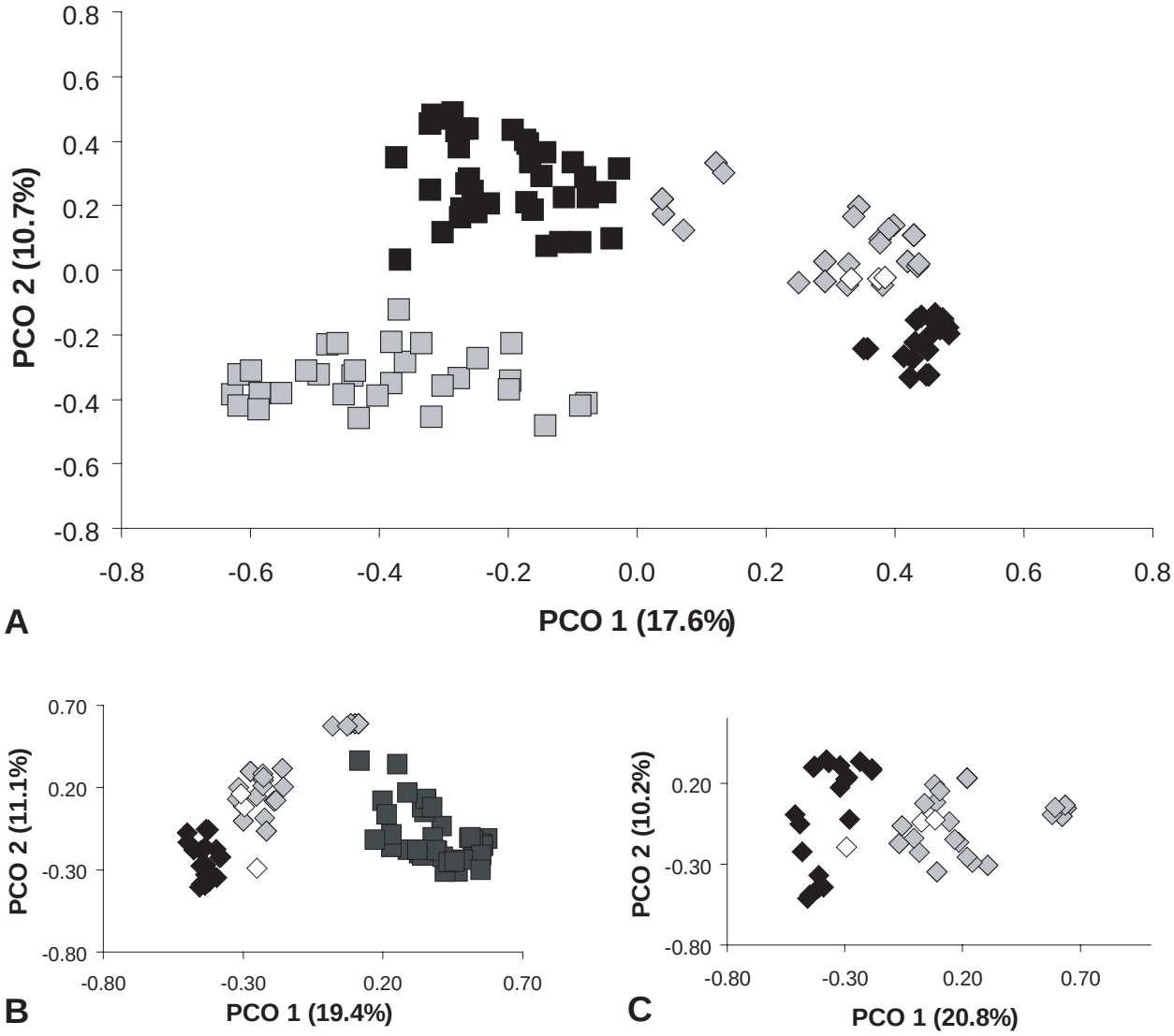
C. digitata
C. 'hyperborealis'
C. microphylla
C. purpurea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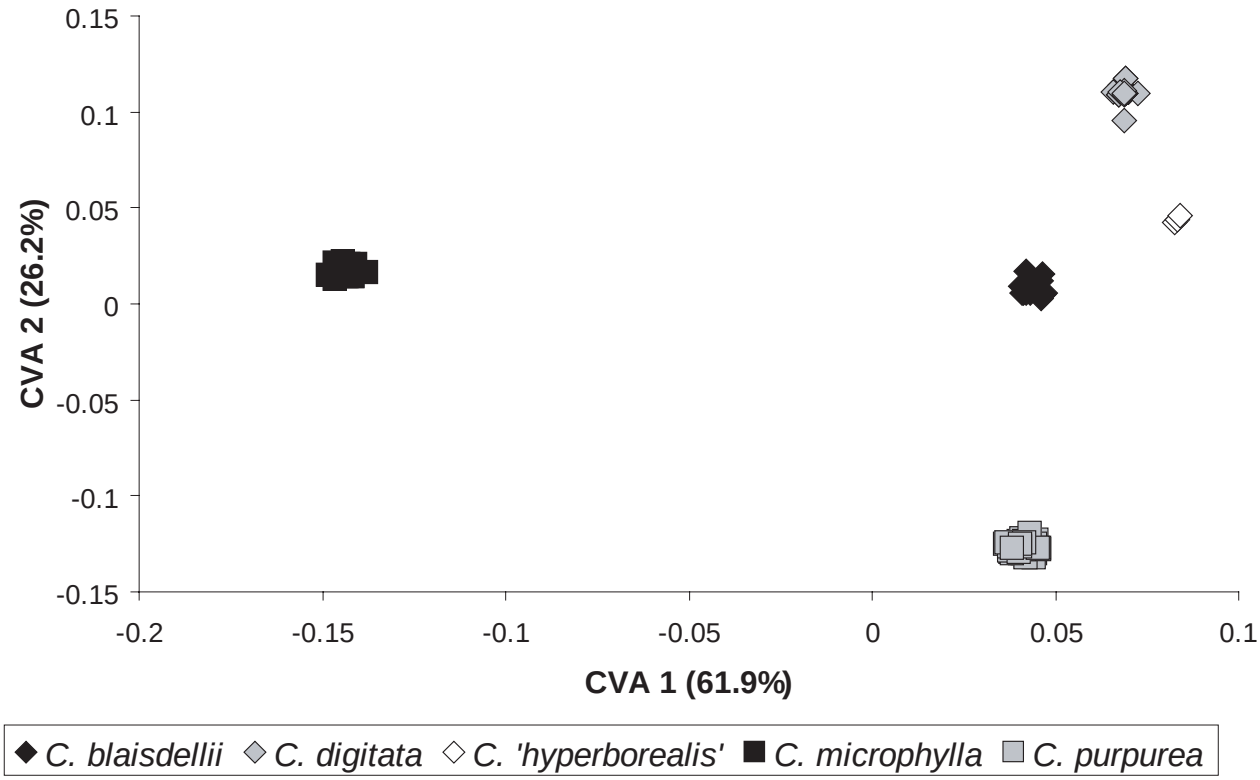
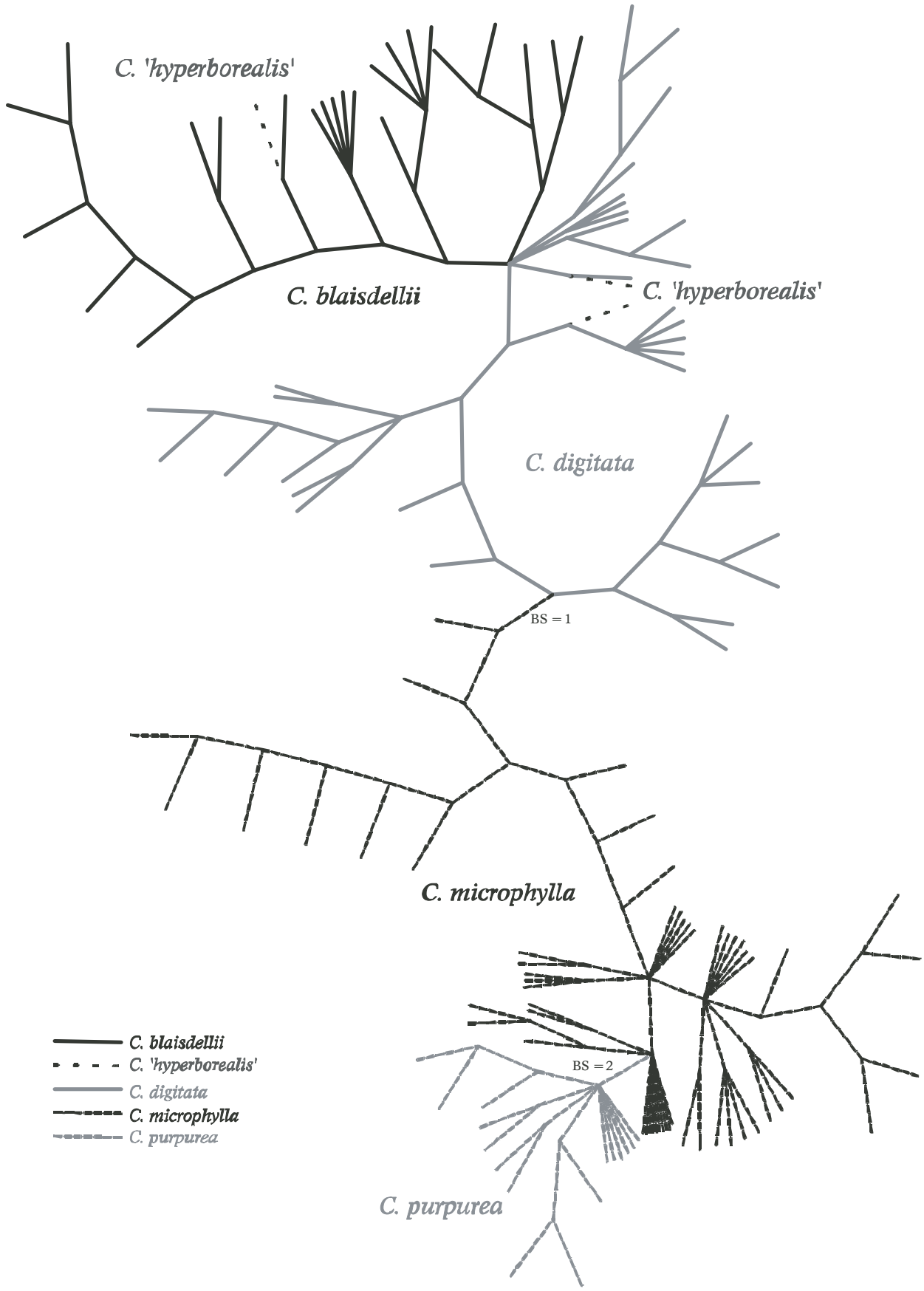
-0.8
PCO 2 (10.7%)
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
PCO 1 (17.6%)
A
0.70
0.20
-0.30
-0.80
PCO 2 (11.1%)
-0.80
-0.30
0.20
0.70
PCO 1 (19.4%)
B
0.20
-0.30
-0.80
PCO 2 (10.2%)
-0.80
-0.30
0.20
0.70
PCO 1 (20.8%)
C
C. blaisdellii
C. digitata
C. 'hyperborealis'
C. microphylla
C. purpurea


0.15
0.1
0.05
0
-0.05
-0.1
-0.15
CVA 2 (26.2%)
-0.2
-0.15
-0.1
-0.05
0
0.05
0.1
CVA 1 (61.9%)
C. blaisdellii
C. digitata
C. 'hyperborealis'
C. microphylla
C. purpurea


C. 'hyperborealis'
C. blaisdellii
C. 'hyperborealis'
C. digitata
BS = 1
C. microphylla
BS = 2
C. purpurea
C. blaisdellii
C. 'hyperborealis'
C. digitata
C. microphylla
C. purpurea

PAPER IV

Contrasting phylogeographic patterns inferred for two alpine sister species *Cardamine resedifolia* and *C. alpina* (Brassicaceae) in Europe

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Abstract

The present study explores intraspecific phylogeography of two alpine sister species *Cardamine alpina* and *C. resedifolia* (Brassicaceae). Both species are confined to siliceous bedrock and disjunctly distributed, the former restricted to the Alps and Pyrenees, the latter with a wider distribution in several European mountain ranges, spanning from the Sierra Nevada in the south-west to the Balkan in the south-east. We used amplified fragment length polymorphism (AFLP) data to address the degree of genetic differentiation of disjunct populations, to explore genetic patterns within the contiguous Alpine and Pyrenean ranges, and to compare the phylogeographic patterns of these two species. Accessions of the closely related arctic *C. bellidifolia* were analyzed as well, to test for the shared phylogeographic histories (immigrations or dispersal events) between the arctic and alpine areas. Two genetically strongly divergent lineages were resolved in the snow-bed *C. alpina*, corresponding to the Alps and Pyrenees, respectively. While multiple glacial refugia were invoked for the Pyrenees, very low genetic variation and virtually no phylogeographic structure observed in the Alpine range implied a single refugium in the Maritime Alps and rapid postglacial colonization of the Alps accompanied by a strong founder effect. The glacial and postglacial history of *C. resedifolia* is much more complex, since the genetic lineages proposed do not correspond to the main geographic disjunctions. The data suggested the existence of a largely widespread and continuous gene pool along with several geographically more restricted lineages, and secondary contacts between them. Differences in

ecological demands (snow-bed *C. alpina* vs *C. resedifolia* with broader ecological amplitude) are probably the major factors underlying such contrasting patterns. The arctic *C. bellidifolia* formed a very divergent lineage with no intraspecific variation, contradicting a scenario of its recent, postglacial immigration from the Alps or Pyrenees, or a recent, postglacial speciation event.

Key words: Alps, amplified fragment length polymorphism (AFLP), Arctic, Corsica, genetic diversity, Pleistocene glaciations, Pyrenees, Sierra Nevada

Introduction

Many phylogeographic studies have been undertaken in recent years with the major goals to understand the Quaternary history of plant species in Europe, to locate glacial refugia, describe postglacial (re)colonizations, and to infer general biogeographic patterns. Much attention has been focused on Alpine (e.g., Tribsch & Schönswetter 2003, Schönswetter & Tribsch 2005, Schönswetter et al. 2005, Bettin et al. 2007, Naciri & Gaudeul 2007) and arctic (Abbott & Brochmann 2003, Brochmann et al. 2003, Schönswetter et al. 2006b) plants, as well as on widespread tree species (e.g. Heuertz et al. 2004, Lumaret et al. 2005, Magri et al. 2006, Grivet et al. 2006). Based on the synthesis of fossil pollen records, palaeoenvironmental and genetic data it has been documented that Pleistocene climatic changes have had profound impact on species geographic distribution and genetic composition (Hewitt 2000, 2004). Plant species experienced repeatedly latitudinal and altitudinal range shifts, leaving behind traces in their intraspecific genetic structure. While lowland and lower altitude temperate species typically contracted and fragmented their ranges during cold periods into isolated refugia located mainly (but not exclusively) in more southern regions (Taberlet et al. 1998, Stewart & Lister 2001, Magri et al. 2006, Médail et al. 2007), alpine and arctic cold-adapted species may have survived in periglacial regions at the edge of the mountains or ice sheets, on ice-free protruding nunataks, or may have expanded to adjacent tundra habitats (Stehlik 2003, Schönswetter et al. 2005b). As the ice sheets retreated and the climate became milder during the interglacial and postglacial periods, populations migrated out of refugia and recolonized suitable sites at higher latitudes and altitudes (Hewitt 2000, 2004).

Previous phylogeographic studies have shown that molecular data can provide sound evidence on past biogeographic processes, e.g., they allow to locate glacial refugia and to infer postglacial migration routes. Processes such as area fragmentation, population isolation, extinction, colonization or long-distance dispersal greatly affect intraspecific genetic constitution, and produce specific patterns of genetic diversity and divergence. Area fragmentation and vicariance in refugia are expected to cause genetic differentiation and formation of genetically distinct lineages (Hewitt 2004). Refugial source populations usually harbour higher genetic diversity than populations of recolonized areas that have undergone bottleneck and founder effects, although secondary contacts of vicariant lineages from different refugia may lead to increased genetic diversity also in recolonized areas (melting spots, see Petit et al. 2003). Molecular data can shed light also onto the origin and timing of

disjunct distributions, which can be either due to vicariance or long-distance dispersal (Schönswetter & Tribsch 2005, Kropf et al. 2006).

Floristic similarities between the arctic and Alpine floras, expressed by the abundance of species with arctic-alpine distribution or sister relationships between Alpine and arctic species, suggest that there have been ancient or more recent (late-glacial and postglacial) contacts between these floras (Hultén & Fries 1986). Tundra vegetation documented to have occurred between the Scandinavian and the Alpine ice sheets in the cold periods of the Pleistocene may have facilitated migration and dispersal between the respective areas (Frenzel et al. 1992). A few recent phylogeographic studies that aimed to unravel the glacial history of arctic-alpine species show that different (post-)glacial scenaria can be encountered. The shared presence of species in the arctic and Alpine range can be explained by the postglacial immigration either from the Alps into northern Europe (and/or other arctic regions) or, conversely, from the arctic areas into the Alps (Schönswetter et al. 2003a, 2004a, 2006a), as well as by glacial survival in interconnecting lowland areas north of the Alps (Schönswetter et al. 2006a).

For the present study we selected two closely related alpine plant species, *Cardamine resedifolia* L. and *C. alpina* Willd. (Brassicaceae). Their sister relationship has been recently shown in a study that included almost all European diploids of the genus (Lihová et al. 2006). Both species are perennial herbs growing on siliceous bedrock, they are diploid with 2n = 16 (Kučera et al. 2005), and show similar dispersal abilities (possessing small and light seeds but without any morphological adaptations for dispersal over long distances). Data on their mating systems are lacking, as no detailed studies in this respect have been done so far. Observations based on genetic analyses (homo- vs heterozygosity) and growth experiments (K.K. Shimizu, unpubl.) suggest self-compatibility and selfing, but outcrossing cannot be ruled out. The two species differ, however, in habitat preferences and the overall distribution areas. *C. resedifolia* occupies moist screes and rock crevices above the timberline (rarely descending also to upper altitude coniferous mountain forests, pers. observ.) in several mountain ranges of the European Alpine System: the Sierra Nevada, Pyrenees, Cordillera Cantabrica, Massif Central, northern Apennines, Alps, Corsica, Sudety Mts., Rila Mts., Pirin Mts, and the Southern Carpathians (Markgraf 1958**,** Rico 1993, Jalas & Suominen 1994, Marhold 1995; only a single undated specimen is known from the Eastern Carpathians deposited in the herbarium BP). The species is thus rather disjunctly distributed, fragmented among mountain ranges, while the distribution in the two major ranges, the Alps and

Pyrenees, is largely contiguous. Typically it is found in the altitudinal range of 1500 m up to 3500 m. Morphologically it is well characterized by having pinnatisect stem leaves with auricles at base (plants with entire leaf blades can be found in the Southern Carpathians as well as in the Alps and were sometimes treated as *C. gellida* Schott, cf. Schulz 1903). The other species, *C. alpina* is restricted to the Pyrenees and Alps, and is typically found on sites such as snow beds and banks of mountain lakes, avoiding dry rocks, and reaching the altitudes of 1800 m to 3000 m (Markgraf 1958, Rico 1993, Jalas & Suominen 1994). Stem leaves are undivided with entire margin, rarely shallowly lobate, but lack auricles at leaf base. *C. alpina* has a counterpart species with circumarctic distribution, *C. bellidifolia* L., which is morphologically very similar and both are quite often treated as two subspecies (arctic *C. bellidifolia* subsp. *bellidifolia* and alpine subsp. *alpina*; e.g. Rico 1993, Jalas & Suominen 1994, Elven et al. 2006). The main focus of the present study is on the two alpine species, but a few samples of the arctic *C. bellidifolia* were included as well.

Major goals of this study were to explore the infraspecific phylogeography of *C. resedifolia* and *C. alpina*, and to elucidate their glacial survival and postglacial migration histories. Studies reconstructing phylogeographic histories of plants from different mountain ranges simultaneously, trying to unravel patterns both within and among the sub-areas, and to deduce on past contacts vs. isolation of respective populations, are still rather scarce (see e.g. Kropf et al. 2006). Here we expected to shed more light on this aspect, investigating two related species, which display different distribution patterns and ecological preferences. Highly polymorphic amplified fragment length polymorphism (AFLP; Vos et al. 1995) markers, proven reliable and efficient in previous phylogeographic studies, have been used. We aimed to answer the following specific questions: (i) What are the degree of genetic differentiation and relationships among populations from different mountain ranges (Pyrenees, Alps, Sierra Nevada, Corsica, Southern Carpathians…etc.)? Are they descendants of distinct refugial populations? Did they originate via recent dispersal or past vicariance events? (ii) Can be the genetic patterns within the contiguous Alpine and Pyrenean ranges related to species persistence in glacial refugia located in peripheral (periglacial) areas? (iii) Are there substantial differences in the intraspecific phylogeographic patterns of the two studied species? If so, can they be attributed to their different ecological demands, and thus habitat availability during the cold stages? (iv) Finally, can we trace the origin of the circumarctic *C. bellidifolia* within the European mountain ranges (or vice versa), or do we face a different Pleistocene scenario?

Material and methods

Sampling

We sampled 27 populations of *Cardamine alpina* and 33 populations of *C. resedifolia*, covering the geographic ranges of the species (Table 1, Fig. 1). Samples of *C. alpina* were collected both in the Pyrenees and Alps; *C. resedifolia* was sampled across several mountain ranges of its area, including the marginal and isolated ones (Sierra Nevada Mts., Corsica, Făgăraş Mts. in the Southern Carpathians). In addition, four Norwegian and one Iceland population of *C. bellidifolia* were included to address their affinities to populations from the European Alpine System. Leaf material of (5)6-8(10) individuals per population was dried in silica gel and used for DNA extraction. Voucher specimens of *C. resedifolia* and *C. alpina* are deposited in the herbarium SAV, those of *C. bellidifolia* are in O.

DNA isolation and AFLP fingerprinting

Total genomic DNA was extracted following the CTAB (2x cetyltrimethyl ammonium bromide) method (Doyle & Doyle 1987) with minor modifications. The quality of the extracted DNA was checked on 1 % TAE-agarose gels. The extracts were quantified photometrically and aliquots were adjusted to 100-200 ng/µl. The AFLP procedure followed the general protocol provided by Applied Biosystems (Applied Biosystems 2005, AFLP® Plant mapping protocol) with some adjustments as specified here. Restriction of genomic DNA was performed separately from the ligation. Restriction was performed for 3 hrs at 37 °C in a 10 µl volume using 5 U *Eco*RI (Fermentas), 2 U *Mse*I (New England Biolabs), 2 µl 10x Tango buffer (Fermentas), and 5 µl of the DNA extract (i.e. ca 500-1000 ng). Afterwards a ligation mix in a volume 5 µl per sample was added, and the reactions were further incubated at 16 °C for 12 hrs. Aliquots of the ligation mix contained 1 U T4 DNA ligase (Fermentas), 1.5 µl T4 DNA ligase buffer (incl. ATP), and 1 µl of each adaptor pairs (Applied Biosystems). Ligated DNA fragments were checked on 1 % TBE-agarose gels, and diluted 1:10 with TE buffer (10 mM Tris, 0.1 mM EDTA).

Preselective and selective amplifications (run in Mastercycler ep gradient S, Eppendorf) were run according to the Applied Biosystems protocol, using provided master mixes (“core mix”),

but the reactions were carried out in a 10 µl volume (i.e. reduced by 50 %). PCR cycle parameters followed those given in the protocol. Products of preselective amplification were checked on 1 % TBE-agarose gels, and diluted 1:15 with TE buffer. An initial screening of selective primers with three selective nucleotides revealed that the number of fragments was very low. Diploids of the genus *Cardamine* have a very small genome size, being at the lower range of the values known for vascular plants (Greilhuber et al. 2004). Therefore, as next we employed *Mse*I selective primers with only two selective nucleotides (-CT and -CA). The final primer combinations, yielding appropriate number of clear peaks, were: *Eco*RI -AAG-(VIC), *Mse*I -CT; *Eco*RI -ATC-(6-FAM), *Mse*I -CT; *Eco*RI -AGC-(NED), *Mse*I -CT (Applied Biosystems). The amplification products were pooled in the ratio 1:1:1 and submitted for the fragment analysis to the BITCET Consortium, Department of Molecular Biology, Comenius University, Bratislava (ABI 3100 Avant capillary sequencer). Size calibration was done using the internal size standard GeneScan –500 LIZ® (Applied Biosystems).

Raw AFLP data were collected and sized using the GeneScan 3.7 software (Applied Biosystems). The AFLP profiles were scored using the software Genographer 1.6.0 (available at http://hordeum.msu.montana.edu/genographer/), which was modified to allow for import of files from ABI 3100 processed in GeneScan 3.7. Only well-scorable and unambiguous fragments in the size range 75-500 bp were recorded, and coded as presence (1) or absence (0). Fragment scoring was done separately for *C. resedifolia* (250 individuals) and *C. alpina* (203 individuals), respectively, and the samples of *C. bellidifolia* (40 individuals) were scored together with a selection of accessions of both *C. resedifolia* and *C. alpina.* Thus, three data matrices were assembled: *C. resedifolia* dataset, *C. alpina* dataset, and the arctic-alpine dataset. To estimate reproducibility of the data, ca. 10 % of the samples were replicated. AFLP profiles of the control replicates were scored independently, and then compared each other to express AFLP reproducibility (error rate) and exclude ambiguous markers (Bonin et al. 2004).

Data analyses

Within each of the three AFLP datasets, the genetic distance and relationships among populations and individuals were first explored by principal coordinate analyses (PCoA, Krzanowski 1990) and by neighbour-joining trees. The analyses were run on the whole datasets, but in certain cases also on some of the main groups resolved, in order to recover

their substructure. PCoA was performed in the software SYN-TAX 2000 (Podani 2001) using Jaccard's coefficient to calculate pairwise genetic similarities, and plotted by the SAS statistical package (SAS 2000). Neighbour-joining trees, based on the Nei & Li (1979) genetic distance, were constructed in PAUP* version 4.0b10 (Swofford 2001). Group support was assessed by bootstrap analyses with 5000 replications.

The AFLP datasets were subjected also to cladistic parsimony analyses, using TNT (Goloboff et al. 2003). Heuristic searches were performed with 1000 random addition sequences and TBR branch swapping, saving ten trees per replication. The resulting trees were swapped on with TBR saving up to 100 000 trees altogether. Collapsing rule was set to minimum length = 0. Random seed was set to "time". Jackknife resampling studies (Farris et al. 1996) were performed with 5000 replicates (10 random entry orders and 10 trees saved each repetition), with 36 % deletion, cut-off value of 50 %, and absolute frequencies as output.

Furthermore, a rather complex genetic structure found in the *C. resedifolia* dataset was analyzed by Bayesian model-based clustering methods implemented in the software STRUCTURE 2.2 (Pritchard et al. 2000, Falush et al. 2007) and BAPS 3.2 (Corander et al. 2006). The program STRUCTURE 2.2 uses a Markov chain Monte Carlo (MCMC) algorithm to cluster genetically similar individuals on the basis of multilocus genotype data. We used a model with no admixture and assuming independence of allele frequencies among populations. *K* value (a user-defined number of clusters) was set from 1 to 12. To test the stability of the results, ten runs were performed for each $K = 1$ to 6, while three runs for each $K = 7$ to 12. The length of the burn-in period was set to 50 000, and the MCMC chains after burn-in were run for additional 500 000 replicates. As a complement to the MCMC Bayesian clustering, a Bayesian analysis based on stochastic optimization was performed using BAPS 3.2. It estimates the highest probability partition, i.e. the optimal number of clusters and assignment of the analyzed individuals. Both the frequencies of AFLP fragments and the number of genetically divergent groups are treated here as random variables. The analysis was repeated three times for each the maximum number of clusters $K = 30$, 25, and 20. Latch et al. (2006) advocate using both STRUCTURE and BAPS programs for inferring the number of clusters and assignment of individuals to clusters; according to them the greatest confidence in results is attained when results are arrived at independently by two different methods.

Genetic diversity within populations was assessed by the total number of AFLP fragments (N_{tot}), the percentage of polymorphic fragments (P(%)), and by the average proportion of pairwise differences between individuals (Nei's gene diversity, Nei 1987) using the R-script

AFLPdat (Ehrich 2006). In addition, Shannon's diversity index implemented in POPGENE 1.32 (Yeh et al. 1997) was computed. As a measure of population divergence we calculated the number of private fragments (those restricted to a given population), and the frequency-down-weighted marker values (DW) as proposed by Schönswetter & Tribsch (2005) and implemented in AFLPdat. Analyses of molecular variance (AMOVA) based on Euclidean pairwise distances were run with Arlequin 2.000 (Schneider et al. 2000).

Results

The reproducibility of AFLP profiles, as assessed by the control replicates, was high. Scoring of AFLP profiles in the *Cardamine alpina* dataset yielded identical AFLP multilocus phenotypes for each replicate pair; in the *C. resedifolia* dataset 99.5 % reproducibility was achieved, and one apparently ambiguous fragment was discarded.

Cardamine alpina *dataset*

With the three primer combinations used, 225 unambiguously scorable fragments were generated in 203 individuals (27 populations) of *Cardamine alpina*. 90 (40 %) of them were monomorphic, seven fragments were restricted to single individuals (autapomorphic fragments), and one fragment was present in all but one individual. The number of fragments per individual ranged between 125 and 154, with the mean being 130. Altogether we detected 51 different AFLP phenotypes, thus several individuals showed identical profiles.

The neighbour-joining tree (NJ, Fig. 2), parsimony analysis (not shown), and PCoA (Fig. 3A) revealed two main and clearly differentiated groups, corresponding to the two geographic areas – the Alps and Pyrenees (separated with 100 % bootstrap support in the NJ analysis and 100 % jackknife support in the parsimony analysis). Genetic differentiation between the Pyrenean and Alpine populations was confirmed also by the hierarchical AMOVA. As much as 88.52 % of the overall genetic variation was assigned to variation between the Alpine and Pyrenean populations, 7.11 % to variation among the populations within the groups, and only 4.37 % to variation within the populations. The Pyrenean populations had 60 private fragments, 13 of them fixed, while the Alpine ones possessed 22 private fragments, 18 of them fixed. Three well-supported clusters could be recognized within the Pyrenean group; the

two westernmost populations (CAN, PAN) clustered together, while the other two clusters corresponded to the two eastern populations, respectively. Genetic variability found within the Alpine range did not show any geographic structuring; it was subtle and rather random (Figs. 2, 3).

Genetic diversity was generally much higher in the Pyrenean populations (Table 1, Fig. 1), where 16 different AFLP phenotypes were detected in the four sampled populations, while 35 AFLP phenotypes were found among the 23 Alpine populations. In the Pyrenees, identical AFLP profiles were found only within the same population, while in the Alps identical AFLP phenotypes were often detected also across several not adjacent populations. Interestingly, one population from the Maritime Alps (CFn) showed significantly higher intrapopulational diversity than any other Alpine population: $D_{Nei} = H_{Sh} = 0.03$ and 8 % of polymorphic fragments vs. the Alpine average (excluding CFn) being $D_{Nei} = H_{Sh} = 0.005$ and 1.2 P(%). PCoA performed on the Alpine populations only (Fig. 3B) showed that individuals from the CFn population spanned the whole phenetic space, i.e. contained a significant portion of the variation present in the Alpine populations. The genetically most variable populations of *C. alpina* exhibited also the highest DW values and the highest numbers of private fragments, illustrating their genetic divergence (Pyrenean populations and CFn, see Table 1).

Cardamine resedifolia *dataset*

With the three primer combinations used, 192 unambiguously scorable fragments were generated in 247 individuals (33 populations) of *Cardamine resedifolia*. 33 (17 %) of them were monomorphic, 16 fragments were restricted to single individuals (autapomorphic fragments), and three fragments were present in all but one individual. The number of fragments per individual ranged between 65 and 88, with the mean being 76. Altogether we detected 194 different AFLP phenotypes; identical phenotypes were found within several populations but not across individuals from different populations.

Ordination graph of PCoA suggested presence of several genetic groups (Fig. 4). Two large and rather loose groupings appeared on the front side of the ordination diagram, separated along the first axis: (1) the accessions from the SW Alps (Maritime, Ligurian, Grajic, Cottic Alps; here named as SW Alpine group) clustering on the lower right side, and (2) the accessions from populations spanning a large geographic area (Sierra Nevada-Pyrenees-Corsica-Alps) on the left side. A few individuals were found in intermediate positions.

Furthermore, (3) five populations from the central-northern part of the Alps, here designated as N Alpine group, formed a rather compact group differentiated along the second and third axis; (4) the two Southern Carpathian populations were separated along the third axis, and (5) genetic distinction was suggested also for a population from the Hohe Tauern Mts. (CIM) but including also two individuals from a population from the Niedere Tauern Mts. (GOR). With an aim to get more detailed insights into the genetic structure within the first two large groupings, we performed a separate PCoA only on that subset of individuals. However, no further resolution or internal structure was obtained (figure not shown).

The unrooted NJ tree (Fig. 5) resolved several clusters, and the groups seen in the ordination formed here separate clusters with high (100 % for the Southern Carpathian populations, 91 % for the N Alpine group) to moderate bootstrap support (64 % for the SW Alpine group, 59 % for the population CIM, incl. two GOR individuals). A certain extent of genetic differentiation, in addition, was suggested also for a population from southern Corsica (LBa) forming a separate cluster, and another population from the Hohe Tauern Mts. (KAT) resolved on a long branch. The parsimony analysis gave trees of length 977 that were congruent to the NJ analysis and thus suggested the same subdivision into genetic groups (figure not shown).

Results of the Bayesian clustering are shown in Fig. 6. As for the STRUCTURE analyses, the assignment of individuals into clusters across replicate runs provided stable results only for $K = 2$. The two groups inferred corresponded to (1) the Southern Carpathian populations, the population CIM, the N Alpine group, the SW Alpine group, and to (2) the group that encompassed remaining populations spanning a large geographic area (Sierra Nevada-Pyrenees-Corsica-Alps). Thus, this division reflects the first axis of PCoA. At higher K we found groups roughly corresponding to the groups along the second and third axes of the PCoA analysis, indicating that there is further substructure in the dataset, but the clustering varied among the replicate runs. BAPS optimal partition estimate showed eight clusters, which, however, differed in their composition from those resolved by STRUCTURE at $K = 8$. Not all individuals sampled in one locality were attributed to the same cluster; several populations appeared heterogeneous (see Fig. 6), as it was found also in the NJ tree (Fig. 5).

Nei's gene diversity (D_{Nei}), Shannon's diversity index (H_{Sh}), and the percentage of polymorphic fragments (P(%)) indicate that low genetic diversity is present mainly in the populations at the edge of the distribution range (Sierra Nevada, Southern Carpathians), as well as in central and eastern parts of the Alps (Table 1, Fig. 1). High diversity, on the other

hand, is concentrated in the SW Alps (Maritime and Ligurian Alps), a few other Alpine populations, and in Andorra. Frequency-down-weighted marker values (DW) expressing population divergence are high in the Sierra Nevada, Southern Carpathians, Central Pyrenees, SW Alps, southern Corsica, and in the Alpine populations SIMP and CIM.

Arctic-alpine dataset

AFLP profiles obtained in *Cardamine bellidifolia* were very distinct from those of *C. resedifolia* and *C. alpina*, and thus the scoring was rather difficult and was done only for one primer combination (*Eco*RI -ATC-(6-FAM) and *Mse*I -CT). Altogether 139 unambiguous fragments were scored in 168 individuals, 13 (9 %) of them were monomorphic. AFLP profiles resolved in *C. bellidifolia* were highly uniform; 37 out of the 40 examined individuals originating from five localities showed identical AFLP multilocus phenotypes, and the other three individuals displayed two different phenotypes that, however, differed by the presence of only one additional fragment. The number of private fragments for *C. bellidifolia*, *C. resedifolia* and *C. alpina* were 20, 26 and 24, respectively. Examination of fragment sharing among the species revealed that there were much more fragments shared by *C. resedifolia* and *C. alpina* than by *C. bellidifolia* and any of the two Alpine species: 39 fragments were uniquely shared by *C. resedifolia* and *C. alpina* (i.e. fragments absent in *C. bellidifolia*); 6 fragments were uniquely shared by *C. bellidifolia* and *C. alpina* (absent in *C. resedifolia*); 4 fragments were uniquely shared by *C. bellidifolia* and *C. resedifolia* (absent in *C. alpina*). Expectedly, the NJ tree (Fig. 7) resolved three well-supported and distinct clusters corresponding to the three species; especially the cluster of *C. bellidifolia* was separated by a very long branch. The parsimony tree (not shown) was of length 276, and had high jackknife support values for the branches corresponding to the three species (100 % for *C. bellidifolia*, 99 % for *C. alpina* and 97 % for *C. resedifolia*.).

Discussion

Many European plant species, which are confined to the alpine belt and prefer specific bedrock, display geographic disjunctions on different spatial scales (Aeschimann et al. 2004). The European high mountain system forms a discontinuous alpine range, thus providing a complex system of habitats that harbour more or less differentiated intraspecific lineages or

distinct species (Comes & Kadereit 2003, Kropf et al. 2006). When considering disjunctions between, as well as within a specific mountain range, both ecological and historical factors are to be taken into account. Studies exploring the origin of infraspecific disjunctions and the phylogeographic history of such species are indeed challenging, as they indicate complex patterns of vicariance and dispersal events associated with Pleistocene glaciations (e.g. Schönswetter et al. 2005a, 2006a, Kropf et al. 2006, Piñeiro et al. 2007). The present study contributes to this topic and exemplifies two contrasting cases of glacial survival and colonization patterns of disjunctly distributed sister species.

Phylogeography of Cardamine alpina *– a deep genetic split between the Pyrenean and Alpine populations*

Populations of the snow-bed species *Cardamine alpina* were resolved in two genetically strongly divergent groups, corresponding to the areas of the Pyrenees and the Alps, respectively. The split between these two mountain ranges explains as much as 88 % of the overall genetic variation, and each group is characterized by a high number of private AFLP fragments. The level of genetic divergence as found here is indeed enormous, so we can conclude that this disjunction is old. Certainly there have been no recent contacts or dispersal events, i.e. populations from the respective areas must have remained isolated, each responding to climatic changes of the last glaciation period independently. Although the percentages of variance expressing differentiation among proposed lineages are not directly comparable across studies and depend also on within-group variation, intraspecific disjunctions observed in other plant species usually displayed much lower genetic differentiation than found here (see e.g. Schönswetter et al. 2004a, Kropf et al. 2006, Ehrich et al. 2007, Mráz et al. in press, Ronikier et al., in press, but see Albach et al. 2006). The high genetic divergence as found in *C. alpina* is rather unusual, and can be expected rather for interspecific comparisons than for intraspecific ones. We might therefore speculate whether the Pyrenean and Alpine populations of *C. alpina* belong to the same taxon (species), but the simple, strongly reduced morphology of *C. alpina* does not provide any clues in this respect. Still, we may hypothesize that the two lineages that we identified represent cryptic biological species. Cases of divergent evolution at the diploid level have been reported in arctic *Draba*, where morphologically indistinguishable conspecific populations showed genomic incompatibilities, and were supposed to represent cryptic biological species (Grundt et al.

2006). In this context it may be worth to explore reproductive isolation in *C. alpina* through crossing experiments between populations from the Alps and Pyrenees.

Phylogeography of Cardamine alpina - *contrasting genetic patterns in the Alps and Pyrenees*

Although the Alpine range of *Cardamine alpina* is much larger and was better sampled than the Pyrenean one, clear differences in genetic diversity and its geographic structuring were observed between these two mountain ranges. Overall diversity as well as within-population diversity was much higher in the Pyrenees than in the Alps (Fig. 1, Table 1). Three distinct lineages, presumably descendants from distinct glacial refugia, were retrieved. The two westernmost geographically adjacent populations were probably derived from the same refugial population; population CAN displayed a subset of genetic variation present in the geographically adjacent PAN population, and thus may be a result of a dispersal event accompanied by the founder effect (Figs. 2, 3A). The remaining two investigated Pyrenean populations were clearly differentiated from each other, and we assume that this is due to the restricted gene exchange caused by the isolation of the respective refugial populations. The pattern observed in the Pyrenean populations favours survival in multiple refugia, a finding that is in line with the fact that the Pyrenees were less glaciated than the Alps (Frenzel et al. 1992), and thus the peripheral areas provided suitable habitats for alpine, snow-bed species such as *C. alpina*. For the present study we had only four populations of *C. alpina* from the Pyrenees at disposal, but denser sampling may provide more detailed assessments of intraspecific genetic patterns and would allow finer inferences on glacial survival and recolonization routes in the Pyrenees. Contrary to the Alps, the Pyrenees have been poorly studied from the phylogeographic perspective at regional scales (see e.g. Segarra-Moragues et al. 2007). Further studies are needed to recover individual phylogeographies of alpine species in this mountain range, and to find common patterns of population survival and postglacial recolonization history.

In contrast to *C. alpina* from the Pyrenees, very low genetic variation and virtually no phylogeographic structure were observed in its much larger Alpine range. Although a few clusters were resolved in the neighbour-joining tree, they were defined on the basis of only a few differentiating fragments, and did not show any reasonable geographic pattern. Identical AFLP profiles were resolved in multiple individuals, found not only within populations, but frequently also in distant populations. Interestingly, a single population from the Maritime Alps (Col de Fenestre, CFn) harboured relatively high diversity, being comparable to the

Pyrenean populations, and importantly, it comprised AFLP phenotypes that were a representative sample of the variation present in the whole Alpine range (Fig. 3B). This population, situated at the SW periphery of the Alps, is apparently located in the area where *C. alpina* may have survived the Würm glaciation and from where it probably migrated and colonized the entire Alpine range. It well coincides with the location of the southwestern-Alpine peripheral refugium that has been supported in several studies on alpine species of siliceous bedrock (Schönswetter et al. 2005b). A few other adjacent populations from this largely unglaciated area were sampled as well, but noticeably all displayed as low variation as found in the central Alps covered by the ice sheet. Even the closest sampled population LFn (a few hundreds meters downwards) is characterized by a single AFLP phenotype that, based on the clustering results (Fig. 2), seems to be a direct descendant of the genetic pool of the population CFn. It appears probable that *C. alpina* survived the last glaciation in a really small refugial area and that the (re-)colonization processes were accompanied by a strong founder effect resulting in an extremely low diversity and no geographic structure across the entire Alps. Similar patterns showing almost no genetic variation in the Alpine distribution range, and colonization from a single glacial refugium, have been reported e.g. in *Androsace brevis* (Schönswetter et al. 2003b) or *Ranunculus pygmaeus* (Schönswetter et al. 2006a). We can speculate about the distribution area of *C. alpina* before the last glaciation period, and thus suggest two possible biogeographic scenaria. This species may have been originally restricted to the SW Alps (except for the Pyrenees), and reached the current distribution in the Alps by relatively rapid eastward migration only in the postglacial period. Recently, it has been shown that even plants lacking adaptations for long-distance dispersal may overcome large distances and spread efficiently (see e.g. Alsos et al. 2007, Piñeiro et al. 2007). Alternatively, *C. alpina* may have had a wider Alpine distribution already before the last glaciation, but subsequently it may have experienced massive population extinctions and survived only in the SW Alps. In the postglacial period it may have spread again and recolonized previously occupied habitats. The area of the SW Alps provides a diversity of high altitude habitats and at the same time it has a favourable location nearby the large area that remained free of ice during the last glacial maximum (Casazza et al. 2007).

Complex phylogeographic history of Cardamine resedifolia – *genetic lineages resolved do not correspond to geographic disjunctions*

The other investigated species, *Cardamine resedifolia*, shows wider distribution area in Europe and has several disjunctions between different mountain ranges (Jalas & Suominen 1994), and thus we expected to find rather diverse phylogeographic patterns throughout its area. If the disjunctions reflected old vicariance, i.e. past area fragmentation and isolation of populations in distinct glacial refugia, genetically differentiated lineages in accordance with their geographic origin would be resolved. Alternatively, if some disjunctions were due to recent dispersal events, low genetic variation caused by founder effects, strong genetic affinity to the source area and little divergence would be detected (Hewitt 1996). The origin and history of the isolated populations at the edge of the current distribution area (the Sierra Nevada, Southern Carpathians, also Corsica) were among the most challenging questions in this study, as well as glacial survival and postglacial recolonization of the larger and continuous areas in the Alps and Pyrenees. At first sight, the genetic lineages resolved in *C. resedifolia* did not correspond to the major geographic areas sampled, and thus we apparently do not face here a simple vicariance scenario (in contrast e.g. to the study of Kropf et al. 2006). Only the Southern Carpathian populations from the Făgăraş Mts. were assigned to a distinct lineage. Very low genetic diversity revealed in the two sampled populations (despite the large population size) may be due to their location at the species area periphery and associated with genetic drift and inbreeding, as suggested e.g. for *Saxifraga cernua* (Kapralov et al. 2006). In fact, habitats suitable for high-alpine species in respect of the altitude (i.e. those above 2000 m) are rather fragmented in the Carpathians (Pawłowski 1970: Figs. 2, 3), and thus the Carpathian populations are probably much more isolated from each other than the Alpine ones. On the other hand, the Carpathians were much less affected by glaciations than the Alps and Pyrenees. Only local glaciers restricted to main mountain ridges were present there, and it is assumed that climatic conditions during the last glacial maximum may have promoted range expansion of alpine and high-mountain plants as well as gene exchange between currently isolated populations, in contrast to the situation in the Alps (Pawłowski 1970, Frenzel et al. 1992). In recent phylogeographic studies on *Hypochaeris uniflora*, considerably higher within-population variation was found in the Carpathians than in the Alps. Populations in the Alps were apparently genetically impoverished through multiple founder effects (Mráz et al., in press). More exhaustive sampling of *C. resedifolia* in the Southern Carpathians and in Bulgarian mountains (Pirin Mts., Rila Mts.; not represented here) is clearly needed to give more details on phylogeographic history of this species in its southeastern range.

On the contrary to the Southern Carpathian populations, neither Corsican populations nor the one from the Sierra Nevada formed distinct lineages, but they were resolved within the widespread genetic group spanning a large geographic area (Figs. 1, 4; Sierra Nevada-Pyrenees-Corsica-Alps). Only one population was sampled in the Sierra Nevada, with all the eight analyzed individuals characterized by the same AFLP phenotype. The population was very small, it consisted of only a few individuals (less than 50) growing under a moist rock. It is questionable whether the lack of genetic variation is just due to the small population size and inbreeding within that particular population, and whether other populations from the Sierra Nevada would display higher genetic diversity. Kropf et al. (2006) recently investigated phylogeography of four alpine species co-occurring in the Sierra Nevada and other high mountains (Pyrenees, Alps, Massif Central). Contrary to the present study, they found clearly differentiated intraspecific lineages corresponding to the mountain ranges, including the one comprising populations from the Sierra Nevada. Considering also palaeoclimatic and palaeoecological data they suggested that the patterns observed can be attributed to vicariance events, i.e. that populations in the Alps, Pyrenees and the Sierra Nevada represent vicariant relicts of ancestral populations widely distributed in intervening areas during the cold periods of the Pleistocene. The existence of cold steppe/tundra occupying the areas between the Pyrenees and Alps, as well as in eastern parts of Spain may have allowed almost continuous distribution of cold-adapted species, which became more fragmented postglacially. The biogeographic scenario as proposed by Kropf et al. (2006) would explain also the existence of the widespread genetic lineage retrieved in *C. resedifolia* (the lineage Sierra Nevada-Pyrenees-Corsica-Alps) with only ambiguous internal structure. *C. resedifolia* is a species occasionally descending to coniferous forests to the altitudes as low as 1700 m (pers. observ.), and thus it may have survived in a system of not strongly fragmented or isolated populations distributed across the eastern Iberian Peninsula from the Sierra Nevada to the Pyrenees, as well as through south-eastern France, south of the Alps, reaching the northern Apennines. It should be noted that *C. resedifolia* currently occupies also the Massif Central (although not included in the present study), and thus at least the contact between the Pyrenees and the W Alps appears very likely. Nevertheless, and contrary to the species studied by Kropf et al. (2006), the current area disjunctions of *C. resedifolia* were not reflected in our AFLP data. We assume that the AFLP markers retrieved here may have extracted a large portion of ancestral AFLP variation for which not enough time elapsed to be sorted into the isolated lineages. Other high-resolution markers (microsatellites, work under

progress) should clarify if our hypotheses presented here can be proved, and may provide more details on the peculiar phylogeographic history of *C. resedifolia*.

There are few clues on the source area(s) for the colonization of Corsica in the present data. Geographically closest are populations in the SW Alps and in the northern Apennines (not sampled here). Since the Mediterranean sea level was considerably lower during the glaciations than today, dispersal from the continent may have been facilitated (Frenzel et al. 1992). The geographic proximity, however, is not decisive. AFLP data of *Bupleurum stellatum* showed that the Corsican population of this species originated via dispersal from source populations in the Eastern Alps and not from the SW Alps (Schönswetter & Tribsch 2005). Considerable genetic diversity in the Corsican populations may suggest that either multiple dispersals occurred (counteracting genetic depauperation due to a founder effect) or that genetic variation was restored in isolation through mutation accumulation.

Hypothethical glacial and postglacial history of Cardamine resedifolia *in the continuous Alpine range*

Cardamine resedifolia has a largely continuous distribution area in the Alps. Twenty-one populations sampled across this range fall into four genetically differentiated groups, although some populations appeared heterogeneous, i.e., contained individuals assigned to different clusters (see below for more discussion). The pattern observed apparently has a geographic component, but its phylogeographic interpretation is not straightforward. Previous phylogeographic studies of Alpine species confined to siliceous bedrock resolved mostly similar patterns, allowing identification of major peripheral refugial areas along the eastern, southern and southwestern border of the Alps (Schönswetter et al. 2005b). Somewhat surprisingly, the genetic structure found in our study does not show much congruence with the known patterns. One of the lineages is formed by populations distributed in the northern siliceous parts of the central Alps (here named as N Alpine group), i.e. in the strongly glaciated central parts of the Alps, and cannot be related to any of the postulated peripheral refugia unequivocally. A plausible explanation may be that the lineage had its source area in a southern peripheral refugium, but the connection had either been disrupted (e.g. the respective genotypes were swamped by another colonization route from the west) or relevant populations were just not sampled. More detailed sampling in this area may give a more conclusive answer. The population at the Simplonpass (SIMP) is geographically rather close to the Penninic and Grajic Alps that were inferred as presumed southern refugia

(Schönswetter et al. 2005b). It shows also increased variation; this is, however, due to the presence of a single genetically divergent individual most likely brought either through dispersal or admixture of two parallel lineages (see below). Otherwise, all those northern populations displayed very low diversity, so neither the patterns of within-population variation do allow indications of a specific colonization route. Another central Alpine population located more to the east but still well within the highly glaciated area (CIM, Hohe Tauern) was recognized as genetically divergent (Fig. 5). The seven AFLP phenotypes identified in the eight sampled individuals were closely related to each other, and apparently are monophyletic. It does not show decreased variation, in fact it displays average-level within-population diversity. We therefore may ask, does this population indicate a case of nunatak survival? Cases documenting *in situ* glacial survival on ice-free mountains above the ice sheet in the central parts of the Alps are really rare (Stehlik 2003, Bettin et al. 2007). *C. resedifolia* does not belong to Alpine species commonly reaching the highest altitudes, and neither it grows in really extreme habitats such as exposed summits or ridges. We therefore doubt if it was able to survive harsh environmental conditions on nunataks. Another finding that should be discussed in this respect is the clustering of the population CIM with the Southern Carpathian populations in the neighbour-joining tree with a high bootstrap support of 95 % (Fig. 5). Even though the Southern Carpathian populations are clearly divergent, as indicated by their long branch, the genetic affinity is apparent. The distinctiveness of the CIM population and the genetic link to the Southern Carpathian populations might point to its origin through long-distance dispersal. Especially in recent years much evidence is accumulating that long-distance dispersal events are not rare, and may have contributed to the present-day distribution patterns to a much larger extent than envisaged (e.g., Schönswetter et al. 2002, Alsos et al. 2007, Piñeiro et al. 2007).

Genetically differentiated are also six populations sampled in the SW Alps (Maritime, Ligurian, Cottic, Grajic Alps), showing substantial diversity both at the within-population level and in the region as a whole. This finding is not surprising at all, since it coincides well with the presence of the southwestern Alpine peripheral refugium in this area, confirmed for several other Alpine species (Schönswetter et al. 2005b, see also Schönswetter & Tribsch 2005, Schönswetter et al. 2004b). The SW Alpine populations appear to have been in contact and partly even intermingled at the population level with the widespread genetic lineage (Sierra Nevada-Pyrenees-Corsica-Alps). The latter lineage represents a more or less continuous gene pool found across the major part of the range of *C. resedifolia*; within the

Alps it was found in the western (Dauphiné Alps), southern as well as the easternmost parts of the Alps. The four populations sampled in the Maritime and Ligurian Alps (part of the SW Alpine lineage) in fact harbour a few genetically divergent individuals attributable to the widespread lineage. The co-occurrence of such divergent AFLP phenotypes can be explained either by the meeting and admixing of different genetic lineages (recolonization routes coming from distinct refugia) or by long-distance dispersal events. The first scenario appears more plausible, since the pattern of population admixture concerns several geographically adjacent populations, and vice versa, AFLP phenotypes from the genetic pool of the SW Alpine lineage were found in one Pyrenean and two Western Alpine populations belonging to the widespread genetic lineage. AFLP phenotypes found in some of such heterogeneous populations are really well distinct (e.g. pop. BRA, SES, CLo), while the other (e.g., MOI, LFn, see Fig. 5) appear in intermediate positions between the main lineages (widespread vs SW Alpine one). Heterogeneity vs. partial homogenization in such populations may be maintained by the mating system (selfing or outcrossing) that prevents or conversely promotes the mixing (homogenization) after arrival of a distinct individual/genotype. Genetically heterogeneous populations, without the presence of intermediate individuals, were reported also in *Comastoma tenellum* (Schönswetter et al. 2004a) or in *Veronica alpina* (Albach et al. 2006). The latter case refers to a genetically clearly distinct group that does not show clear geographic coherence, and hence to certain extent resembles the here distinguished widespread lineage. Despite the partial overlap (genetic and geographic) of the SW Alpine and widespread lineage, it seems that the SW Alpine group emerged in geographic isolation in adjacent refugia, and the meeting of differentiated genotypes occurred postglacially, as a result of postglacial range shifts. Interestingly, there appear to be secondary contacts of the widespread lineage also with the N Alpine one, as manifested by the heterogeneity of two populations at the edge of the area occupied by the latter group (pop. SIMP, PJO, Figs. 1, 6). The patterns of within-population diversity across all sampled populations are thus strongly affected by the heterogeneous nature of such populations being in contact zones, and representing hotspots of genetic diversity.

Comparison between Cardamine resedifolia *and* C. alpina – *similar and contrasting phylogeographic patterns*

The two investigated species, *Cardamine resedifolia* and *C. alpina*, are closest relatives and show two similar features in their current distribution patterns: they both exhibit disjunctions

in their distribution ranges, and their distributions within the Alps and Pyrenees are largely continuous. Despite these similarities and their common ancestry, we detected here highly contrasting phylogeographic patterns. Probably the only resemblance in their glacial and postglacial history regards the area of the SW Alps (especially the Maritime and Ligurian Alps) that harbours much genetic diversity in both species, and apparently has played an important role as a glacial refugium. This is in line with many previous phylogeographic studies, and the finding that this area is a biodiversity hotspot both at the genetic (intraspecific) and species level (endemism). The low impact of glaciations in this area allowed glacial survival of many plants, and also induced population divergence and speciation (Schönswetter et al. 2005b, Casazza et al. 2007). Otherwise, clearly incongruent patterns were found: while clear-cut spatial structure was found in *C. alpina* allowing the inference of a rather straightforward phylogeographic scenario, the glacial and postglacial history of *C. resedifolia* was undoubtedly much more complex. The data suggested the existence of a largely widespread and continuous gene pool along with several geographically more restricted lineages, and also indicated quite common secondary contacts between them. Further investigations are needed, both in respect of additional molecular markers and denser sampling, to characterize historical processes that led to the present-day patterns in *C. resedifolia*. Differences in ecological demands are probably the major factors underlying such distinct patterns, which reflect different responses to climatic oscillations during the Pleistocene. While *C. resedifolia* as a species with a broader ecological amplitude may have found favourable habitats across relatively large areas at the periphery of mountain ranges as well as in interconnecting areas, *C. alpina* must have been much more restricted.

Genetic diversity in populations is affected not only by historical (population isolation, bottleneck, founder effect), but also by contemporary factors (breeding system, genetic drift). AFLP data of *C. alpina* support our assumption that this is a predominantly selfing species. A very low percentage of the overall AFLP variation was attributed to the within-population component. Even within the relatively more variable Pyrenean populations, identical AFLP phenotypes were found in each of the sampled population. We therefore assume that the low genetic variation observed is not only due to bottlenecks experienced by populations during postglacial recolonizations, but also due to autogamy. Selfing is supported also for *C. resedifolia*, but at least a low level of outcrossing is suggested as well, considering the presence of genetically intermediate individuals found in populations located in the contact

zones of the inferred genetic lineages. Further detailed studies employing also co-dominant markers would be desirable to address these questions more precisely.

The arctic-alpine relationships – a distinct position of the circumpolar Cardamine bellidifolia *and lack of variation in the north*

Strong morphological resemblance of the circumpolar *Cardamine bellidifolia* to the Alpine-Pyrenean *C. alpina* suggested their phylogenetic relatedness, and this was reflected also in taxonomic treatments by several authors who recognized them at the subspecific levels (e.g. Jalas & Suominen 1994, Elven et al. 2006). Recent molecular phylogenetic analyses based on DNA sequencing supported the view that these two species, together with *C. resedifolia*, represent closely related taxa (Carlsen et al., submitted). Our AFLP data have brought two striking findings for *C. bellidifolia* – its placement as a very divergent lineage well separated from the accessions from Central and southern Europe, and its extremely low genetic variation. The strong divergence observed for the arctic populations excludes the scenario of their recent postglacial immigration from the Alps or Pyrenees, as it was suggested e.g. for *Ranunculus glacialis* (Schönswetter et al. 2003a). Neither it supports past continuous distribution in the area between the Alpine and Scandinavian ice sheets, its postglacial disruption and migration northwards and southwards (to the Alps) as suggested for *Minuartia biflora* (Schönswetter et al. 2006a). Thus, a recent, postglacial speciation event of the lineage of *C. alpina* and *C. bellidifolia* is not supported by our data, and the pattern found contradicts also the subspecific taxonomic concept. Postglacial colonization of the arctic areas must have occurred from other source areas than Central or southern Europe, putatively located in Eastern Europe, Asia or in North America (Abbott & Brochmann 2003). The second finding, the lack of genetic diversity in *C. bellidifolia*, is not surprising. The very low genetic variation in the formerly glaciated areas in the north was already reported for other plant taxa, e.g. for *Ranunculus glacialis*, *R. pygmaeus*, and *Arabis alpina* (Schönswetter et al. 2003a, 2006a, Ehrich et al. 2007). Such patterns are caused by strong founder effects and genetic drift experienced during the recolonization process. More detailed sampling of *C. bellidifolia* throughout its distribution and employment of other markers (microsatellites, Carlsen et al., in prep.) are needed to unravel the origin, glacial and postglacial history of this arctic species.

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Figure legends:

Fig. 1. Sampled populations of *Cardamine resedifolia* and *C. alpina*. List of populations along with the abbreviations used is given in Table 1. Symbol colours designate genetic groups as resolved by AFLP data: five tentative groups in *C. resedifolia* and two in *C. alpina*. Within-population genetic diversity (expressed by Nei's gene diversity and the percentage of polymorphic loci) is indicated by the symbol size. Two-colour symbols on the map of *C. resedifolia* indicate genetically heterogeneous populations, with the colour proportion corresponding to the number of individuals attributed to the respective lineages.

Fig. 2. Midpoint-rooted neighbour-joining tree based on AFLP data of 203 individuals (27 populations) of *Cardamine alpina*. Numbers above branches indicate bootstrap values. Individual labels include population abbreviation (see Table 1) and a unique accession number.

Fig. 3. **A**. Principal coordinate analysis based on AFLP data of 203 individuals (27 populations) of *Cardamine alpina*. Symbols designate geographic origins of the accessions (see Table 1): Alps (circles), Pyrenean pop. PESS (stars), MOL (hearts), PAN (spades), CAN (cubes). The first three axes extract 62.21 %, 7.01 % and 5.65 % of total variation. **B**. Principal coordinate analysis based on AFLP data of 176 individuals (23 populations) of *Cardamine alpina* from the Alps. Individuals from the population CFn (see Table 1), harbouring large genetic diversity, are highlighted. The first three axes extract 22.07 %, 20.85 % and 12.48 % of total variation.

Fig. 4. Principal coordinate analysis based on AFLP data of 247 individuals (33 populations) of *Cardamine resedifolia.* Colours designate five genetic groups as recognized here in the ordination space: N Alpine group (green), SW Alpine group (red), Southern Carpathian populations (black), a population from the Hohe Tauern Mts. (CIM, yellow), and the remaining populations (blue). The first three axes extract 20.22 %, 10.56 % and 8.02 % of total variation.

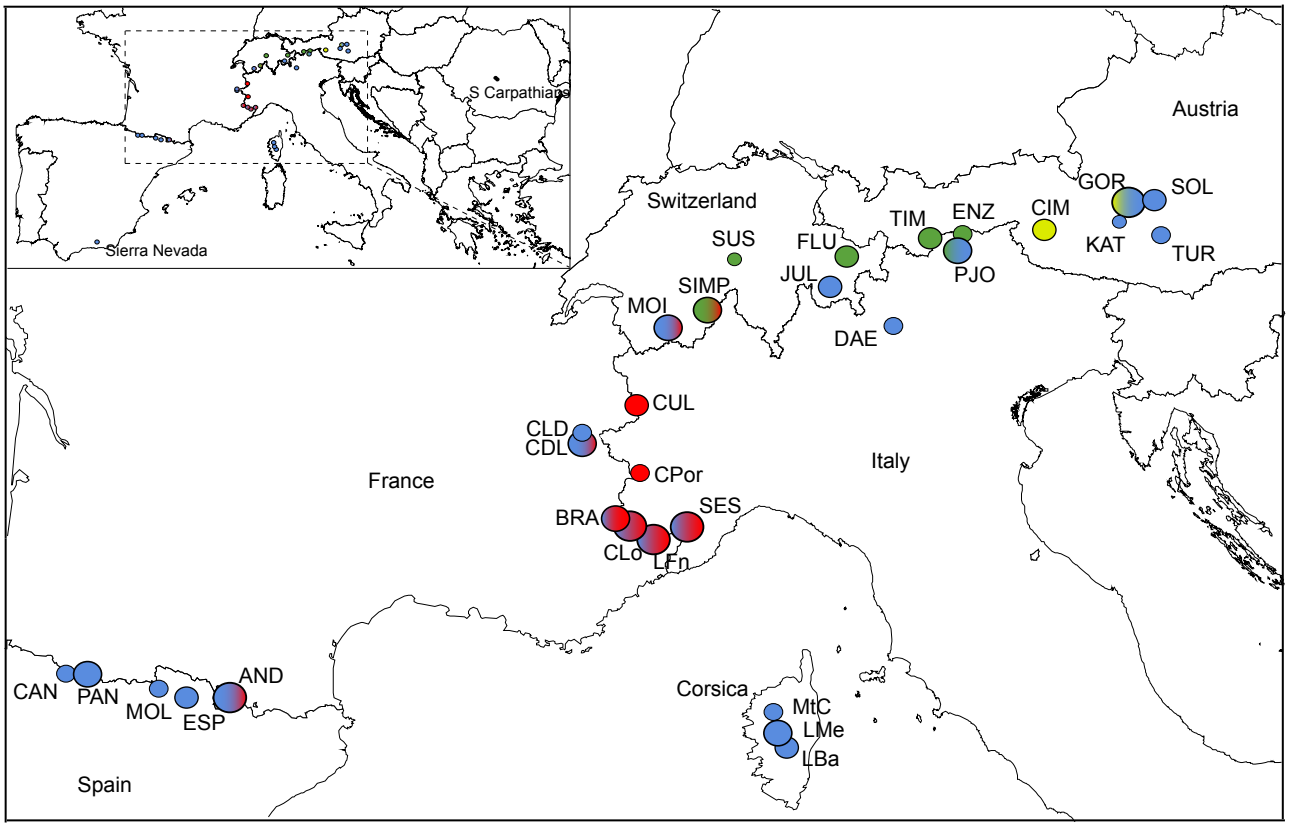
Fig. 5. Unrooted neighbour-joining tree based on AFLP data of 247 individuals (33 populations) of *Cardamine resedifolia*. Clusters corresponding to genetically coherent groups as suggested by PCoA are encircled, including their bootstrap values: N Alpine group, SW

Alpine group, Southern Carpathian populations, a population from the Hohe Tauern Mts. (CIM), and the remaining populations.

Fig. 6. Genetic structure of *Cardamine resedifolia* (247 individuals from 33 populations) as resolved by Bayesian clustering of AFLP phenotypes, based on algorithms implemented in STRUCTURE and BAPS. Each individual is represented by a vertical bar, which is coloured according to its assignment to a respective group. The upper row shows assignment into two groups as estimated by STRUCTURE (the only stable assignment); the lower row corresponds to the most probable partition obtained by BAPS. Population abbreviations follow Table 1; their geographic origin is indicated in the uppermost row (SNe means Sierra Nevada; S Carp. means Southern Carpathians).

Fig. 7. Unrooted neighbour-joining tree based on AFLP data of *Cardamine bellidifolia* (40 individuals from five populations), and a selection of *C. resedifolia* (69 individuals from nine populations) and *C. alpina* (59 individuals from eight populations) accessions. The tree is based on 139 AFLP fragments generated by one primer combination (*Eco*RI -ATC-(6-FAM) and *Mse*I -CT). Bootstrap values are indicated for the three main clusters, and two subclusters of *C. alpina*.

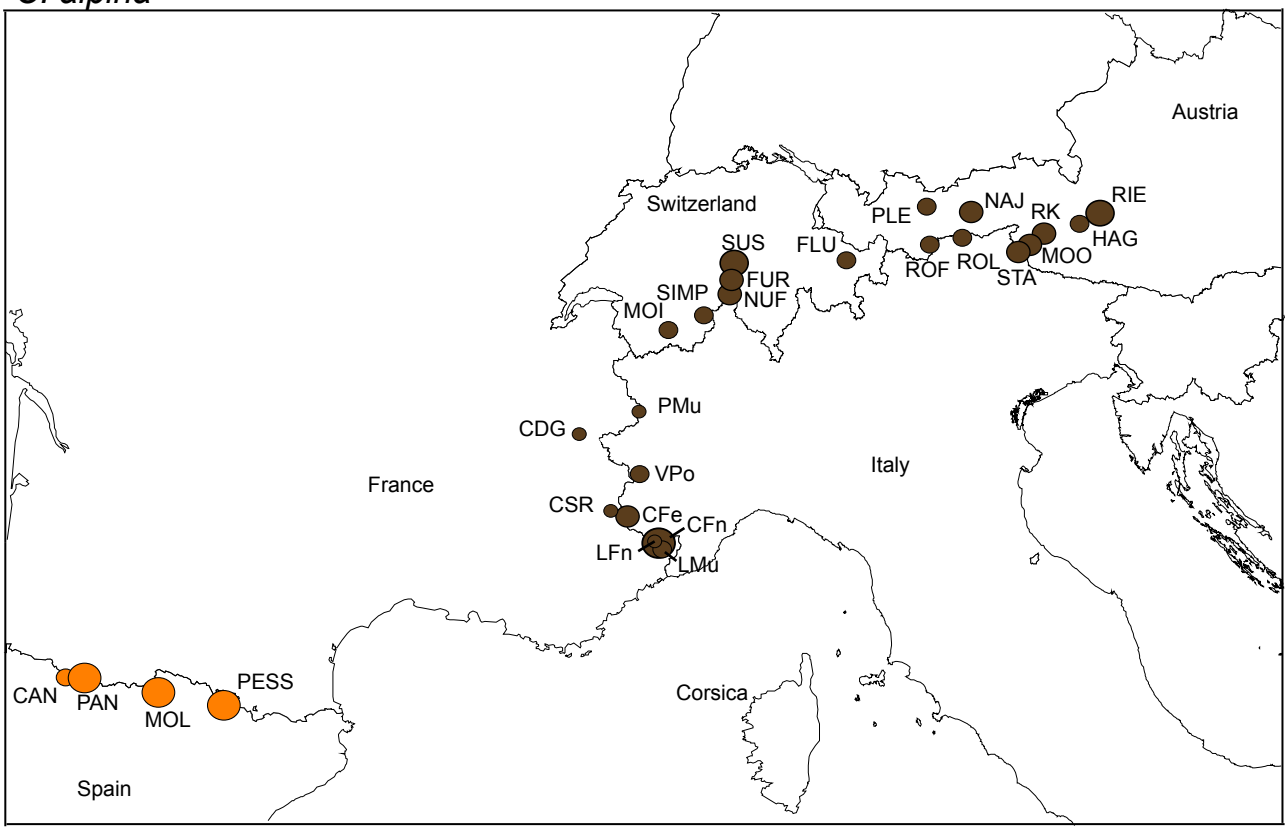
C. resedifolia



◯ DNEI=0.08-0.12; P(%)=20-25 ◯ DNEI=0.04-0.05; P(%)=10-12 ○ DNEI=0-0.01; P(%)=0-3

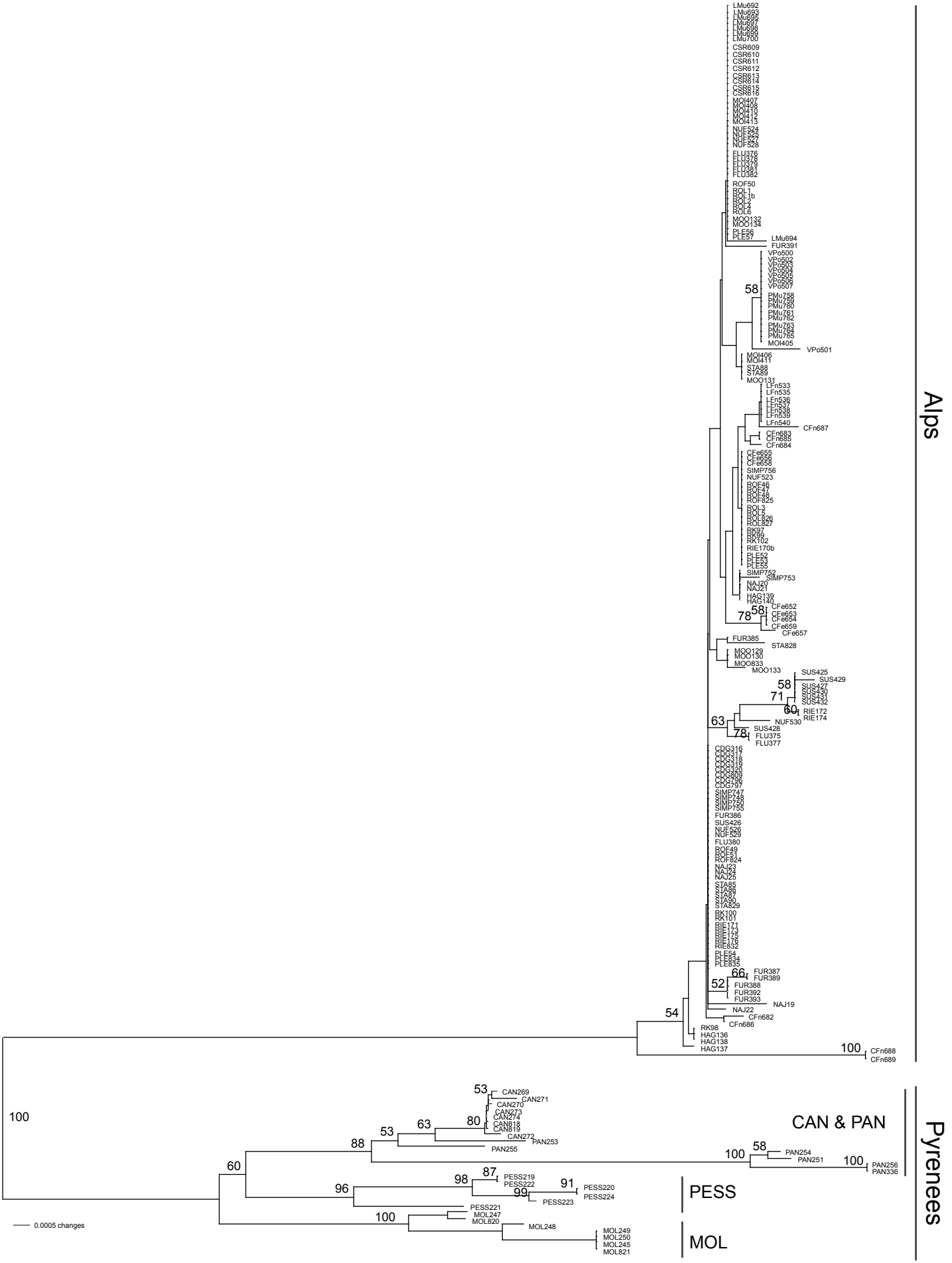
◯ DNEI=0.05-0.07; P(%)=14-21 ○ DNEI=0.01-0.03; P(%)=4-10

C. alpina

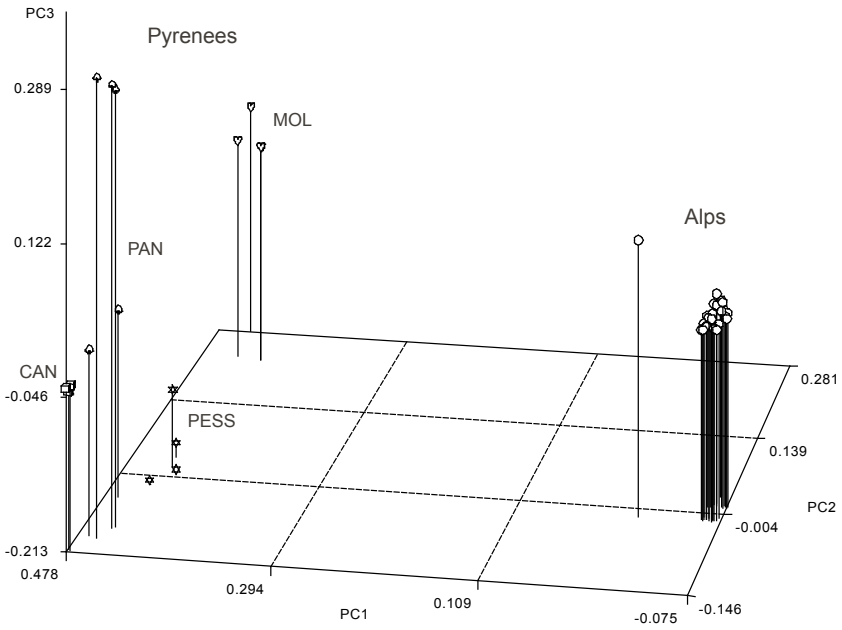


◯ DNEI=0.03-0.08; P(%)=6.7-16.5 ◯ DNEI=0.006-0.008; P(%)=1.3-2.2 ○ DNEI=0; P(%)=0

◯ DNEI=0.011; P(%)=2.6-3.1 ○ DNEI=0.002-0.005; P(%)=0.4-1.3


Alps
Pyrenees
CAN & PAN
PESS
MOL
0.0005 changes

A


PC3
Pyrenees
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MOL
Alps
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PAN
CAN
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PESS
0.281
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PC2
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-0.213
0.478
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PC1
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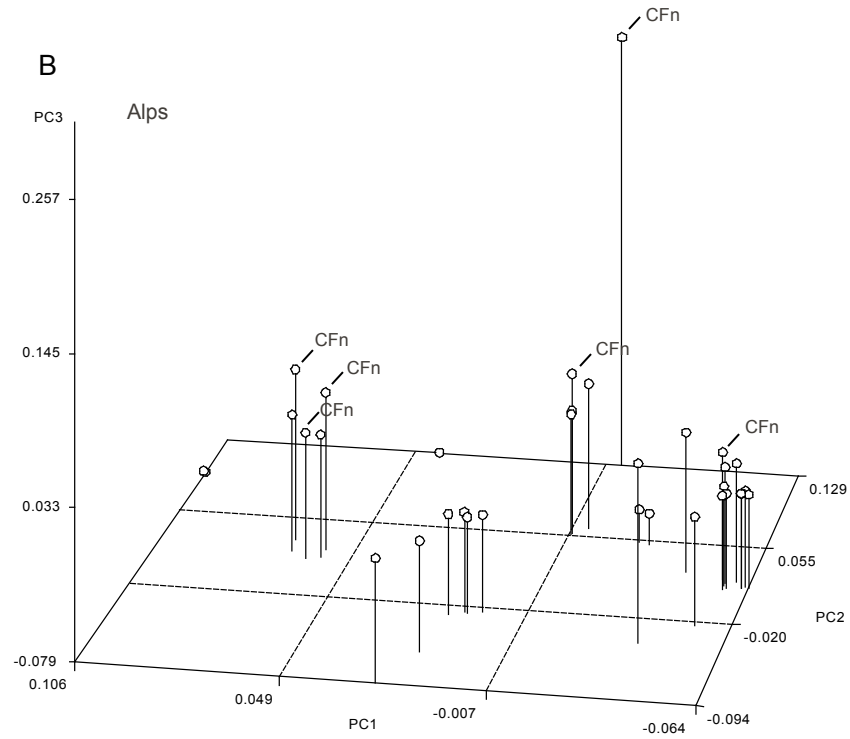
-0.075
-0.146

B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PC3
Alps
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0.145
CFn
CFn
CFn
CFn
CFn
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0.129
0.055
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-0.079
0.106
0.049
PC1
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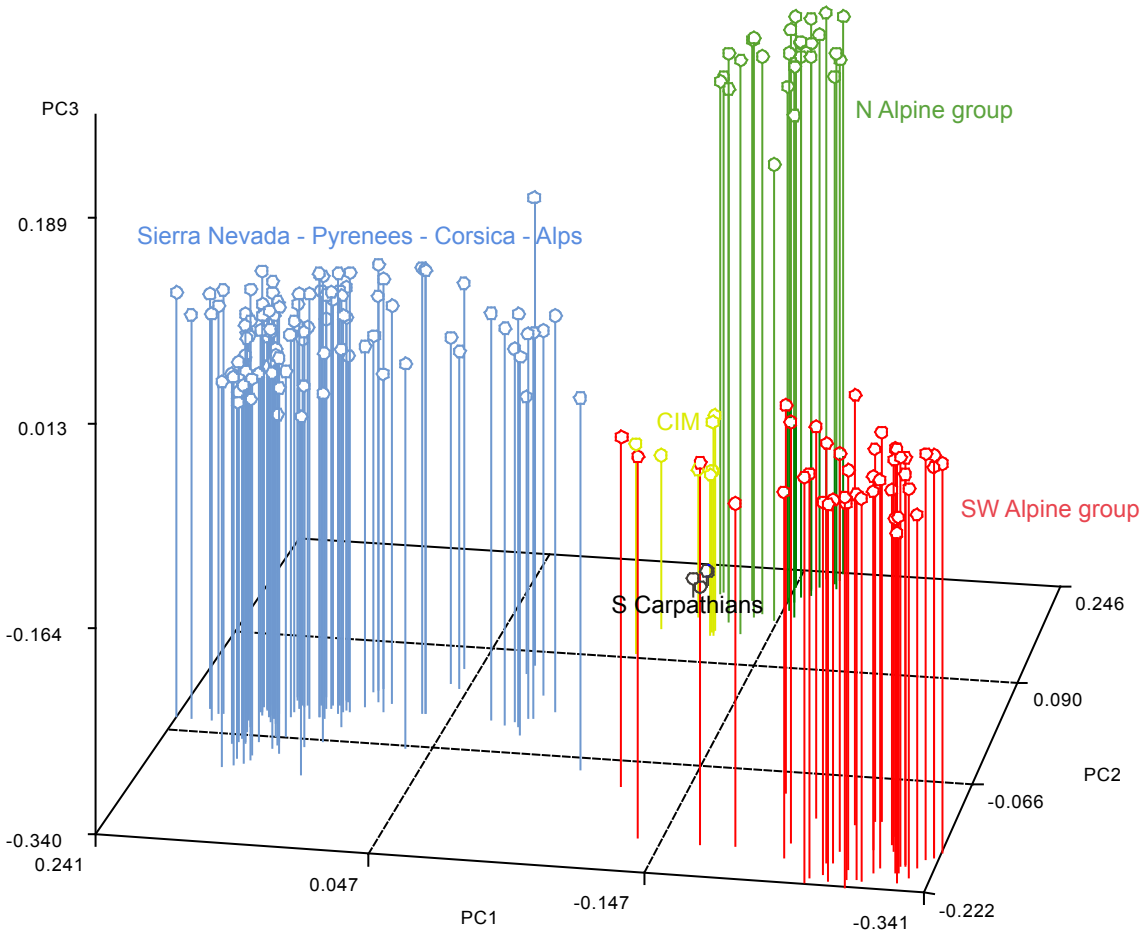
-0.064
-0.094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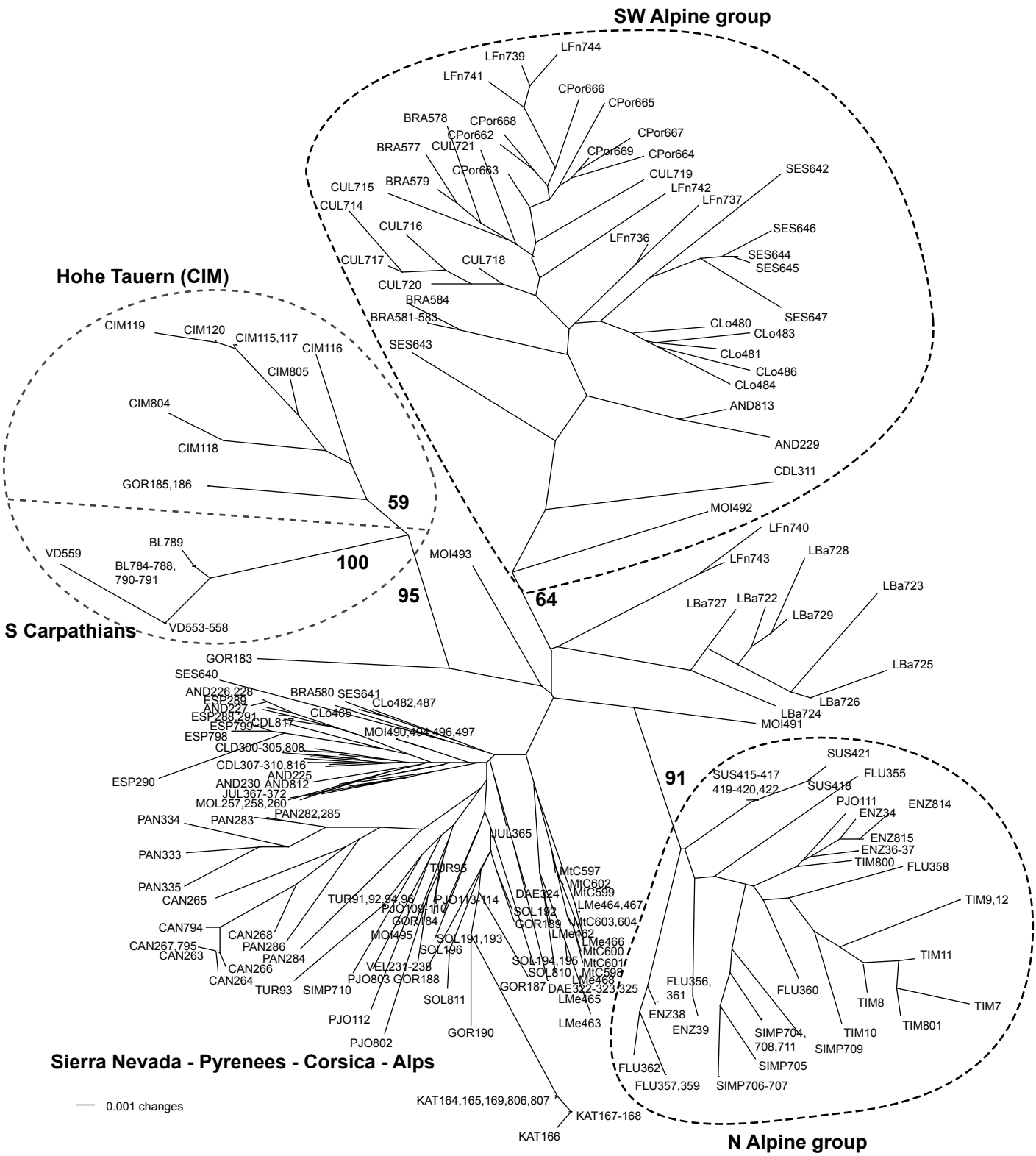
-0.340
0.241
0.047
-0.147
-0.341
PC1
0.246
0.090
-0.066
-0.222
PC2
Sierra Nevada - Pyrenees - Corsica - Alps
N Alpine group
CIM
SW Alpine group
S Carpathians


SW Alpine group
Hohe Tauern (CIM)
S Carpathians
Sierra Nevada - Pyrenees - Corsica - Alps
N Alpine group
0.001 changes
59
100
95
64
91
LFn744
LFn739
LFn741
CPor666
CPor665
BRA578
CPor668
CPor662
CPor667
BRA577
CUL721
CPor669
CPor664
CPor663
CUL719
SES642
CUL715
BRA579
LFn742
LFn737
CUL714
CUL716
SES646
LFn736
SES644
SES645
CUL717
CUL718
CUL720
BRA584
SES647
BRA581-583
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CLo483
SES643
CLo481
CLo486
CLo484
AND813
AND229
CDL311
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LFn743
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LBa723
LBa727
LBa722
LBa729
LBa725
LBa726
LBa724
MOI491
MOI493
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CIM120
CIM115,117
CIM116
CIM805
CIM804
CIM118
GOR185,186
BL789
VD559
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790-791
VD553-558
GOR183
SES640
AND226,228
ESP289
AND227
ESP288,291
ESP799
ESP798
ESP290
BRA580
SES641
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CLD300-305,808
CDL307-310,816
AND225
AND230
AND812
JUL367-372
MOL257,258,260
PAN282,285
PAN334
PAN283
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CAN265
CAN794
CAN268
CAN267,795
CAN263
PAN286
PAN284
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TUR93
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GOR188
VEL231-238
MOI495
SOL196
TUR95
TUR91,92,94,96
PJO109,110
PJO113-114
GOR184
SOL191,193
SOL811
GOR190
JUL365
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SOL194,195
SOL810
GOR187
DAE322-323,325
MtC597
MtC602
MtC599
LMe464,467
MtC603,604
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MtC601
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LMe465
LMe463
KAT164,165,169,806,807
KAT167-168
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SUS421
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FLU355
PJO111
ENZ814
ENZ34
ENZ815
ENZ36-37
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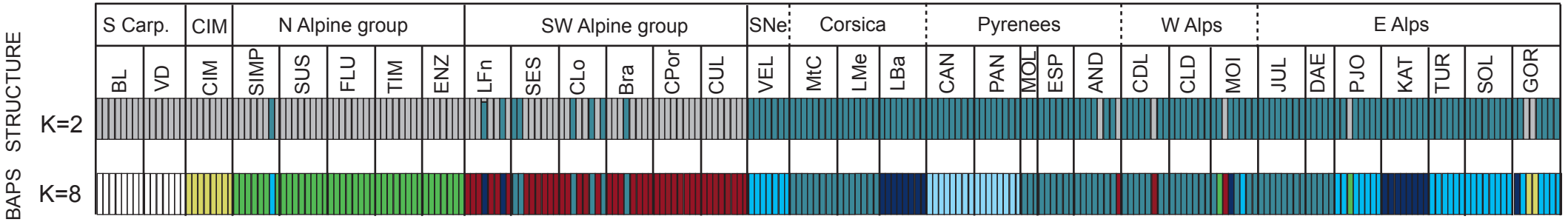
BAPS STRUCTURE
K=2
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S Carp.
CIM
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SW Alpine group
SNe
Corsica
Pyrenees
W Alps
E Alps
BL
VD
CIM
SIMP
SUS
FLU
TIM
ENZ
LFn
SES
CLo
Bra
CPor
CUL
VEL
MtC
LMe
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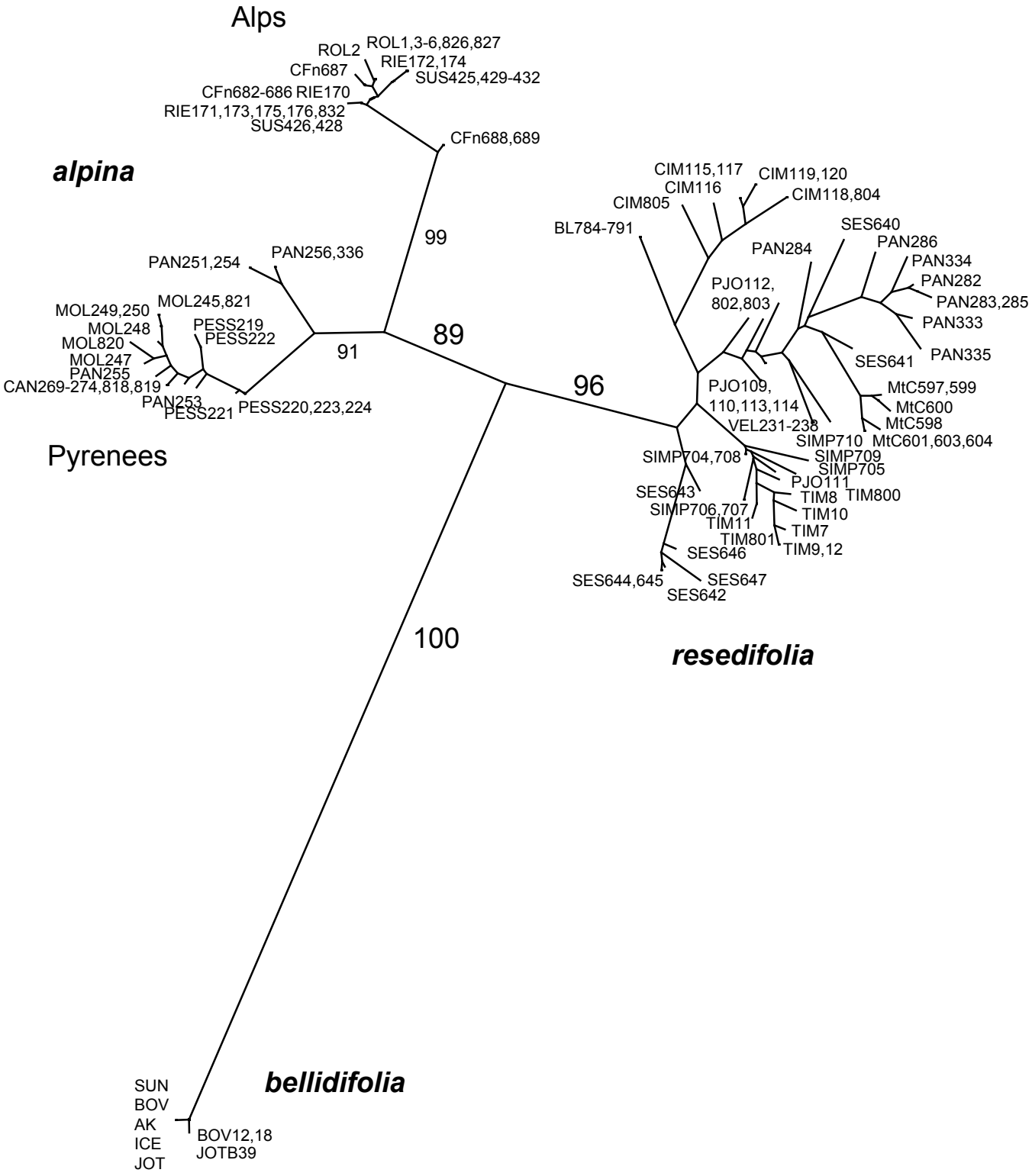
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CFn687
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SUS426,428
CFn688,689
alpina
99
CIM115,117
CIM116
CIM119,120
CIM118,804
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TIM10
TIM11
TIM7
TIM801
TIM9,12
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SES647
SES642
100
resedifolia
SUN
BOV
AK
ICE
JOT
bellidifolia
BOV12,18
JOTB39

PAPER V

Microsatellites for three distantly related genera in the Brassicaceae

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Key words: SSR, *Draba*, *Smelowskia*, *Cardamine,* cross-species transfer, cross-genus transfer

Running title: Microsatellites for Brassicaceae

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Abstract

Microsatellites are important genetic markers both in population genetics and for delimitation of closely related species. However, to develop microsatellites for each target organism is expensive and time consuming. In this study, we have therefore developed 71 new microsatellite primers for the genus *Draba* and tested cross-species and cross-genus transfer success of these primers for two other genera in the Brassicaceae; *Cardamine* and *Smelowskia*. Furthermore, 15 previously developed microsatellites were tested for amplification in these three genera. The microsatellite markers that amplify across these genera may be useful for other genera in the Brassicaceae as well.

Microsatellite markers are popular tools for studies in population genetics, molecular ecology and systematics. However, it is expensive and time consuming to develop these markers for each study organism. Thus, it is desirable if they can be transferred across species and genera. Barbará *et al*. (2007) reviewed 64 microsatellite primer notes published the last ten years and found a transfer success in eudicot plants of 60% and 10% in cross-species and cross-genus tests within families, respectively. In the Brassicaceae, cross-species transfer rate of microsatellites for *Arabidopsis* (DC.) Heynh. and *Brassica* L. has been high; 43-100% among *Arabidopsis* species, and 65-90% among *Brassica* species (Clauss *et al*. 2002; Lowe *et al*. 2002; Suwabe *et al*. 2002; Van Treuren *et al*. 1997). Clauss *et al*. (2002) tested *Arabidopsis* microsatellites for cross-genus transfer to the closely related *Boechera* A. Löve & D. Löve and found a success rate of 79%. Tests of amplification of microsatellite loci among genera that are more distantly related than *Arabidopsis* and *Boechera* have also been performed, with varying success, e.g. few markers amplified microsatellites in both *Brassica* and *Arabidopsis* in a study by Westman and Kresovich (1998).

A cross-transfer approach was used in this study to find microsatellite markers for three genera in this family: *Draba* L., *Smelowskia* C.A. Mey and *Cardamine* L. Of the three main clades found in a family-scale phylogenetic study of the Brassicaceae, *Cardamine* and *Smelowskia* were placed in the same clade as *Arabidopsis*, while *Draba* was placed in the same clade as *Brassica* (Beilstein *et al*. 2006). However, none of these genera were closely related to each other within the clades. Here we developed microsatellite primers for *Draba nivalis* and tested them for amplification and variation within all three of our target genera. In addition, we tested primers previously developed for *Arabidopsis* and *Brassica* for the same genera.

Four microsatellite enriched libraries (CA, GA, AAG and CAG) were produced by GIS (Genetic Identification Services, Chatsworth, CA) using genomic DNA from *Draba*

nivalis. GIS then ligated restricted DNA, enriched for a microsatellite motif, into the BamH1 cut site of a pUC19 plasmid. The recombinant plasmids were then electroporated into *E. coli* strain DH5∀. Glycerol was added after recovery incubation in SOC broth. To isolate colonies for sequencing, cells from the glycerol stock were spread on X-gal/IPTG/ampicillin-LB agar plates. The colonies were sequenced using the primers A: 5'- AGGAAACAGCTATGACCATG -3' and B: 5'- ACGACGTTGTAAAACGACGG -3'. Some colonies were processed by GIS and others were processed by the authors using the following PCR conditions: 0.5 μM dNTP, 2.5 μM $MgCl_2$, Taq buffer, 0.4 units Taq enzyme, 0.25 μM primer A and B and H_2O to a total volume of 10 μL. Cycling was carried out as follows: 95 °C for 2 min; 25 times 94 °C for 30 sec, 57 °C for 30 sec, 72 °C for 30 sec; and 2 min extension. The products were purified with 10x diluted ExoSAP-IT (USB Corporation, Cleveland, Ohio, USA) before cycle sequencing with 10x diluted BigDye (Applied Biosystems, Foster City, California, USA) in 25 cycles of 10 sec at 96 ºC, 5 sec at 50 ºC, and 240 sec at 60 ºC. The samples were then cleaned using Sephadex (GE Healthcare, Buckinghamshire, U.K.) before they were added formamide and analyzed on an ABI 3100 or ABI 3700 DNA Sequencer (Applied Biosystems, Foster City, California, USA).

Primers were partly designed by GIS using DesignerPCR, version 1.03 (Research Genetics, Inc.), and partly by the authors using a combination of Primer3 (http://frodo.wi.mit.edu/) and by manually choosing primers from the sequences. All primers were amplified with 0.5 μM dNTP, 2.5 μM $MgCl_2$, Taq buffer, 0.4 units Taq enzyme, 0.2 μM reverse primer, 0.04 μM forward primer with M13 oligo, 0.2 μM labeled M13 primer (5'-CACGACGTTGTAAAACGAC-3') and 1.0 μL diluted DNA template in a 10 μL solution. Details of the different cycling conditions can be found in Table 1. Formamide and the size standard GeneScan 500 LIZ (Applied Biosystems, Foster City, USA) were added to the samples, which were genotyped on an ABI 3100 or ABI 3700 DNA Sequencer.

All loci were tested for variation between two closely related species of each genus; *Smelowskia porsildii* and *S. borealis*, *Cardamine blaisdellii* and *C. digitata*, and *Draba nivalis* and *D. fladnizensis*. Both *Cardamine* species in this study are polyploid and may therefore have more than two fragments. For *Draba*, the loci were also tested for variation between two geographically distant (Alaska and Norway) individuals of *D. nivalis*. The previously developed primers were initially selected from microsatellite markers that had amplified for several *Arabidopsis* species, for *Arabidopsis* and *Brassica*, or for several *Brassica* species. Many primers were tested, but only primers that amplified for at least one of the species are published here because of possible errors in the PCR protocol (a list of primers that did not amplify in the present study are available upon request).

Seventy-one of the markers developed from the microsatellite library amplified in *D. nivalis*, and were tested for variation within *D. nivalis*, and among the other species. We found that 50 of these loci were variable within *D. nivalis* (when including two loci from marker DnA123; Table 1). Sixty-eight loci were variable between *D. nivalis* and *D. fladnizensis*; 97% cross-species transfer. Twelve of the primers designed for *Draba* amplified and gave variable fragment lengths between species in *Cardamine*; 17% cross-genus transfer. For *Smelowskia* four loci amplified, and two of these showed variation between the two *Smelowskia* species; 6% cross-genus transfer.

Five of the 15 previously published microsatellite loci amplified for all species in all genera. None were variable within *D. nivalis*, seven were variable within *Draba*, eight were variable within *Smelowskia,* and six were variable within *Cardamine* (Table 1).

We thus found several microsatellite primers that are well suited for studies in three genera of the Brassicaceae. Rates of cross-genus transfer are similar to those reported by Barbará *et al*. (2007). However, the cross-species transfer rate, while similar to previous reports for the Brassiaceae (Clauss *et al*. 2002; Suwabe *et al*. 2002), are higher than for many

other plant groups. We observed that primers amplifying in more than one genus often amplified in all three genera, suggesting that amplification in other genera of the family can be expected. Many of the loci may be suitable for population studies because they display variation within species, while other are less variable and may be better suited for studies of interspecific variation.

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9

Table 1: Characterization of 87 polymorphic microsatellite markers in three genera of the Brassicaceae; *Cardamine*, *Draba*, and *Smelowskia*. Fragment lengths are based on one individual of each species, except for *D. nivalis,* where two individuals were tested. The microsatellite markers are named according to motif; DnA = CA, DnB = GA, DnC = AAG and DnD = CAG. "-" indicates no PCR product, or nonspecific products. "gel" indicates that PCR fragments have been visualized on an agarose gel, but the exact fragment length is unknown

Marker names	PCR conditions[1]	Forward primer	Reverse primer	Fragment length (bp)					
				D. nivalis	*D. fladnizensis*	*C. blaisdellii*	*C. digitata*	*S. porsildii*	*S. borealis*
DnA112	TD48	GGCGTTACAACAGAAATTC	GTCTATTCAGAAAGCCGTATAG	271, 273	269, 279	-	-	-	-
DnA115	TD48	ATTTCCCAAGAATGTGTTTACG	ATGAAGGAGCAGGTAGCATCTA	155, 173	130, 134	-	-	-	-
DnA117	TD48	TTGTATTCATCGGTTGTGTATC	ACCTGGAAGCACTGGTTC	271, 277	265, 267	224	219, 227	-	-
DnA118	TD48	TTACCGGAAGCGTAGAAA	ACGCACACACATTCACAAATC	225	219, 229	-	-	-	-
DnA119N	TD48	GCCTCTATCGCTAGAGATTTACACG	CCTGTGACGGATATTGGGACG	261, 266	-	-	-	-	-
DnA123[9]	TD48	TCATCCTTCATCATCACACC	GAGAGAGACGGGATTCACTC	178, 182; 201, 215	-	-	-	-	-
DnA124	TD48	GCAAACCCAGATAAACAAATTC	TCGGATGATGAAGATGATAATG	278	276, 280	-	-	-	-
DnA127	TD45	GTCCCGTGACTTGCTCTC	GGAAGATCTGATGCCAGAGAAGG	129, 175	-	-	-	-	-
DnA131	SSR55	TCATGGACTCACATCTAATCC	AGACGGTGGTTCTCTCGA	144	146	-	-	-	-
DnA136	SSR55	GGACGGTGGTTTCCTCTATAGTGC	CGACCTCAATCCCGAAACACG	267	261, 267	-	-	-	-

DnA138	TD48	CTTCCTGCGACATCACTCAAAC	TACGGATTGGAGAGAATTCTGAGC	238, 255	214, 218	162, 164	164	-	-
DnA201	TD48	CCACAACCCTCTTTATTAAATTACC	CCAATGACCGTTTTGGTGAT	165, 169	165	-	-	-	-
DnA202	TD48	ATCCTGAACACACACACAC	ACTATTCCTTTCATGGCGTG	145, 151	157	-	-	-	-
DnA206	TD48	GCCACAACCCTTCTTTATTAAATTACC	CACAACTGGAACCATTGACG	226, 228	226, 228	-	-	-	-
DnA207	TD48	TACACACGCGTTTTCAGAC	GGGACGGTGGTTTCCTTTAT	180	162, 180	-	-	-	-
DnA208	TD48	GATGCAAACTCGAACTCCTTG	TCAAGTTTTCCAAGCTCAATTTC	230, 254	230, 240	-	-	-	-
DnA210	TD48	TGCACACAAGCATCAAAACA	AAGAGGGTCTTAGGCGGATA	180, 346	346	-	-	-	-
DnA213	TD48	GTGGCAATTTGCTTCCAACC	GGGATCAGGTTTGAGGATGA	228, 243	240, 246	-	-	-	-
DnA214	TD48	CCTCACATTGTCTCTGATACAC	GCTCGAAGAAGCGATATTGG	214, 223	200, 216	178, 180	181, 183	-	-
DnA218	TD48	CGGATTGGAGAGAATTCAGAG	CCTTTTCTTCCTGCGACATC	245, 263	253	251	253, 255	-	-
DnA219	TD48	CCCATGAGATGAAAGTGAAGAGTAGG	ATGGACAACTCCGTGTAGGC	246, 262	252	-	-	-	-
DnA222	TD48	GTGGCAATTTGCTTCCAACC	GCGCAGTGAGATGGATTTCTGG	156, 160	-	134, 146	141, 149	gel	150
DnA8	TD48	CTTTGGTGGTCTTCCTTG	ATACGATTCCGAGTATTACCTC	gel	gel	214	202, 216	-	-
DnA9	TD48	TCTTGCCTTTTGACTTTTG	CAAATCTGTCTCACATCTCTC	203, 214	207, 216	-	-	-	-
DnB10	TD48	GTTCTTCTTGTTGGAGAGACAA	CTTGCTGAAACCAATAATCTTG	178, 198	-	-	-	-	-
DnB101	TD48	TGGCTTACCATTGCTGTCC	CCGCATTGTGTTGTTCTTG	239, 267	227, 241	203, 221	205, 225	gel	211, 251
DnB105	SSR55	CGAAACACTTGCCCTACGA	ACCGATTCTTCAACCTCACC	207	197, 199	-	-	-	-

DnB106	TD48	TGCGCGCAGAGACAAAGGAG	GAATCCGCCATAGCCGAGGTTG	309, 321	272, 292	178, 194, 220	178, 194, 220	-	-
DnB107	SSR56	GAGGTAAACTCGTGAAGTCTTG	CGAAGTGATGGGACAGATAA	129, 175	-	-	-	-	-
DnB109N	TD48	GAGCCCTATCAGACTGGC	CAAGAACGGAAAAGAAGTATGT	193, 217	181	-	-	-	-
DnB112	TD48	TGGGTACAGAAGAGAGAAAGTC	GGCTTCAATGCTTTAGATGTAG	275, 284	-	-	-	-	-
DnB116	TD48	GAGGAAAAGAGGCAGAAAAG	GCTAAAGGGACTTGATGAGC	292, 294	266, 284	-	-	-	-
DnB123a	TD48	CAGTGCAAAATGCGTGAAT	GCGTGGAGATAGAGAAAGAGC	154, 170	154, 160	130, 138, 148	133, 138, 148	-	-
DnB134	TD48	GCTTCATCCGACGATTTCTC	CGAGCGAGCCTAATGTTTTC	231, 235	-	-	-	-	-
DnB140	SSR58	GCTATTTATGTTGGCGTGTTG	TATCGCTGCTTCCACTAACC	305, 317	-	-	-	-	-
DnB2	TD48	ATGTTGATGAGCCCAAGTC	CGCCGATTTACAGTTACAAG	167, 175	161, 163	-	-	-	-
DnB200	TD48	AGGAGGGATGCTCCTCAGAT	CGGCGAAGTGATGGGACAG	144	144	-	-	-	-
DnB201	TD48	CAAATGAATTCGGGCCTAGA	TCCGGTATGCTTGTTGTTTG	251, 271	245, 259	-	-	-	-
DnB202	TD48	GGTCACGGCTTGATCCTTC	TCTTCACGAGGAACATAAACTTCTC	128, 131	-	-	-	-	-
DnB203	TD48	AAAAAGTAACCTGCGCATCAC	GAGCTCCATGTCGATCTTCC	233, 241	-	-	-	-	-
DnB204	TD48	CCCAAGACAAAATCAGAAAAGG	TCAACGTGTTGAGTGTCAATTC	253	237	-	-	-	-
DnB206	TD48	GAGGAAAAGAGGCAGAAAAGC	TGCTGACACAATCGTTTTACG	141, 170	141, 164	-	-	-	-
DnB207	TD48	GGACGGCTGCATTTTCAC	TCAGCTTCACACCAAACAATTC	241, 258	222, 229	227	229	213	225
DnB209	TD48	GTTACTTCTTCAGTCGCTTG	AGGAAAATTAGAGTCCATCACG	228	222	-	-	-	-

DnB210	TD48	CGAGAAAGAGAGCTGGAAAAC	TTCAGGATGCTTGCTCTTTG	216, 224	236	-	-	-	-
DnB212	TD48	GACCACAACATGTCAAAACCAAC	TCTTCTCACCGGAGACTTGC	205, 233	221	-	-	-	-
DnB214	TD48	TTCGTCTTCTTGAGCACTGG	CGGAATTCAACCCCAATAGC	205, 231	191, 205	-	-	-	-
DnB216	TD48	TCGACGATCGGAGACAGG	CGGCAAAAGATGATTTCCTCGTG	144, 155	-	-	-	-	-
DnB219	SSR52	TGCATCCATTAAACCCTAAAGC	AATCCGCCGGAATAGTAAGC	250, 266	234, 256	-	-	-	-
DnB220	TD48	GCAAAGCAGAGCGTAGAATGG	ACTCGGACGTCTCAATCAGC	193, 210	201, 224	144, 188	147, 191	186, 341	176, 341
DnB227	TD48	GTTGTTGTGTCCTCCTCTCG	TATCACTCGCCACCGTAAGC	256, 259	172, 174	-	-	-	-
DnB228	TD48	GCTCATACGTTTAATCTCTCCAAAG	CTCCATCTGATCAAGTCTCTCC	133, 140	138, 147	-	-	-	-
DnB229	TD48	GTTCTTGGTGTGGGAATGAG	CTTCCTCTTCGCCGTTTTC	167, 181	175	-	-	-	-
DnB233	TD48	CGACGATCGGAGACAGG	GCAAAAGATGATTTCCTCGTG	145, 149	140, 147	-	-	-	-
DnB234	TD48	TCTCCTCCGGTGTCATCTCC	ATTCCCACGTTTCCCTGTTC	204, 237	202	228, 234	-	-	-
DnB237	TD48	GTGTTGCTGGGGATTATTCG	CCGGTTTACTCACCAAAAGC	212	196, 204	-	-	-	-
DnB238	TD48	CCTCCTTTGTCCACCATCTC	GCCATTCTTTTGTCCTTTTG	262, 268	300, 320	-	-	-	-
DnB239	SSR58	GTGAACATCGACGGCTTCTC	AACCAAAATCCGCAAAAGG	155, 171	153, 197	-	-	-	-
DnB240	TD48	CGATTAAGCAGCCCTCGTG	CTATCCCATCAAGCCGTAAAG	211, 215	172, 174	-	-	-	-
DnB241	TD48	AATCCACTAAACGGCAATCG	TCTCGGTTCTAATGGGCTTG	210	212, 214	-	-	-	-
DnB244	TD48	GGAGAAATGCAAAAACCCGTCG	CGATCAACCAACCCTCAAGC	187, 200	-	-	-	-	-
DnB245	TD48	GGTACGACTCTAGAGGATCC	CAGTAGACGTCCATTGCCTCG	174	152, 170	-	-	-	-
DnB246	TD48	CCAAGTTTCGTCTGTTGGATTG	CGATCGGAGACATGATTTGAG	185, 193	172, 178	-	-	-	-

DnB247	TD48	GTCGGTTCACGAAGTCAATACAG	AAGGAGATCTGGCGGACAG	318, 340	-	-	-	-	-
DnB3	TD48	GCCGTTGTATTGTAGAGTGAG	ACTGGGTCCTCGCTAAAC	273	266, 270	262	264	-	-
DnB7N	TD48	TGGCATTTAATGGAGGAACCAATTTAC	TTCCGACAAGAAGAAAGAGAGAGAG	238, 250	-	-	-	-	-
DnC5	TD48	AAAGCTACGCTCTTTTCAA	ACCAAATACTTTTGAGTAGTCC	264, 280	218	-	-	-	-
DnC7	TD48	CCTCCTCCGTTACCGAAAT	CCTCCTCCTCCACCTTACG	203	203	-	-	-	-
DnD2	TD48	GAACCAGAACTCGAACCTATGCG	GCAGAGGTGGTAGATACGTCTC	205, 221	189, 207	-	-	-	-
DnD6	SSR55	CAGAGGTGCCAGGTATGC	CCGAACACTTATGACTGCC	285, 291	261, 285	-	-	-	-
DnD11	TD48	CTTGCCAGAACCATAACCTC	GGTGGATGCTTTTGATTTTC	119, 142	-	-	-	-	-
AthCTRI[5, 7, 8]	TD51	TATCAACAGAAACGCACCGAG	CCACTTGTTTCTCTCTCTAG	157	151, 162	157	157	163	130,135
AthGAPAb[3, 7]	TD51	CACCATGGCTTCGGTTACTT	TCCTGAGAATTCAGTGAAACCC	171	168	gel	gel	157	157
AthGAPAab[5]	TD51	TCTGGTCACCATGGCTTCGG	GCCACCTTAAGCTTGGCCTC	412	410, 416	-	-	-	-
AthSO191[2, 8]	TD51	TGATGTTGATGGAGATGGTCA	CTCCACCAATCATGCAAATG	171	168, 174	195, 198	195, 198, 202	-	-
AthSO392[2, 7,8]	TD51	GTTGATCGCAGCTTGATAAGC	TTGGAGTTAGACACGGATCTG	140	140	160, 175, 187	155, 167, 181, 196	159	159

BRMS008[4]	TD49	AGGACACCAGGCACCATATA	CATTGTTGTCTTGGGAGAGC	129	127, 129	124	124	124	124
BRMS033[4]	TD51	GCGGAAACGAACACTCCTCCCATG T	CCTCCTTGTGCTTTCCCTGGAGACG	288	288	237, 241, 243	237, 241, 243	gel	gel
BRMS037[4]	TD51	CTGCTCGCATTTTTTATCATAC	TACGCTTGGGAGAGAAAACTAT	179	-	188	173, 188	gel	gel
BRMS044[4]	TD51	AGGCGAGGAGAAGACAACACAA	TACGGGTGGTTTGAATCAGCAG	440	440	-	-	-	-
ICE14[3, 8]	TD51	TCGAGGTGCTTTCTGAGGTT	TACCTCACCCTTTTGACCCA	240	240	267	261, 270	-	-
ICE4[3, 7]	TD51	CACGAGGAATCTGGCATGGTCG	AGCGATTGCAAGCGGCTCAAG	195	195	183, 199	183, 199	195	189
MR187[6,8]	SSR51	GAGTTTTGGTTCCACCATTA	CCCTTCAGCCTTTGATAAAT	-	-	149, 167, 171, 197	184	gel	gel
nga1145[5]	TD51	CCTTCACATCCAAAACCCAC	GCACATACCCACAACCAGAA	-	-	260, 265	256, 260, 265	250	gel
nga129[2]	TD51	TCAGGAGGAACTAAAGTGAGGG	CACACTGAAGATGGTCTTGAGG	161	153, 161	-	-	173	173
SSL2[3, 7]	TD51	CATGTACTGGGATTCAGTGTCC	CGTCCTTTGTGTGGTTACACG	-	-	gel	gel	281,303	gel

[1]PCR conditions: The number in the PCR condition name indicates amplification temperature (T_a), $SSRT_a$: 95 °C for 2 min, (94 °C for 30 sec, T_a for 30 sec, 72 °C for 45 sec) 35 times, final extension at 72 °C for 20 min, finalizing at 4 °C. TDT_a: 95 °C for 2 min, (94 °C for 30 sec, T_a+10 for 30 sec, - 1 °C per cycle, 72 °C for 45 sec) 10 times, (94 °C for 30 sec, Ta for 30 sec, 72 °C for 45 sec) 30 times, final extension at 72 °C for 20 min, finalizing at 4 °C. [2](Bell, Ecker 1994). [3](Clauss *et al.* 2002), [4](Suwabe *et al.* 2002), [5](Ponce *et al.* 1999), [6](Uzunova, Ecke 1999), [7]Has been further investigated for *Smelowskia* by Carlsen *et al*. (2007), [8] Has been further investigated for *Cardamine* by Jørgensen *et al*. (2007), [9] Primer amplified two different polymorphic loci.