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# MOLECULAR EVIDENCE FOR ALLOPOLYPLOID SPECIATION AND A SINGLE ORIGIN OF THE NARROW ENDEMIC *DRABA LADINA* (BRASSICACEAE) [1]

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*Draba ladina* (Brassicaceae) is a small alpine flower endemic to the Swiss Alps. It occurs exclusively at elevations between 2600 and 3000 m and is restricted to less than a dozen mountains in the Lower Engadin. Morphological characters and polyploidy suggest a hybrid origin. Potential diploid progenitor species are distributed widely and often occur sympatrically. To study the evolutionary history of *D. ladina* we assessed intra- and interspecific sequence variation at noncoding chloroplast DNA loci and nuclear rDNA ITS sequences in *D. ladina* and its presumed progenitor species *D. aizoides, D. dubia,* and *D. tomentosa*. A single ITS (Internal Transcribed Spacer) genotype was found in each of *D. aizoides* and *D. dubia* and two in *D. tomentosa.* Additivity of ITS sequences of *D. aizoides* and *D. tomentosa* was found in *D. ladina,* supporting the hypothesis of an allotetraploid origin. Intraspecific cpDNA variation was found in all diploid species, but not in *D. ladina.* The single chloroplast DNA haplotype found in the latter was closest to one cpDNA haplotype found in *D. tomentosa,* suggesting that *D. tomentosa* was the maternal parent. These results suggest that *D. ladina* is a relatively young, presumably postglacial, taxon with a single allopolyploid origin.

**Key words:** Brassicaceae; cpDNA; hybridization; ITS nucleotide sequences; polyploid evolution; speciation.

Polyploid evolution has received great attention from botanists in recent decades because of its ubiquity in plants (Grant, 1981; Masterson, 1994) and because polyploidization may be a significant mode of speciation (Leitch and Bennett, 1998). Within the last decade, the application of molecular markers has changed our concepts about polyploids (i.e., lack of genetic variation or single origin of polyploids) and has provided significant new insights into the evolutionary dynamics of polyploid speciation in plants. Polyploid species are often polyphyletic with two or more independent origins (Soltis, Doyle, and Soltis, 1992), especially in polyploids with wide geographic distribution. In many cases their diploid relatives have large geographical distributions as well (Ehrendorfer, 1980), suggesting that polyploids are typically polyphyletic when their parental species co-occur on a large geographic scale.

The study of polyploid evolution has been facilitated by the development of several molecular techniques that help to identify progenitor species of allopolyploid taxa and assess genetic variation within both progenitors and polyploids. Nuclear genetic variation, which is often assessed through allozyme electrophoresis, Randomly Amplified Polymorphic DNA analysis (RAPD), or sequencing, may identify parental species because plants of polyploid or hybrid origin typically exhibit additivity of parental genomes (Soltis, Doyle, and Soltis, 1992). In comparison to allozyme and RAPD data, sequences from nuclear genes, such as the nuclear ribosomal Internal Transcribed Spacer (ITS) not only allow the identification of parents, but may provide information about the age of the hybrid species.

The application of ITS sequences to the study of hybrid speciation, however, may be hindered by the particular mode of ITS sequence evolution (Wendel, Schnabel, and Seelanan, 1995). ITS sequences are part of the ribosomal DNA (rDNA), which in higher plants is grouped into arrays that contain hundreds or even thousands of repeats. Evolutionary processes, usually referred to as concerted evolution, are thought to homogenize these repeats such that only a single copy is present (Zimmer et al., 1980). In polyploids of hybrid origin, additivity of parental rDNA sequences is often observed (Soltis, Doyle, and Soltis, 1992). However, interlocus concerted evolution may homogenize repeats from different arrays, effectively removing one parental rDNA type from the hybrid genome (Wendel, Schnabel, and Seelanan, 1995). Conclusions based on the presence of a single rDNA type, i.e., that species of presumed hybrid origin are in fact autopolyploids, may therefore be premature. How fast one of the parental rDNA repeat types may be removed from the hybrid genome and by what evolutionary forces, however, is currently unresolved.

Maternal inheritance of the chloroplast genome predominates in angiosperms (Harris and Ingram, 1991; but see Testolin and Cipriani, 1997) making chloroplast DNA (cpDNA) sequences an ideal marker for the identification of maternal species in studies of polyploid speciation. The large variation in the average rate of evolution between different genes and between coding and noncoding regions of the chloroplast genome allow the choice of suitable regions for study. Rapidly evolving sequences, such as the coding *matK* gene (Brochmann, Nilsson, and Gabrielsen 1996), and noncoding cpDNA sequences, such as the *trnL* (UAA) intron and the intergenic spacer between the *trnL* (UAA) 3′ exon and the *trnF* (GAA)

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gene (Taberlet et al., 1991), are considered ideal markers for the study of phylogenetic relationships among closely related taxa, due to their high average rate of evolution. They have been used intensively for studying relationships at lower taxonomic levels (Kim and Jansen, 1994; Mes and 'T Hart, 1994; Kita, Ueda, and Kadota, 1995; Gielly and Taberlet, 1996; Kim, 'T Hart, and Mes, 1996; Mes, Vanbrederode, and 'T Hart, 1996; Mes, Wijers, and 'T Hart, 1997) and are considered an extremely valuable tool for the differentiation of closely related taxa (Gielly and Taberlet, 1994). Despite the presumably conservative mode of cpDNA evolution, several cases of intraspecific variation have been reported (Rajora and Dancik, 1995; Demesure, Comps, and Petit, 1996; Jordan, Courtney, and Neigel, 1996; Soltis et al., 1996). Such intraspecific variation offers great possibilities for the study of allopolyploid evolution because it may indicate repeated origin of hybrid species (Brochmann et al., 1998).

The tetraploid *Draba ladina* is a narrow endemic to the Swiss Alps and was first described in 1919 [ Braun-Blanquet, 1919 (1920)]. It is restricted to few (<12) mountains of the Ofenpassgruppe in the Lower Engadin (Unterengadin) east of Zernez where it grows in dolomite crevices and in scree on limestone at altitudes between 2600 and 3000 m. Schulz (1927) suggested that *D. ladina* is a hybrid between *D. hoppeana* and *D. tomentosa* but gave no indication how this conclusion was reached. Markgraf (1958) refused this hypothesis, stating that it was based solely on the yellowish flower color, which was intermediate between the yellow flowers of *D. hoppeana* and the white flowers of *D. tomentosa*. However, *D. hoppeana* does not occur in the area [Markgraf, 1958; and personal observations, but see Braun-Blanquet, 1919 (1920)]. Buttler (1967) rejected a recent hybrid origin of *D. ladina,* stating that such hybrids were always sterile, and considered an ancient allopolyploid origin unlikely. He preferred the hypothesis that *D. ladina* represents a western outpost of an asian Draba species group, and further proposed that *D. ladina* survived the last glaciation period in the Alps and later failed to expand its geographic range after the retreat of the glaciers (Buttler, 1967). In 1969, Buttler suggested an allopolyploid origin for *D. ladina* based on morphological and caryological data and identified the diploid *D. aizoides* and *D. tomentosa* as potential parental species. *Draba ladina* occurs in sympatry with its proposed diploid progenitors, *D. aizoides*, *D. tomentosa*, and another white-flowered species, *D. dubia* [ Braun-Blanquet, 1919 (1920); Markgraf, 1958; and personal observations]. These species share the same chromosome number ($2n = 2x = 16$; Hess, Landolt, and Hirzel, 1967) and are typical members of the alpine flora where they often co-occur (Welten and Sutter, 1982).

In this study, we used a molecular approach involving sequencing of chloroplast and nuclear rDNA to reconstruct the evolutionary history of *D. ladina*, to verify its polyploid origin (auto- or allopolyploid), to identify the parental species in the case of an allopolyploid origin, to assess whether it evolved once or repeatedly, and to estimate the age of the species.

## MATERIALS AND METHODS

***Plant material and DNA isolation***—Leaf material was collected from six populations of *D. aizoides*, two populations of *D. tomentosa*, two populations of *D. dubia*, and two populations of *D. ladina*. Fresh leaf material was stored in silica gel at ambient temperature. Additional samples of *D. aizoides* from six populations were taken from older herbarium collections (Table 1, Fig. 1). DNA was extracted from leaf material stored in silica gel or from herbarium material by the method of Doyle and Doyle (1987).

***CpDNA amplification and sequencing***—Aliquots of these DNA samples were then used as templates in polymerase chain reactions (PCR). Primers c and f of Taberlet et al. (1991) were used to amplify the *trnL* (UAA) intron, the *trnL* (UAA) 3′ exon, and the intergenic spacer between the *trnL* (UAA) 3′ exon and *trnF* (GAA). PCRs were carried out in a total reaction volume of 50 μL, containing 1× reaction buffer (Promega, Catalys AG, Wallisellen, Switzerland),1.5–2.5 mmol/L $MgCl_2$, 0.3 μmol/L of each primer (Microsynth GmbH, Balgach, Switzerland), 100 μmol/L of each dNTP (Boehringer Mannheim, Rotkreuz, Switzerland), and 2.5 Units of Taq Polymerase (Promega, Catalys AG, Wallisellen, Switzerland). The thermocycling profile consisted of an initial denaturation step at 94°C for 2 min, followed by 25 cycles with 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C. PCRs were performed either on a PTC-100 temperature cycler (MJ Research Inc., Watertown, Massachusetts) or on a GeneAmp PCR System 2400 (PE Biosystems, Foster City, CA). PCR fragments were purified with a QIAquick PCR purification kit (Qiagen, Basel, Switzerland) to remove unincorporated primers and dNTPs. Primers c, d, e, and f of Taberlet et al. (1991) were used to sequence both strands of the PCR fragment to unambiguously identify all sites. Sequencing was done by the dideoxy method (Sanger, Nicklen, and Coulson, 1977) using AmpliTaq DNA Polymerase FS and fluorescent labelled dNTP's (PE Biosystems, Foster City, CA). Sequencing reactions were set up according to the supplier's recommendations (Perkin-Elmer, 1995) and purified using ethanol precipitation at room temperature. Automatic DNA sequencing was performed on an ABI PRISM™310 Genetic Analyzer (PE Biosystems, Foster City, CA). Both strands were sequenced at least once for each species and DNA fragment, to unambiguously identify all sites. CpDNA sequences were obtained from ten individuals of *D. dubia*, 20 of *D. ladina*, and ten of *D. tomentosa*. Sequences of nine *D. aizoides* haplotypes (DA1-DA9) found among 53 individuals (unpublished data) were included in the analysis.

***ITS amplification, cloning, and sequencing***—Nuclear rDNA-gene spacers ITS1 and ITS2 were amplified by polymerase chain reaction (PCR) using the primers ''ITS 2,'' ''ITS 3,'' ''ITS 4,'' and ''ITS 5'' (White et al., 1990). 5–25 ng of DNA were submitted to amplification in a total volume of 50 μL, containing 1× reaction buffer, 1.5–2 mmol/L $MgCl_2$, 0.4 μmol/L of each primer, 200 μmol/L of each dNTP and 0.5 U of Pfu DNA polymerase (Stratagene AG, Basel, Switzerland) or 1.2 U of AmpliTaq Gold (Perkin Elmer Europe B.V., Rotkreuz, Switzerland). The typical thermocycling profile consisted of 25 cycles with 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C. With AmpliTaq Gold Polymerase, a preheating step of 8 min at 94°C was necessary. All PCR reactions were performed on a PTC-100 temperature cycler.

To test for intra-individual ITS sequence variation, corresponding PCR products (primers ''ITS4'' and ''ITS5'') of three *D. ladina* were cloned. PCR products were purified with the QIAquick PCR purification kit and subsequently cloned into the Sma1 site of Bluescript $KS^+$, using *E. coli* strain DH5α as host (Stratagene AG, Basel, Switzerland). The plasmid preparation was performed based on the alkaline lysis method of Birnboim and Doyle (1979). The universal primers SK and KS and primer ''ITS3'' were used for sequencing three clones each of three *D. ladina*. PCR-amplified ITS sequences of other taxa were sequenced as described above. ITS sequences other than from *D. ladina* were obtained from 20 individuals of *D. aizoides* (including at least one individual per population and per chloroplast DNA haplotype), eight of *D. dubia*, and ten of *D. tomentosa*.

TABLE 1. Population, locality, and sample information of *Draba* sampled for cpDNA and ITS analyses. (*N* = number of individuals analyzed; hm = herbarium material; sg = silica-gel-dried leaf material).

| Pop. code | Species | *N* | Location | Habitat | Elevation | DNA source | Collection date |
|---|---|---|---|---|---|---|---|
| BFL | *D. aizoides* | 1 | Balmfluh near Günsberg | Crevices on limestone | 700 | hm | 14 May 1983 |
| BRH | *D. aizoides* | 2 | S slope of Höch Gumme, ca. 6 km NE of Brienz | S-SW exposed scree on limestone | 2100–2200 | hm | 15 August 1984 |
| DAV | *D. aizoides* | 4 | Strelapass, 3 km NW from Davos-Platz, GR | Dolomite crevices | 2360 | sg | 22 August 1996 |
| | *D. aizoides* | 1 | S slope of Schiahorn, NW Davos-Platz | S exposed meadow on dolomite | 2350 | hm | 1 July 1994 |
| FLA | *D. aizoides* | 5 | SE slope between Fuorcla Laschadurella and Ils Cuogns, GR | Crevices and scree on limestone | 2650–2850 | sg | 30 July 1997 |
| | *D. ladina* | 5 | | | | | |
| GEM | *D. aizoides* | 1 | Lämmerenalp, 2 km W Gemmipass | Meadow and scree on limestone | 2320 | hm | 14 August 1981 |
| | *D. aizoides* | 2 | Gemmipass | Scree on limestone | 2400 | hm | 12 July 1994 |
| | *D. aizoides* | 2 | Gemmi-Passhöhe | Open meadow on limestone | 2300 | hm | 9 August 1985 |
| OFP | *D. aizoides* | 2 | Ofenpass | — | — | hm | 10 June 1979 |
| PNA | *D. aizoides* | 5 | Piz Nair, Fuorcla Schiattain | Scree on limestone | 2875 | sg | 26 July 1996 |
| SAM | *D. aizoides* | 4 | Zeblaswiese above Samnaun | Rock of limestone | — | hm | 11 July 1977 |
| | *D. aizoides* | 2 | Zeblasjoch | Scree on limestone | 2540–2600 | hm | 11 July 1987 |
| STV | *D. aizoides* | 5 | S exposed rocky slope, Stragliavita, 5 km ENE Zernez, GR | Scree on limestone | 2500–2650 | sg | 30 July 1997 |
| VDB | *D. aizoides* | 5 | Fuorcla Val dal Botsch, 11 km E Zernez, GR | Crevices and scree on limestone | 2700–2750 | sg | 25 July 1996 |
| | *D. dubia* | 5 | | | | | |
| | *D. tomentosa* | 5 | | | | | |
| | *D. ladina* | 5 | | | | | |
| VDH | *D. aizoides* | 8 | Between Liapey d'Enfer and Serra Neire, ENE Les Haudères, Val d'Hérens, VS | Open sites in rocky meadow | 2500 | sg | 8 August 1996 |
| | *D. dubia* | 2 | | | | | |
| | *D. tomentosa* | 5 | | | | | |
| ZIN | *D. aizoides* | 3 | Sorebois, 2.5 km WNW of Zinal | Open meadow on limestone | 2450–2800 | hm | 1 July 1982 |
| | *D. aizoides* | 2 | Lirec, 2 km NE von Zinal | Meadow and scree | 2500–2900 | hm | 30 June 1982 |

***Sequence analyses***—Sequence files obtained from the ABI PRISM™310 Genetic Analyzer (PE Biosystems, Foster City, CA) were imported separately for each individual into Sequence Navigator version 1.0.1 (PE Biosystems, Foster City, CA) and complementary strands were aligned using the Clustal V option as provided in the program. Ambiguous sites were checked manually and corrected by comparing the electropherograms from both strands. Consensus sequences were obtained for each individual. Multiple alignments based on consensus sequences were carried out in Sequence Navigator, using Clustal V. Minor adjustments were made manually to minimize the number of inferred insertions/deletions (indels).

PAUP 4.0d64 written by D. L. Swofford was used for phylogenetic analyses, using maximum parsimony. One-base pair (bp) indels were treated as binary presence/absence characters. Longer indels were considered as one independent evolutionary event each and subsequently treated as one polymorphic position each. The presence and frequency of indels suggest that only a parsimony approach should be used for phylogenetic reconstructions, since no reliable genetic distances can be estimated from both substitutions and indels (Gielly and Taberlet, 1994). All characters were specified as unordered and weighted equally. The presence of phylogenetic signal in the sequence matrices was tested using the $g_1$ statistic test based on the skewness of tree-length distributions (Hillis and Huelsenbeck, 1992). The $g_1$ was estimated by generating $10^5$ trees from the complete data set with the random-trees option in the test version 4.0d64 of PAUP provided by D. L. Swofford.

Parsimony analyses were performed using the branch-and-bound search strategy. Bootstrap analyses (Felsenstein, 1985) were run, using 1000 replications to obtain estimates of reliability for nodes.

***Cytology***—Two living plants of *D. ladina* from Fuorcla Val dal Botsch (population code VDB, Table 1) were transferred to the greenhouse for cytological investigation, which was done using root tips. These were pretreated for 0.5 h with colchicine (0.05%), then fixed in ethanol/acetic acid (3:1), and stained and squashed in lacto-propionic orcein. For the determination of chromosome numbers, 5–10 metaphases were counted for both individuals.

## RESULTS

***ITS sequences***—Aligned ITS sequences were 636 bp in length, of which 30 (4.7%) were variable and 24 (3.8%) were parsimony informative. A single ITS genotype was found in *D. aizoides* and *D. dubia*, and two in *D. tomentosa* (four with genotype DT-A and six with genotype DT-B). From two out of three individuals of *D. ladina* for which ITS sequences were cloned prior to sequence analysis, two different ITS sequences could be retrieved from three sequenced clones, each (genotypes DL-A and DL-B, respectively; Table 2). Only genotype

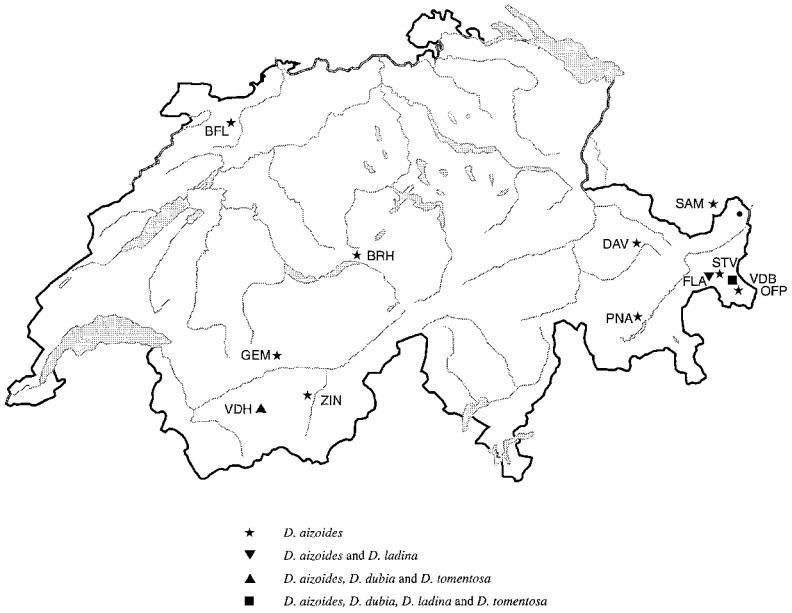


Fig. 1. Map of Switzerland showing the 12 sampling sites for 18 populations of *Draba* spp. Reference codes are given for each population (cf. Table 1).

DL-A was found in the three clones sequenced from the third individual of *D. ladina*. ITS sequences produced a significantly skewed tree-length distribution ($g_1 = -2.02$, $P < 0.01$), suggesting that phylogenetic signal in the data set exists. The exhaustive search option in the parsimony analysis produced a single most-parsimonious tree of 30 steps (consistency index = 1.0; Fig. 2). Two major clades, differing at 23 positions, could be identified: one containing *D. aizoides* and *D. ladina* ITS genotype DL-B, and one including *D. dubia*, both *D. tomentosa* ITS-genotypes, and *D. ladina* ITS genotype DL-A (Fig. 2). The latter is closest to both *D. tomentosa* ITS sequences with one and two transition substitutions, respectively, but more distant to the *D. dubia* genotype (three transitions). A single transversion substitution separates *D. aizoides* from *D. ladina* ITS genotype DL-B.

***CpDNA sequences***—CpDNA sequences varied in length between 765 and 796 base pairs (bp). After alignment the sequence data matrix contained 813 characters. Five multi-bp indels varying in length between five and 19 bp were found (Table 3). Irrespective of length, indels were considered to represent one independent evolutionary event each and subsequently treated as one polymorphic position each. Of the remaining 777 positions, 46 (5.9%) were variable. Of those, 33 (72%) were substitutional mutations, eight (17%) were 1-bp insertions/deletions (indels), and five (11%) were multi-bp indels (Table 3). Intraspecific sequence variation was absent or weak in *D. dubia* (two haplotypes), *D. tomentosa* (four haplotypes), and *D. ladina* (one haplotype), but extensive in *D. aizoides*. Patterns of cpDNA variation in the latter species are discussed in detail elsewhere (unpublished

TABLE 2. Matrix of variable sites (30) for ITS 1, 5.8S rDNA, and ITS 2 sequences of *D. ladina* (genotypes DL-A and DL-B), *D. aizoides*, *D. dubia*, and *D. tomentosa* (genotypes DT-A and DT-B). Sequences are numbered from position 1 with reference to the *D. aizoides* sequence (GenBank accession number AF120721). Only nucleotides that differ from the *D. aizoides* genotype are shown. Dots (.) indicate that the character state is the same as for *D. aizoides*. A dash (-) indicates a 1-bp insertion/deletion mutation.

| | | Variable nucleotide position | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | Genotypes | 46 | 51 | 75 | 112 | 174 | 191 | 227 | 382 | 384 | 392 | 416 | 426 | 450 | 473 | 482 |
| *D. aizoides* | | A | G | G | C | A | A | C | C | A | G | G | A | C | G | G |
| *D. dubia* | | G | A | T | T | C | . | T | T | T | A | C | T | . | A | A |
| *D. ladina* | DL-A | G | . | T | T | C | . | T | T | T | A | C | T | . | . | A |
| *D. ladina* | DL-B | . | . | . | . | . | C | . | . | . | . | . | . | . | . | . |
| *D. tomentosa* | DT-A | G | . | T | T | C | . | T | T | T | A | C | T | T | . | A |
| *D. tomentosa* | DT-B | G | . | T | T | C | . | T | T | T | A | C | T | . | . | A |
| | | 490 | 492 | 499 | 504 | 520 | 523 | 528 | 544 | 551 | 554 | 561 | 570 | 594 | 624 | 625 |
| *D. aizoides* | | C | A | A | C | C | T | G | - | A | G | T | C | C | A | T |
| *D. dubia* | | T | C | G | T | T | C | . | C | C | - | A | G | . | G | A |
| *D. ladina* | A | T | C | G | T | . | C | A | C | C | - | A | G | T | G | A |
| *D. ladina* | B | . | . | . | . | . | . | . | . | - | G | . | . | . | . | . |
| *D. tomentosa* | A | T | C | G | T | . | C | . | C | C | - | A | G | T | G | A |
| *D. tomentosa* | B | T | C | G | T | . | C | . | C | C | - | A | G | T | G | A |

TABLE 3. Matrix of variable sites (46) for cpDNA haplotypes of *D. aizoides*, *D. ladina*, *D. dubia*, and *D. tomentosa*. Sequences are numbered beginning at position 1 with reference to the *D. aizoides* DA1 sequence (GenBank accession number AF120727). Only nucleotides that differ from the DA1 haplotype are shown. Dots (.) indicate that the character state is the same as for DA1. A dash (-) indicates a 1-bp insertion/deletion mutation. I1 to I5 indicate the position of multi-bp insertion/deletion mutations, with 0 indicating a deletion and 1 the presence of an insertion.

| | | Variable nucleotide position | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species | Haplotype | 57 | 128 | 131 | 132 | 138 | 145 | 152 | 202 | 213 | 233 | 266 | 267 | 360 | I1 | 408 | 409 | 410 | 423 | 424 | 446 | 447 | 448 | 470 |
| *D. aizoides* | DA1 | C | G | T | A | G | C | C | A | T | A | T | A | A | 0 | - | - | T | C | T | T | T | - | A |
| *D. aizoides* | DA2 | . | . | . | . | . | . | . | . | . | . | . | . | T | 0 | - | - | . | . | . | . | . | - | . |
| *D. aizoides* | DA3 | . | . | . | . | . | . | . | . | . | . | . | . | . | 0 | - | - | . | . | . | . | . | - | . |
| *D. aizoides* | DA4 | T | A | C | C | . | G | A | G | C | C | . | . | . | 0 | - | - | . | . | . | . | . | T | C |
| *D. aizoides* | DA5 | T | . | C | . | A | G | A | G | C | C | G | . | . | 0 | - | - | . | . | . | - | - | - | C |
| *D. aizoides* | DA6 | . | . | . | . | . | . | . | . | . | . | . | . | . | 0 | - | - | . | . | . | . | . | - | . |
| *D. aizoides* | DA7 | T | A | C | C | . | G | A | G | C | C | . | . | . | 0 | - | - | . | . | . | . | . | T | C |
| *D. aizoides* | DA8 | . | . | . | . | . | . | . | . | . | C | . | T | . | 0 | - | - | . | . | C | . | . | - | . |
| *D. aizoides* | DA9 | . | . | . | . | . | . | . | . | . | C | . | . | . | 0 | - | - | . | . | C | . | . | - | . |
| *D. ladina* | DL | T | . | C | . | A | G | A | G | C | C | G | . | . | 0 | - | C | . | . | . | - | - | - | C |
| *D. dubia* | DD1 | T | . | C | . | A | G | A | G | C | C | G | . | . | 1[1] | A | C | . | G | . | - | - | - | C |
| *D. dubia* | DD2 | T | . | C | . | A | G | A | G | C | C | G | . | . | 1[2] | A | C | . | . | . | - | - | - | C |
| *D. tomentosa* | DT1 | T | . | C | . | A | G | A | G | C | C | G | . | T | 0 | A | C | . | . | . | - | - | - | C |
| *D. tomentosa* | DT2 | T | . | C | . | A | G | A | G | C | C | G | . | . | 0 | - | C | . | . | . | - | - | - | C |
| *D. tomentosa* | DT3 | T | . | C | . | A | G | A | G | C | C | G | . | . | 0 | A | C | . | T | . | - | - | - | C |
| *D. tomentosa* | DT4 | T | . | C | . | A | G | A | G | C | C | G | . | . | 0 | - | - | - | . | . | - | - | - | C |

data). Thirty-eight of the 46 variable positions were parsimony informative. A significantly skewed tree-length distribution ($g_1 = -0.58$, $P < 0.01$) is evidence for phylogenetic signal in the data set. The branch-and-bound search option produced four shortest trees of 61 steps with a consistency index of 0.82 (uninformative sites excluded) (Fig. 3). Three well-diverged groups were consistently recovered. Two of them were formed exclusively by *D. aizoides* haplotypes. The third group consisted of one *D. aizoides* haplotype (DA5) and the remaining *Draba* haplotypes. *Draba ladina* formed a clade with two *D. tomentosa* haplotypes (DT3, DT4) based on two synapomorphic transversion mutations (A ↔ T, position 524; C ↔ A, position 541). Whether this group is sister to *D. dubia* (DD2) or *D. tomentosa* (DT1) remains unresolved in the parsimony analysis (Fig. 3). ITS and cpDNA sequences have been deposited in GenBank (accession numbers AF120721–120726, AF120738–120744).

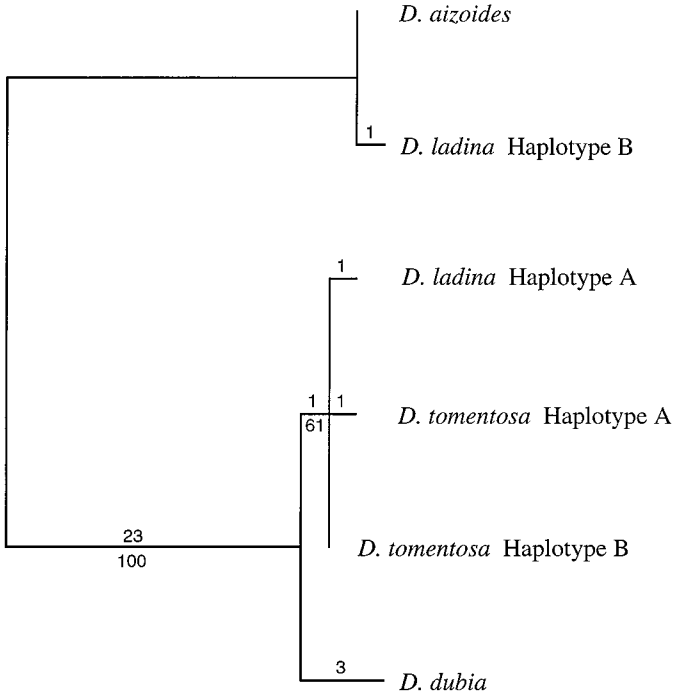


Fig. 2. Single most-parsimonious tree derived from the analysis of 636-bp ITS sequences of *D. aizoides*, *D. dubia*, and *D. tomentosa*. The tree is rooted at the midpoint. Numbers above branches indicate the number of base substitutions. Bootstrap support is given as percentages based on 1000 bootstrap replications below branches. Tree length = 30, consistency index = 1.00, $g_1 = -2.02$ ($P < 0.01$).

***Cytology***—The two investigated plants of *D. ladina* showed the same chromosome number of $2n = 32$ chromosomes. The basic chromosome number within *Draba* is mostly $x = 8$ (Brochmann, Soltis, and Soltis, 1992a), the two plants are therefore tetraploid, which corresponds with the indication in Merxmüller and Buttler (1965).

## DISCUSSION

***The evolution of D. ladina***—Our results clearly support a hybrid origin of *D. ladina*, as suggested by Schulz (1927) and Buttler (1969), because the presence of two distinct ITS sequences in the tetraploid *D. ladina* is not compatible with an autopolyploid origin. The rDNA sequences found in *D. ladina* are most closely related to those of the presumed (Buttler, 1969) parental species, *D. aizoides* and *D. tomentosa*. Additivity of rDNA sequences in the allopolyploid is intuitively expected and has often been found (for a review see Rieseberg and Brunsfeld, 1992). However, as shown by Wendel, Schnabel, and Seelanan (1995), bidirectional interlocus concerted evolution may effectively remove one of the parental rDNA repeats from the hybrid genome. Although the exact nature of this process is not well understood, it appears that homogenizing of ITS sequences via bidirectional interlocus concerted evolution requires many sexual generations for completion (Wendel, Schnabel, and

TABLE 3. Extended.

| Variable nucleotide position | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 507 | 522 | 523 | 524 | 537 | 541 | 559 | I2 | 589 | I3 | 612 | I4 | 654 | 655 | 685 | I5 | 748 | 749 | 752 | 753 | 765 | 796 | 803 |
| G | T | T | A | A | C | C | 0 | A | 0 | G | 0 | T | A | C | 1[7] | C | A | G | C | A | G | G |
| . | - | - | . | . | . | . | 0 | . | 0 | . | 0 | . | . | . | 1[7] | . | C | . | . | . | . | . |
| . | - | - | . | . | . | . | 0 | . | 0 | . | 0 | . | . | . | 1[7] | . | C | . | . | . | . | . |
| T | . | - | . | . | . | . | 1[3] | . | 0 | T | 0 | G | . | T | 0 | A | . | T | T | . | . | . |
| T | . | - | . | - | . | . | 1[4] | C | 0 | T | 0 | G | . | . | 1[7] | . | . | . | . | . | . | . |
| . | - | - | . | . | . | . | 0 | . | 0 | . | 0 | . | . | . | 1[7] | . | . | . | . | . | . | . |
| T | . | - | . | . | . | G | 1[3] | . | 0 | T | 1[6] | G | . | T | 0 | A | . | T | T | . | . | . |
| . | . | - | . | . | . | . | 0 | . | 0 | . | 0 | . | . | . | 1[7] | . | . | . | . | . | . | . |
| . | - | - | . | . | . | . | 0 | . | 0 | . | 0 | . | . | . | 1[7] | . | C | . | . | . | . | . |
| T | . | . | T | - | A | . | 1[4] | . | 0 | T | 0 | G | . | . | 1[7] | . | . | . | . | . | . | . |
| T | . | - | . | - | . | . | 1[4] | . | 0 | T | 0 | G | . | . | 1[7] | . | . | . | . | . | . | . |
| T | . | - | . | - | . | . | 1[4] | . | 0 | T | 0 | G | . | . | 1[7] | . | . | . | . | . | . | A |
| T | . | - | . | - | . | G | 1[4] | . | 0 | T | 0 | G | . | . | 1[7] | . | . | . | . | . | . | . |
| T | . | - | . | - | . | . | 1[4] | . | 1[5] | T | 0 | G | G | . | 1[7] | . | . | . | . | C | C | . |
| T | . | . | T | - | A | . | 1[4] | . | 0 | T | 0 | G | . | . | 1[7] | . | . | . | . | . | T | A |
| T | . | . | T | - | A | . | 1[4] | . | 0 | T | 0 | G | . | . | 1[7] | . | . | . | . | . | C | A |

[1] CCCATCCCCAACTAT.
[2] CCCATCTATCCCCAACTAT.
[3] CAAATGAGAGAT.
[4] CAAATGAGAAAT.
[5] ATAGA.
[6] ATCATT.
[7] CTACTT.

Seelanan, 1995; Brochmann, Nilsson, and Gabrielsen, 1996). The presence of two parental ITS sequences in *D. ladina* may indicate a relatively recent origin of the species. The absence of one parental rDNA repeat in one of the studied individuals of *D. ladina* is most likely a consequence of the small number of clones sequenced and is thus no evidence for the loss of one parental repeat due to bidirectional interlocus concerted evolution.

The cpDNA data per se do not provide conclusive evidence about the maternal progenitor of *D. ladina*. Intraspecific cpDNA sequence divergence in *D. aizoides* was found to be extensive, with three genetically well-differentiated groups of cpDNA haplotypes occurring in Switzerland (unpublished data). Intraspecific cpDNA sequence divergence among different *D. aizoides* haplotypes is similar or larger than interspecific divergence among *D. aizoides, D. dubia,* and *D. tomentosa. Draba aizoides* can be excluded as the maternal progenitor of *D. ladina* because all known haplotpyes are more distant from *D. ladina* than are either *D. tomentosa* or *D. dubia.* The topology of the cpDNA tree (Fig. 3) suggests that the chloroplast genome found in *D. ladina* shared a common ancestor with that found in both *D. dubia* and *D. tomentosa*. Given the small genetic differences between cpDNA haplotypes this could implicate that *D. dubia* instead of *D. tomentosa* was involved in the formation of *D. ladina*. However, strong evidence for the hypothesis that *D. tomentosa* is in fact the maternal species of *D. ladina* comes from indel I1 (Table 3), which is present in both *D. dubia* haplotypes, although in different length variants, but is absent from the chloroplast genomes of *D. tomentosa* and *D. ladina.* Further evidence for the identification of *D. tomentosa* as the maternal species comes from the analysis of ITS sequences, which excludes *D. dubia* from the pool of potential progenitors of *D. ladina,* since the two ITS types found in the hybrid clearly belong to *D. aizoides* and *D. tomentosa.* We therefore conclude that *D. ladina* is of allopolyploid origin with *D. aizoides* as the paternal parent and *D. tomentosa* the maternal parent.

***Single vs. multiple hybridization events and age of D. ladina***—Allopolyploidy results from chromosome doubling following hybridization between two genetically distinct diploid species. This important mechanism of speciation in flowering plants (Grant, 1981) was considered to produce genetically depauperate species because instant reproductive isolation precludes influx of genetic variation from the parental diploid taxa.

Recently, molecular studies on polyploid evolution have found extensive genetic diversity indicative of multiple origins of many polyploid taxa (for a review see Soltis and Soltis, 1993). Recurrent hybridization events have been documented occurring over relatively short time spans and geographic distances (Ashton and Abbott, 1992; Arft and Ranker, 1998). If polyploidization is less rare than has been assumed (Soltis, Doyle, and Soltis, 1992; Soltis and Soltis, 1993), then significant genetic variation may be incorporated into allopolyploid species from genetically distinct parental individuals or populations. In arctic *Draba,* multiple origins of polyploids appear to be the rule rather than the exception (Brochmann, Nilsson, and Gabrielsen, 1996; Brochmann, Soltis, and Soltis, 1992b,c), making polyploid *Draba* a textbook example for recurrent speciation through hybridization (Briggs and Walters, 1997).

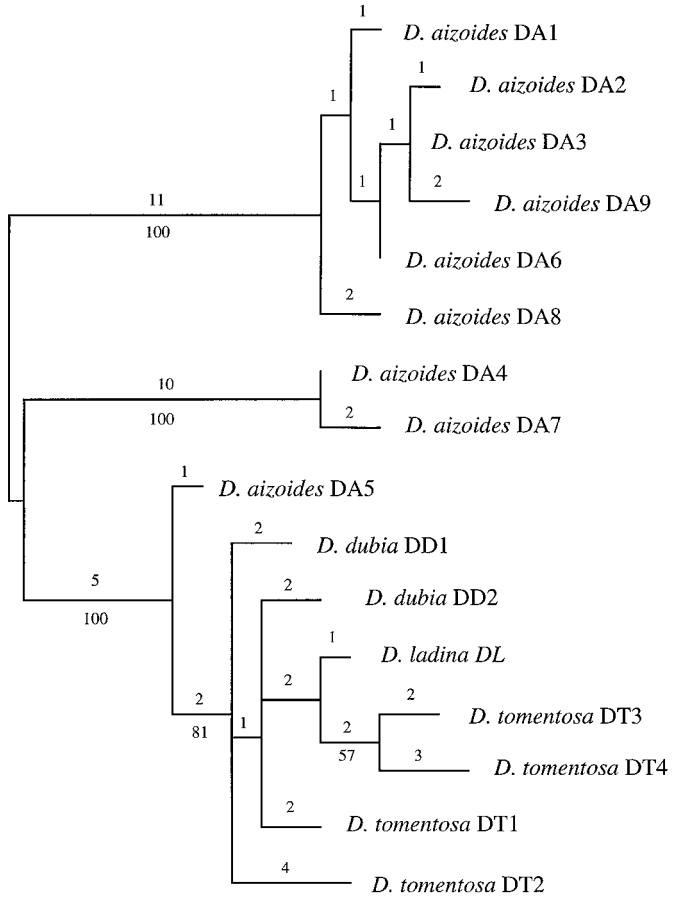


Fig. 3. One out of four most-parsimonious trees based on 813 cpDNA characters (including indels) of *D. aizoides, D. dubia, D. ladina,* and *D. tomentosa.* The tree is rooted at the midpoint. Numbers above branches indicate the number of base substitutions. Bootstrap support is given as percentages based on 1000 bootstrap replications below branches if support is over 50%. Tree length = 61, consistency index = 0.82, $g_1 = -0.58$ ($P < 0.01$).

In *D. ladina,* however, no intraspecific ITS or chloroplast variation was detected despite extensive intraspecific cpDNA variation in *D. aizoides* and moderate intraspecific variation in *D. dubia* and *D. tomentosa,* the presumed progenitor group. Lack of variation in *D. ladina* therefore does not result from choice of an inappropriate cpDNA fragment or from low variation in the progenitor species. Also, the two populations examined represent two geographically distinct locations of this narrow endemic, for which less than 12 populations are known. We therefore propose a single allopolyploid origin for this narrow-endemic species. Otherwise, we would expect to find variation at least among populations, such as in the autopolyploid *Saxifraga osloensis* (Brochmann, Nilsson, and Gabrielsen, 1996).

Sequence divergence between ITS and cpDNA sequences found in *D. ladina* and its progenitor species may be due to sampling error (i.e., insufficient sampling of progenitor sequences), or because the genome of *D. ladina* has diverged from the progenitor's genomes. The observation that both parental rDNA repeat types are still present in the allopolyploid and that none has yet been eliminated from the genome through bidirectional interlocus concerted evolution should allow us to estimate the age of *D. ladina.* Unfortunately, however, estimates of mutation rates for noncoding cpDNA sequences and for ITS sequences (Baldwin, 1992; Suh et al., 1993) are either unknown or differ widely among lineages. Thus one cannot reliably estimate the age of *D. ladina* based on the sequence divergence observed. Similarly, the time scale necessary for bidirectional interlocus concerted evolution to remove one parental rDNA repeat from the hybrid genome is unknown. There seems to be general agreement, however, that many sexual generations are necessary for homogenization to be complete (Wendel, Schnabel, and Seelanan, 1995; Brochmann, Nilsson, and Gabrielsen, 1996). The generation time of *D. ladina* is unknown. Our personal observations, however, suggest that plants can grow to a very old age, probably >20 yr. Taking all this circumstantial evidence into account, we propose a relatively recent, probably postglacial origin for *D. ladina.*

***Geographic range***—*D. ladina* is a narrow endemic. This pattern is in marked contrast to many other polyploids, for which a wider geographic range relative to their diploid progenitors has been reported (Soltis and Soltis, 1991). Three factors may account for the restricted range of *D. ladina*: the presumed single origin, low dispersal capacity of its seeds, and its restriction to dolomite crevices and scree on limestone at elevations between 2600 and 3000 m. A similar conclusion was reached in the allopolyploid *Cardamine schulzii,* which evolved within the last century and failed to expand its geographic range because of specific habitat requirements (Urbanska et al., 1997).

Repeated evolution, which has often been reported in the arctic-alpine genus *Draba* (Brochmann, Soltis, and Soltis, 1992a, b, c ) and has been suggested for the alpine *D. dolomitica* (Buttler, 1969), could also lead to a wide geographic distribution. Indeed, the parental species of *D. ladina, D. aizoides,* and *D. tomentosa,* are widely distributed in the Alps and co-occur frequently (Welten and Sutter, 1982, and personal observations). Hybrids between the two species have been reported from various regions in the Alps (Markgraf, 1958). However, our molecular results provide no evidence that the polyploidization necessary for the repeated evolution of *D. ladina* has occurred more than once.

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