Multiple Hybrid Formation in Natural Populations: Concerted Evolution of the Internal Transcribed Spacer of Nuclear Ribosomal DNA (ITS) in North American *Arabis divaricarpa* (Brassicaceae)

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DNA sequence variation of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA from *Arabis holboellii*, *A. drummondii*, and its putative hybrid *A. divaricarpa* was analyzed to study hybrid speciation in a species system geographically covering nearly the entire North American continent. Based on molecular systematics the investigated species are better combined under the genus *Boechera*. Multiple intraindividual ITS copies were detected in numerous accessions of *A. divaricarpa*, and, to a minor extent, in the parental taxa. Comparative phylogenetic analysis demonstrates that reticulate evolution is common. Consequently, concerted evolution of ITS regions resulted in different types of ITS fragments not only in hybrid populations but also in one of the parental taxa, *A. holboellii*. Hybrid formation often occurred independently at different sites and at different times, which is reflected by ITS copies resampling the original parental sequence variation in different ways. Some biogeographic structuring of genetic diversity is apparent and mirrors postglacial migration routes. Hybridization, reticulation, and apomixis are assumed to be the major forces driving speciation processes in this species complex. Analysis of conserved regions and secondary structures of the ITS region provided no evidence that, in this system, hybrid ITS evolution is predominantly driven in a particular direction. However, two regions in the ITS1 and ITS2, respectively, show higher mutation rates than expected from outgroup comparisons. Strong evidence for the occurrence of apomixis in *A. holboellii* and *A. divaricarpa* has come from pollen size measurements and estimations of pollen quality, which favor the hypothesis that *A. drummondii* served as paternal hybridization partner more frequently than *A. holboellii*.

Introduction

Hybridization and polyploidization are thought to represent an important mechanism in angiosperm evolution. In the past, numerous studies on the evolution of polyploid complexes have shown that recurrent formation of allopolyploid and autopolyploid plant species are the rule rather than the exception, and as a consequence approximately 50% of all angiosperms are thought to be of hybrid origin (Arnold 1997). Furthermore, 35% to 50% of all angiosperms are thought to be of polyploid origin (Müntzing 1936; Darlington 1937; Grant 1963; Stebbins 1971; Goldblatt 1980; Masterson 1994). The origin of polyploids and the mechanisms underlying the establishment of newly evolved populations and taxa are among the most challenging questions in plant sciences (Thompson and Lumaret 1992; Ramsey and Schemske 1998; Petit, Bretagnolle, and Felber 1999; Soltis and Soltis 1999). New combinations of favorable genes may provide starting points for new phylogenetic lineages, and hybridization processes are integral to the genesis and maintenance of plant biodiversity. These newly mixed genomes may be more easily stabilized in polyploid taxa, however, and the permanent coexistence of favorable traits and characters may be effectively preserved as fixed heterozygosity (Soltis and Soltis 1993).

Molecular tools have greatly improved our knowledge about hybrid speciation, because such tools allow characterization of parental genomes that can then be followed in the offspring. This characterization could be random (e.g., amplified fragment length polymorphism [AFLP]; random amplified polymorphic DNA [RAPD]) or highly specific (e.g., microsatellites, genomic mapping). In many cases the characterization of strictly uniparentally inherited genomes, such as the plastome in many angiosperms (Harris and Ingram 1991), allows identification of the paternal or maternal crossing partner. Recent studies of the total genome structure of hybrid and polyploid taxa provide new insight into the dynamic nature of complete genomes analyzed either on the basis of artificial hybrids or by comparative mapping (Kowalski et al. 1994; Lagercrantz 1998; Rieseberg, Whitton, and Gardner 1999; Rieseberg and Linder 1999). In their comparative genome analysis of some Brassicaceae, Acarkan et al. (2000) showed that structural rearrangements occurred with a significantly higher frequency in polyploid *Brassica* than in diploid *Arabidopsis thaliana* or *Capsella rubella*.

DNA analyses of the nuclear and plastid genomes have greatly increased the possibility of detecting and distinguishing allopolyploids and autopolyploids, following paternal and maternal genome lineages, and documenting hybridization, introgression, and reticulate evolution. Through such analyses, several polyploid complexes within the Brassicaceae have been characterized in significant detail, including those of the genera *Microthlaspi* (Koch, Mummenhoff, and Hurka 1998; Koch and Hurka 1998), *Draba* (Brochmann 1992; Brochmann and Elven 1992; Brochmann, Soltis, and Soltis 1992; Brochmann, Nilsson, and Gabrielsen 1996; Widmer and Baltisberger 1999; Koch and Al-Shehbaz 2002), *Cochlearia* (Koch, Huthmann, and Hurka 1998; Koch, Mummenhoff, and Hurka 1999), *Yinshania* (Koch and Al-Shehbaz 2000), *Cardamine* (Franzke et al. 1998; Urbanska et al. 1997), *Biscutella* (König 1998;

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Tremetsberger et al. 2002), *Brassica* and related genera (Anderson and Warwick 1999), and *Nasturtium* (Bleeker, Huthmann, and Hurka 1999). In some of these complexes ITS sequence evolution has been analyzed in hybrid systems; the phenomenon of concerted evolution of ITS DNA loci has been demonstrated several times in the Brassicaceae. Concerted evolution describes the molecular process of DNA sequence homogenization among different loci within multigene families (Arnheim et al. 1980; Dover 1982; Avise 1994). DNA sequence homogenization via concerted evolution is driven by two molecular processes, gene conversion and unequal crossing over. The relative contribution of each as a homogenizing agent, however, continues to be the subject of debate (Muir, Fleming, and Schlötterer 2001).

The rDNA loci in particular serve as a classic example of concerted evolution of tandemly repeated gene families and have been analyzed in detail (for references see Buckler, Ippolito, and Holtsford 1997; Muir, Fleming, and Schlötterer 2001). In principle, there are three different ways in which two different ITS copies evolve within a single individual: (1) unidirectional concerted evolution leads to the loss of one copy and fixation of the second (e.g., Wendel, Schnabel, and Seelanan 1995*a*; Koch and Al-Shehbaz 2000); (2) concerted evolution leads to a new ITS type that represents a mixture of the two original ITS sequences (Van Houten, Scarlett, and Bachmann 1993; Wendel, Schnabel, and Seelanan 1995*b*; Mummenhoff, Franzke, and Koch 1997; Franzke and Mummenhoff 1999; Koch and Al-Shehbaz 2000); (3) both ITS copies are still present, which might be mostly the case in young hybridogenous taxa (Kim and Jansen 1994; O'Kane, Schaal, and Al-Shehbaz 1996; Koch and Al-Shehbaz 2000). However, the third possibility has also been described in the genus *Rosa* as a stable situation and has been defined as "nonconcerted" evolution (Wissemann 1999; 2000). In some cases these paralogues are old and might have lost their function and turned into pseudogenes (Buckler and Holtsford 1996; Hartmann, Nason, and Bhattacharya 2001; Muir, Fleming, and Schlötterer 2001). Functional analysis on the transcriptional level provided additional insight into concerted evolution of rDNA (Chen and Pikaard 1997; Chen, Comai, and Pikaard 1998; Frieman et al. 1999; Muir, Fleming, and Schlötterer 2001) and the phenomenon of nucleolar dominance (Volkov et al. 1999).

We used a phylogenetically complex model system of naturally occurring hybrids within North American *Arabis*. Taxonomically, the species under study should be included in the genus *Boechera* (Koch, Bishop, and Mitchell-Olds 1999) and are not related to Eurasian *Arabis* (Koch, Haubold, and Mitchell-Olds 2000). But because of the traditional taxonomic treatment of these taxa herein, we continue to refer to them as *Arabis*: *Arabis drummondii*, a widespread North American perennial, mostly diploid, and *Arabis holboellii*, often apomictic on the triploid chromosomal level and also widespread on the North American continent, the putative parental taxa of *Arabis divaricarpa*. In contrast to *Arabis drummondii*, which contains only limited morphological variation, several variable morphological characters led to the recognition of multiple varieties among *Arabis holboellii* (Rollins 1993). However, *Arabis divaricarpa* is an intermediate morphotype between the parental taxa. Because of the wide distribution of the hybrid and its occurrence in pristine habitats, this system is ideally suited to the study of hybrid speciation on a broad scale and in an evolutionary context. Here, to gain some insight into rDNA evolution in natural populations on a temporal and spatial scale, we have chosen the ITS region of rDNA to study the fate of parental ITS copies in hybrids and their offspring.

This study is based exclusively on plant specimens from several herbaria, and we aim to demonstrate the value of this source of biological variation for fundamental, molecular-based research.

Materials and Methods

Plant Material

Material of all individuals analyzed in this study originated from material of the herbaria in St. Louis, Mo. (MO) and Cambridge, Mass. (GH) and is listed in *Supplementary Material* online. Both herbaria house a broad collection of specimens from North American representatives of the genus *Arabis*. In total we analyzed 402 accessions covering the entire geographic range of the three taxa in North America (fig. 1). In total, 85 and 187 accessions of the parental taxa *A. drummondii* and *A. holboellii*, respectively, have been analyzed. For *A. divaricarpa*, 130 accessions have been investigated. In most cases each accession (population) is represented by a single individual. In a few cases several different individuals from one population (mounted on the same sheet of herbarium paper) have been investigated to look for additional within-population diversity (indicated in Supplementary Material online). However, the primary focus of this study is the total geographic distribution of the taxa; therefore, within-population variations have been examined only sporadically.

DNA Amplification and Sequencing

Total DNA was obtained from approximately 50–75 mg of dried leaf tissue of the specimens from single individuals by a CTAB procedure (Doyle and Doyle 1997). Homogenization of the leaf material was performed using a mixer mill MM 200 (Retsch, Germany) in 2 ml reaction tubes with 3.0 mm glass beads. Polymerase chain reaction (PCR) was carried out using an ABI 9700 (Applied Biosystems). The PCR cycling scheme was 5 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 48°C, and 1 min at 72°C; a 10-min extension at 72°C; and a final hold at 4°C. The oligonucleotide sequences used to amplify the complete ITS region, including the 5.8 S rDNA region, were designed by White et al. (1990) and modified by Mummenhoff, Franzke, and Koch (1997) for ITS4. The forward primer is located at the 3′ end of the 18 S rDNA gene (5′-GGAAGGAGAAGTCGTAACAAGG-3′), and the reverse primer is located at the 5′ end of the 25 S rDNA gene (5′-TCCTCCGCTTATTGATATGC-3′). PCR products were purified with a Boehringer PCR

product purification kit (Roche Molecular Biochemicals). PCR products spanned the entire ITS1, 5.8 S rDNA, and ITS2 regions and were cycle-sequenced directly without cloning using a Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.) and the amplification primers. Products of the direct cycle sequencing reactions were run on an ABI 377XL automated sequencer.

All primary PCR products that resulted in ambiguous ITS sequences were subsequently cloned into the pGEM-T easy-cloning vector (PROMEGA) in order to separate ambiguous sites among the differing intraindividual ITS copies (*A. drummondii*: one of 85 accessions; *A. holboellii*: 36 of 187 accessions; *A. divaricarpa*: 34 of 130 accessions). Five to ten clones per each amplification product have been isolated, and ITS inserts have been reamplified and sequenced using the original ITS primer described above. This sequencing was performed using a Thermo Sequenase cycle sequencing kit (rpn2438 from Amersham Pharmacia Biotech) with IRD700- and IRD800-labeled primers (MWG Biotech, Ebersberg, Germany). Products of these direct cycle sequencing reactions were run on a LICOR L4200S-2 automated sequencer.

Analysis of Ploidy Levels

It is impossible to determine chromosome numbers from herbarium specimens directly. Therefore, we used an indirect procedure to obtain evidence of ploidy distribution in the taxa under study. It has been shown that there is a strong correlation of ploidy levels with morphometric parameters such as stomata density, branching pattern of trichomes, or size of pollen grains. We used pollen grain size as an indicator of ploidy level by measuring length and width of the pollen grains under a microscope. Mature pollen was sampled from flowers of single specimens. We measured 20 to 30 pollen grains per individual examined. The dimensions of *Arabis* pollen grains range from 15 to 36 μm. Additionally, pollen quality was summarized in six categories, for which we estimated the percentage of highly degraded pollen (subsequently converted into percentage of well-developed pollen: 0%, 0%–10%, 10%–50%, 50%–80%, 80%–90%, 90%–100%, based on more than 100 pollen grains) as an indicator for pollen sterility, which is frequently accompanied by apomixis in *Arabis* species.

Data Analysis

Alignment and Outgroup Comparisons

Alignment was performed by hand, and ITS sequence data were submitted to GenBank (AF165313–AF165439). Automated alignments were not necessary because of nearly identical sequence lengths in the ingroup taxa. We used ITS sequences from *Cusickiella douglasii* (AF146515) and *C. quadricostata* (AF146514) (Koch and Al-Shehbaz 2002), *Halimolobus perplexa* ssp. *lemhiensis* (AJ232927) and *H. perplexa* ssp. *perplexa* (AJ232926) (Koch, Bishop, and Mitchell-Olds 1999), and from *Polyctenium fremontii* (AF183109) (Roy 2001) for outgroup comparisons.

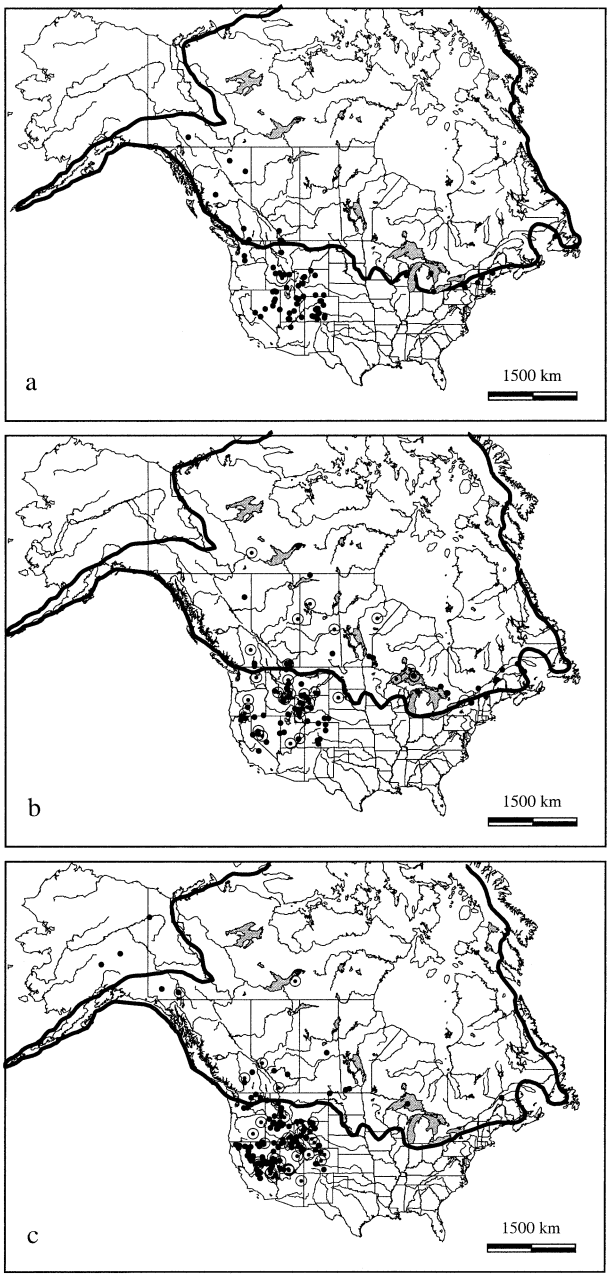

FIG. 1.—Location of accessions investigated. (*a*) *Arabis drummondii*, (*b*) *A. divaricarpa*, (*c*) *A. holboellii*. Accessions for which multiple ITS types have been found in cloned PCR products are marked by circled dots. The maximum extension of the last glacier (Wisconsin glaciation) is indicated by a solid line.

Sequence Conservation and Secondary Structure Analysis of ITS Regions

Secondary structures were generated for the ITS1 and ITS2 regions separately under the RNA folding option using the online version 3.0 of Mfold (Jaeger, Turner, and Zuker 1989; Zuker 1989; Zuker, Mathews, and Turner 1999) and the standard options. The Mfold server is accessible under the URL http://bioinfo.math.rpi.edu/~zukerm/ or alternatively under http://mfold.burnet.edu.au/. The distributions

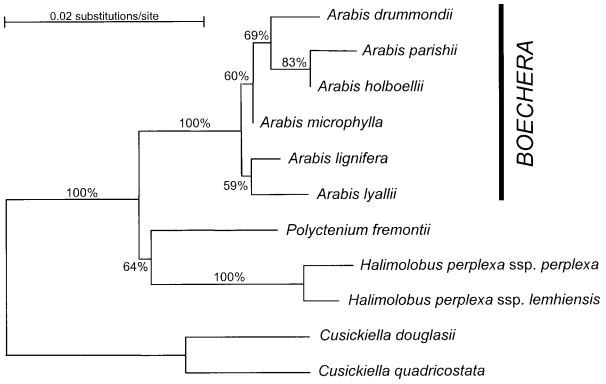

FIG. 2.—Neighbor-Joining distance tree of ITS sequences demonstrating the phylogenetic position of the taxa under study. Bootstrap support is given along the corresponding branches.

of variable nucleotide positions were subsequently mapped onto the plotted secondary structures of the ITS1 and ITS2 from ingroup and outgroup taxa. A sliding window analysis to test recombination and analysis of linkage disequilibrium of the DNA sequences has been performed with DnaSP software version 3.51 (Rozas and Rozas 1999).

Phylogenetic Analysis

To analyze the relative position of the outgroups, a Neighbor-Joining distance analysis was conducted with all five outgroups and sequences from *Arabis drummondii*, (AJ232887), *A. holboellii* (accession 009, *Supplementary Material* online), and some additional related *Arabis* taxa (Koch, Bishop, and Mitchell-Olds 1999: *A. lyallii* AJ232897, *A. lignifera* AJ232898, *A. microphylla* AJ232929, *A. parishii* AJ232901). The analyses were run using TREECON for Windows (version 1b [Van de Peer and De Wachter 1997]). Distance matrices according to Kimura (1980) were generated using the GAPS INCLUDED option. The bootstrap option (1,000 replicates) was used to assess relative support of the branching pattern. Phylogenetic analysis of ITS sequences of the various *Arabis* accessions has been performed in two ways: (1) only sequences of the parental taxa *A. drummondii* and *A. holboellii* were considered in obtaining information about within-species variation; (2) in addition all *A. divaricarpa* sequences were considered to test their phylogenetic position with respect to their parents.

Results

ITS Length Variation, Outgroup-Ingroup Alignment, and Outgroup Comparisons

The length of the ITS regions including the 5.8 S rDNA gene is highly conserved among the taxa studied (ITS1:5.8 SrDNA:ITS2 [length in bp]: *Polyctenium*: 268:164:190; *Cusickiella*: 266:164:193; *Halimolobus*: 267:164:187; *Arabis*: 267:164:190). For phylogenetic comparisons using the outgroups and testing their phylogenetic position, we produced an alignment 626 bp in length (ITS1: 268 bp, 5.8 SrDNA: 164 bp, ITS2: 194 bp) and with 10 gaps included (2 in ITS1 and 8 in ITS2) (see *Supplementary Material* online). It should be kept in mind, however, that only some *Arabis* have been considered for this analysis, and therefore numbers of variable sites from the outgroup analysis differ from the analysis considering all *Arabis* ITS sequences analyzed in this study and described below. Of the 626 nucleotides, 563 were constant. Of the remaining 63 variable sites, 34 were parsimony-informative, and 29 variable positions occurred only once (ITS1: 26 parsimony-informative and 11 unique sites; 5.8 SrDNA: 2 parsimony-informative and 2 unique sites; ITS2: 6 parsimony-informative and 16 unique sites). The Neighbor-Joining distance tree is shown in figure 2 (A parsimony analysis yielded the same topology, data not shown.) *Cusickiella* is the most distantly related taxon compared to the group of *Arabis* species, and the *Halimolobus* and *Polyctenium* taxa investigated herein represent a separate lineage.

ITS Type Variation Within *Arabis drummondii*, *A. holboellii*, and Its Hybrid *A. divaricarpa*

We used an alignment similar to that shown in figure 1 of the *Supplementary Material* to analyze all ITS types detected within *Arabis drummondii*, *A. holboellii*, and *A. divaricarpa* (see *Supplementary Material*). Because of additional DNA length heterogeneity among the ingroup sequences, however, we had to introduce several gaps into the overall alignment. In total we detected 127 different ITS types, which were distributed among the several species as follows: *A. drummondii* with 14, *A. holboellii* with 62, and *A. divaricarpa* with 77 ITS types (table 1; *Supplementary Material* online). Not surprisingly, more than 97% of the accessions of *A. drummondii* have only a single ITS type. In the second parental taxon, *A. holboellii*, however, the number of accessions with only a single ITS copy is 79%, and, therefore it is closer to the hybrid *A. divaricarpa*, in which 62% of accessions show a unique ITS type. The distribution of multiple ITS copies in single individuals is shown in figure 3. These data demonstrate that in *A. divaricarpa* more than 50% of the accessions with intraindividual ITS variation contain three or more ITS copies. In *A. holboellii* accessions with multiple ITS copies, we found predominantly two ITS copies. Finally, only one *A. drummondii* accession contained two ITS types. The distribution of the variable nucleotides along the alignment and among the three taxa is shown in figure 4. This analysis demonstrates that *A. divaricarpa* resamples the variation of the parental taxa with the exception of sites 29, 149, 219, 243, 506, 509, and 605. At those sites, however, the frequency of the variable nucleotide is below 1%, and it is most likely that the corresponding mutation in the parental taxa was not detected because of undersampling. The distribution of variable nucleotide sites also demonstrates that within larger proportions of the alignment (between positions 140 and 267 and between positions 462 and 559) *A. divaricarpa* resamples only variable positions from *A. holboellii*. The remaining alignment shows a mosaic distribution of variable nucleotides in *A. divaricarpa*, which probably originated from either *A. drummondii* or *A. holboellii*.

Table 1
Frequency of Observed ITS Types ($n = 127$) Among *Arabis* Species Under Study

Type	dr	di	ho	Type	dr	di	ho	Type	dr	di	ho	Type	dr	di	ho	Type	dr	di	ho
B	1	—	—	AC	—	—	19	BJ	—	1	1	DG	—	1	—	FH	—	1	—
C	12	6	—	AD	—	3	4	BK	—	1	—	DH	—	1	—	FI	—	—	1
D	3	—	—	AG	—	—	5	BL	—	1	—	DI	—	1	—	FJ	—	—	1
E	11	28	1	AH	—	—	1	BN	—	1	—	DJ	—	2	—	FL	—	1	—
F	—	1	14	AI	—	—	1	BO	—	1	—	DL	—	1	—	FN	—	1	—
G	—	—	5	AJ	—	—	2	BP	—	1	—	DM	—	1	—	FO	—	1	—
H	—	25	88	AK	—	—	1	BR	—	1	—	DN	—	2	—	FQ	—	1	—
I	—	4	8	AM	—	—	1	BT	—	3	17	DO	—	1	—	FR	—	1	—
K	—	3	—	AN	—	—	4	BV	—	—	1	DP	—	1	—	FT	—	1	—
L	18	19	2	AO	—	—	8	BW	—	1	2	DQ	—	1	—	FW	—	1	—
M	1	—	—	AP	—	—	5	BX	—	1	5	DS	—	1	—	FY	—	2	—
N	—	4	—	AQ	—	—	1	CA	1	1	—	DT	—	—	1	FZ	—	—	1
O	—	1	—	AR	—	—	3	CM	1	—	—	EA	—	1	—	GB	—	—	1
P	—	1	—	AS	—	—	1	CQ	1	—	—	EE	—	1	1	GD	—	—	1
Q	—	2	—	AT	—	—	1	CS	—	—	1	EH	—	1	—	GF	—	—	1
R	7	4	2	AU	—	—	7	CT	—	—	1	EI	—	1	—	GG	—	—	1
S	1	6	—	AW	—	—	2	CU	—	1	1	EJ	—	1	—	GM	—	1	—
T	7	12	—	AX	—	—	1	CV	—	1	—	EK	—	3	—	GQ	—	—	1
U	—	2	—	AZ	14	14	2	CX	—	1	—	EL	—	1	—	GR	—	—	1
V	8	3	1	BC	—	1	—	CY	—	2	—	EM	—	1	—	GS	—	—	1
W	—	2	3	BD	—	1	—	CZ	—	1	—	ER	—	—	1	GU	—	—	1
X	—	—	1	BE	—	2	—	DB	—	1	—	EU	—	—	1	GV	—	—	1
Y	—	7	9	BF	—	1	—	DC	—	1	—	EV	—	—	2	GW	—	—	1
Z	—	—	5	BG	—	1	—	DD	—	1	—	EW	—	—	1				
AA	—	—	2	BH	—	1	—	DE	—	1	—	EX	—	—	1				
AB	—	—	5	BI	—	1	—	DF	—	1	—	FB	—	—	1				

NOTE.—dr = *Arabis drummondii*, di = *Arabis divaricarpa*, ho = *Arabis holboellii*.

In phylogenetic analysis with the outgroups and the parental taxa *A. drummondii* and *A. holboellii*, all the *A. drummondii* accessions grouped together and formed a monophyletic assemblage (results not shown). Not unexpectedly, however, in a subsequent analysis including all *A. divaricarpa* populations, these latter accessions were positioned throughout the phylogenetic tree (results not shown).

Pollen Measurements, Pollen Quality, and Ploidy Levels

A canonical discriminant analysis evaluating means of pollen grain length and breadth has been conducted for the three *Arabis* taxa under study. The results are shown in figure 5. Major differences can be detected among the taxa: *Arabis drummondii* is mostly diploid with only a few triploid and tetraploid individuals. In contrast, *Arabis holboellii* shows a remarkably high proportion of triploid individuals. However, according to the pollen size measurements, numerous diploid accessions of *A. holboellii* have been detected. In contrast, the putative hybrid *A. divaricarpa* had very few diploid individuals, and most genomes exist at the triploid level. Remarkably, in the case of *A. drummondii* and *A. holboellii*, the slope of the pollen size distribution is relatively steep compared to *A. divaricarpa*. We took this as strong evidence that in *A. divaricarpa* aneuploids are more frequent than in the parental taxa (fig. 5). This finding is in congruence with cytological data compiled by Rollins (1993). Herein numerous reports of aneuploids and the occurrence of B chromosomes can be found.

Pollen quality may be correlated with male fertility and apomixis (Sharbel and Mitchell-Olds 2001). Among the *Arabis* taxa there are significant differences, as shown in figure 6. In *Arabis drummondii* more than 90% of the accessions had more than 80% well-developed pollen. In *A. holboellii* less than 50% of the accession showed quantities of well-developed pollen greater than 80%, and there is a strong association of ploidy level (diploid versus polyploid) and pollen quality with well-developed pollen found in diploid individuals ($\chi^2 = 55.5$, $df = 4$, $P < 0.001$). In the case of *A. divaricarpa*, most of the pollen was degraded morphologically, and it must be assumed that most of that pollen is sterile.

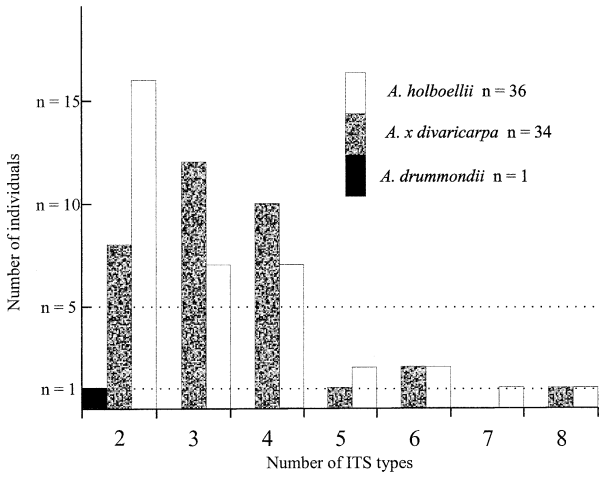

FIG. 3.—Frequency distribution of the number of different ITS types observed in single individuals.

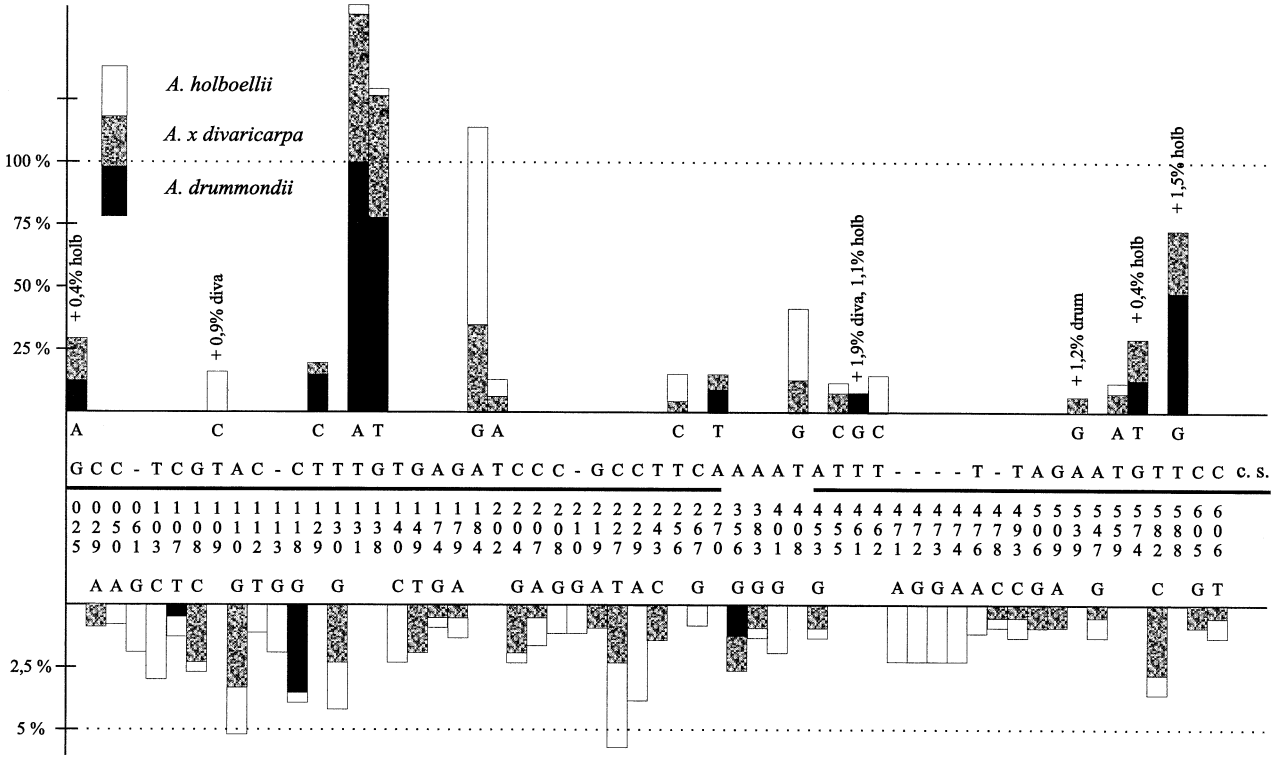

FIG. 4.—Frequency of bases (5) at variable ITS sites among the species under study. Upper histogram shows positions at which a variation of 5% or higher was observed within at least one of the three taxa. Lower histogram represents frequencies below the 5% level. Bases occurring only once in the whole data set were excluded. Bold underscoring on the left of the consensus sequence marks the range of ITS1; that on the right, the range of ITS2.

Sequence Conservation, Secondary Structure of ITS1 and ITS2 Regions, and Pseudogenes

It was not possible to obtain a consensus minimum energy secondary structure of ITS1 types from all taxa under study including the outgroups. Suboptimal foldings also differed to such an extent that the calculation of consensus structures was not possible. Furthermore, suboptimal foldings of the ITS1 of the several taxa differed substantially from one another. However, the energy (dG [kcal/mole]) of each of the several ITS1 structures of the outgroups at 37°C folding temperature is in the same order of magnitude (*Cusickiella* sp.: –98.3; *Polyctenium*: –96.4; *Halimolobus* sp.: –101.6) as for the *Arabis* ITS1 types (mean –100.7, SD = 2.44,) and major proportions of the DNA sequence are highly conserved. Because *Cusickiella* is the most distantly related outgroup in this study compared to *Arabis* (fig. 2), we have chosen the ITS1 of *Cusickiella douglasii* to demonstrate the distribution of variable nucleotide positions (*Cusickiella* versus *Arabis* and *Halimolobus/Polyctenium*) plotted onto its secondary structure (fig. 7*a*). This plot is compared to the distribution of variable sites among *Arabis* accessions only (fig. 7*b*). The position of conserved and variable regions is congruent with those shown in figure 4. The secondary structure analysis (fig. 7*a* and *b*) allows the recognition of four different regions (marked I to IV), which show similar structure among all taxa under study. A highly conserved 20–23 bp motif observed in ITS1 of angiosperms (Liu and Schardl 1994) is present in region III (fig. 7*a* and *b*). Because secondary structures of the ITS1 among the accessions under study provided no further evidence of structured variation within and among taxa, we tested several hypotheses using the distribution of the calculated free energy values of the several ITS1 types. A t-test indicated significant differences in free energy distribution for the species pairs *A. drummondii/A. holboellii* ($P < 0.001$) and *A. divaricarpa/A. holboellii* ($P < 0.001$). The differentiation between *A. divaricarpa/A. drummondii* was not significant.

The secondary structure of the ITS2 was highly conserved among all taxa under study including the outgroups. We found a characteristic structure with four helical regions (fig. 8*a* and *b*). This structure is conserved among green algae and flowering plants (Mai and Coleman 1997; Denduangboripant and Cronk 2001). Within the four helical regions, even the distribution of stem and loops is very similar when *Cusickiella douglasii* ITS2 (fig. 8*a*) is compared with the *Arabis* ITS2 structure (fig. 8*b*). As reported from green algae and flowering plants (Mai and Coleman 1997; An, Friedl, and Hegewald 1999), we also found two conserved motifs in helix II (pyrimidine-pyrimidine juxtaposition pairing) and helix III (GGU trinucleotide motif). Analysis of the distribution of ITS2 free energy provided similar results as for the ITS1 (data not shown). From the distribution analysis of variable sites summarized in figure 4 we concluded that between nucleotide positions 140–267 and 462–559, *A. divaricarpa* resembles nucleotides of *A. holboellii* only, and it is noteworthy that the second region (positions 462–559) located in the ITS2 covers exactly half of the stem of region I, all of region II including the

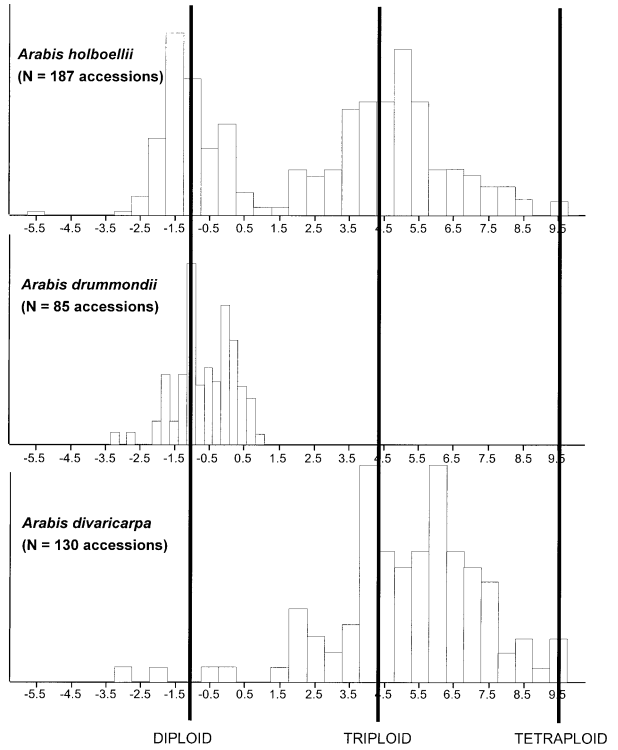

FIG. 5.—Canonical discriminant function 1 evaluated at the means of pollen grain length and breadth.

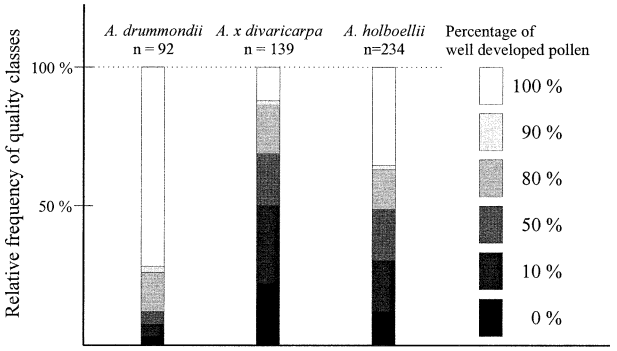

FIG. 6.—Pollen quality in *Arabis drummondii*, *A. divaricarpa*, and *A. holboellii*. The estimated percentage of well-developed pollen was assigned to six classes. Bars show the distribution of these classes among the three taxa.

pyrimidine-pyrimidine juxtaposition, and half of the stem of region III, ending with the conserved GGU trinucleotide motif (indicated in fig. 8*b*). This observation might explain recombination breakpoints located in hairpin return regions. The situation for the ITS1 is not as significant as for the ITS2, and here the "*A. holboellii* typical region" (position 140–167) comprises only the second half of the stem within region II—beginning right after a loop, all of region III including the highly conserved 20–23 bp region, and all of region IV.

The accumulation of mutations is an evolutionary process, which should be reflected when several different taxa are compared. If the number of sites among the different regions varying in *Arabis* is compared to the number of sites varying in the whole data set, some differences are obvious (table 2). Most regions within the alignment evolved as one would expect, with higher diversity (number of mutations/bp) when considering all taxa including the outgroups. These four regions span the flanking regions of ITS1 and ITS2. The most highly conserved regions are within regions II and III of ITS1 and region II of ITS2, all of which contain a conserved motif. Here the number of mutations/bp does not differ significantly between the two data sets (within *Arabis* only versus the total data set).

As outlined above, several sequence variants have been characterized within numerous individuals (fig. 3). However, these paralogues might become pseudogenes, a process driven either by inactivation or transposition of ITS copies to other genomic regions. It can be assumed that if ITS sequence variants occur, they should accumulate random substitutions, which will result in reduced secondary structure stability. The corresponding analysis did not find any evidence for this reduction in secondary structure stability, likely because these new ITS types are too young in an evolutionary sense and, therefore, have not yet accumulated mutations.

Discussion

ITS Type Evolution

We have characterized several paralogues within numerous individuals. These paralogues might become pseudogenes. If ITS pseudogenes occur, they should accumulate random substitutions, which will result in reduced secondary structure stability (Buckler, Ippolito, and Holtsford 1997). This approach has been used successfully to determine ITS types grouped into class I (high secondary structure stability and low free energy) and class II (low secondary structure stability and high free energy: putative pseudogenes) for *Gossypium* and *Aconitum* (Buckler, Ippolito, and Holtsford 1997; Kita and Ito 2000). In these studies the estimated free energy for the class I ITS1 types varied between –97.6 and –85.0 kcal/mole. This is within an order of magnitude of our calculated mean free energy of –100.7 (SD = 2.44). For putative pseudogenes in *Aconitum* the free energy of the ITS1 has been estimated at –3.8 to –63.3 kcal/mole (Kita and Ito 2000). In the examined *A. divaricarpa* hybrid system we found no evidence for pseudogenes. However, the functional fate of multiple ITS copies found in *A. divaricarpa* as well as in *A. holboellii* can only be established with certainty once processed RNA has been investigated via reverse transcriptase PCR (e.g., Muir, Fleming, and Schlötterer 2001). Some correlations have been found between structural properties and the distribution of nucleotide diversity within the ITS2. Here recombination breakpoints are located predominantly in loops at the hairpin returns. A similar situation was not detectable for ITS1, and one might assume that the two regions of the nrDNA operon, separated by the 5.8 S rDNA gene, evolved independently.

Nonetheless, it is clear that during speciation processes among the different taxa, hybridization, introgression, and reticulation resulted in up to eight intra-individual ITS copies in *A. holboellii* and in

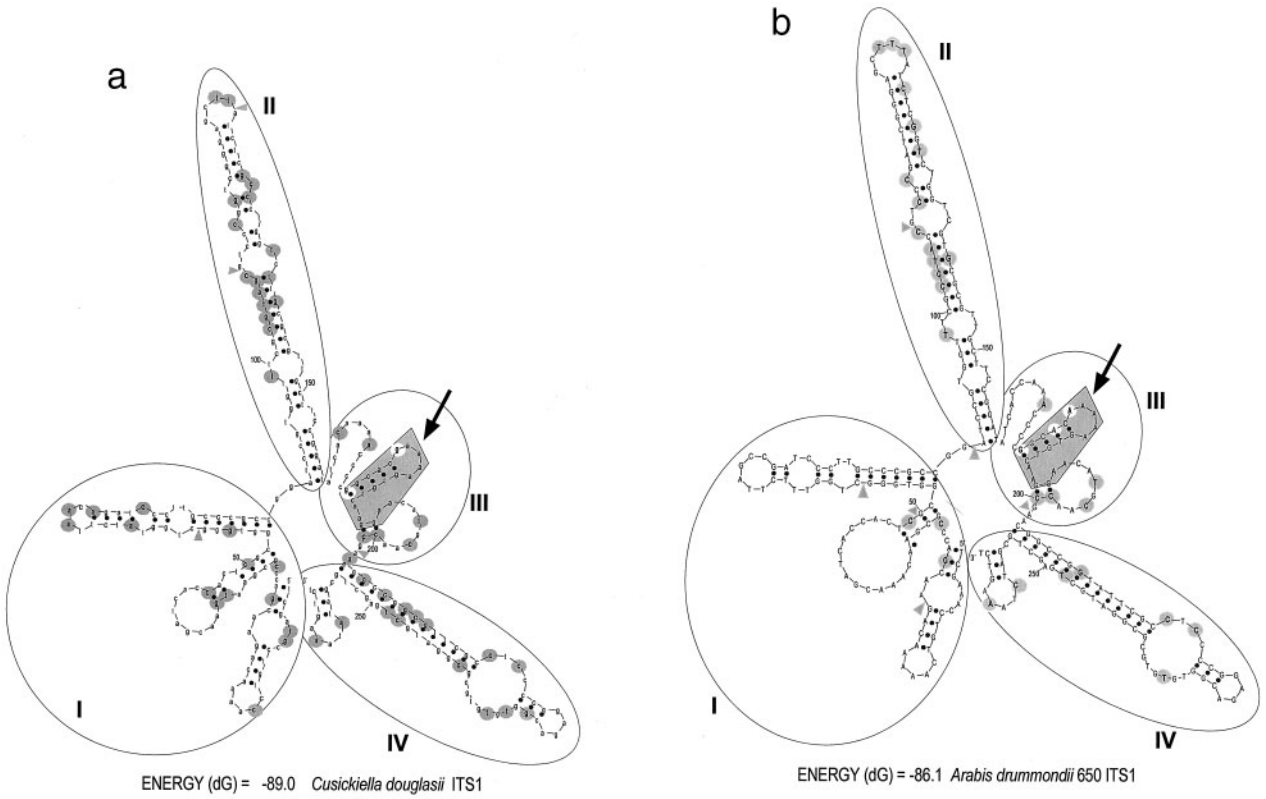

FIG. 7.—Secondary structure model of the internal transcribed spacer 1 (ITS1) of (*a*) *Cusickiella douglasii* and (*b*) and *Arabis drummondii*. All variable nucleotide positions among ingroup and outgroup taxa are highlighted in figure 7*a*. Ingroup variation only is indicated in figure 7*b*. Positions of insertions/deletions are indicated with triangles. The arrow in region III marks the location of the highly conserved region.

A. divaricarpa. If it is taken into account that most of the accessions are triploid (with and without extra chromosomes; cf. Sharbel and Mitchell-Olds 2001), the variation can be explained by intralocus variation with the two parental genotypes as the original source. Nonetheless, interlocus variation is also likely. Different modes of concerted evolution of ITS regions detected within a particular species group have been reported and discussed extensively (Wendel, Schnabel, and Seelanan 1995*a*; 1995*b*; Koch and Al-Shehbaz 2000; 2002). The same is true for the *Arabis* species hybrid system analyzed here. The morphologically defined hybrid *A. divaricarpa* is represented (1) with accessions possessing only a single ITS sequence typically found in *A. drummondii* accessions (57 accessions), (2) with accessions possessing only a single ITS type typically found in *A. holboellii* (24 accessions), (3) with accessions possessing multiple ITS types, of which at least one resamples an original *A. drummondii* ITS type but no original *A. holboellii* ITS type (7 accessions), (4) with accessions possessing multiple ITS types, of which at least one resamples an original *A. holboellii* ITS type but no original *A. drummondii* ITS type (6 examples), (5) with accessions possessing multiple ITS types, of which both parental original ITS types are still present (18 accessions), (6) with accessions possessing a single intermediate ITS type (no example found), and (7) with accessions possessing several intermediate ITS types (one accession). These different findings can be explained by dominance of one parental ITS type in cases (1) and (2). In our analysis, dominance of *A. drummondii* has been more frequently found in *A. drummondii* than in *A. holboellii*, which is also suggested by cases (3) and (4). However, dominance is not as strong as in cases (1) and (2), and additional "intermediate" ITS types have been observed. Interestingly, even here more *A. drummondii*–dominated accessions have been detected than *A. holboellii*–dominated accessions. The situation described in case (5) might demonstrate very recent hybridization, whereas cases (6) and (7), with differing complexity, illustrate evolutionary processes affected by recombinations and gene conversions leading to mosaic ITS types. The high frequency of accessions showing ITS types of case (5) suggests that many accessions of *A. divaricarpa* have been constituted "recently" and can be explained by postglacial migration, range extension, and subsequent hybridization.

The situation for *A. holboellii* is more complex. Even in this species we detected strong evidence for hybridization, introgression, and reticulation, although not as extensive as in *A. divaricarpa*. Most likely, this difference is due to existing classification. Both parental species, *A. drummondii* and *A. holboellii*, and the hybrid have been recognized based on morphological characters (fruit and trichome characteristics). It is possible, however, that after hybridization and constitution of some populations of *A. divaricarpa*, introgression and reticulation involving *A. holboellii* has also taken place. In addition, more complex reticulation patterns might be present in *A. holboellii* only. Such introgression and reticulation should have resulted in

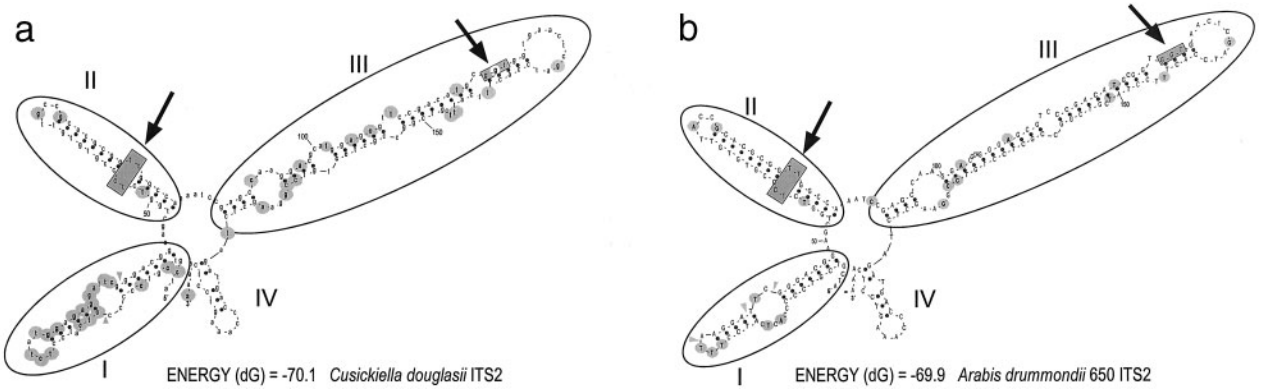

FIG. 8.—Secondary structure model of the internal transcribed spacer 1 (ITS2) of (*a*) *Cusickiella douglasii* and (*b*) and *Arabis drummondii*. All variable nucleotide positions among ingroup and outgroup taxa are highlighted in figure 7*a*. Ingroup variation only is indicated in figure 7*b*. Positions of insertions/deletions are indicated with triangles. The arrows in regions II and III marks the location of the highly conserved pyrimidine-pyrimidine juxtaposition and the GGU trinucleotide motif.

morphotypes more similar to *A. holboellii* but showing increased molecular variation. This hypothesis is supported by taxonomical-morphological investigations characterizing several varieties within an *A. holboellii* species group (Rollins 1993). Preliminary morphometric analyses (Koch and Dobeš, unpublished) support this view. Additional evidence came from pollen size measurements as an indicator of ploidy levels (fig. 5). Accordingly, most accessions carrying multiple ITS types (either *A. holboellii* or *A. divaricarpa*) turned out to be triploid (as inferred from pollen size measurements), with and without extra chromosomes. The only exceptions are diploid accessions (accession numbers 489, 736, 741, 753, 754, 761, and 762). Interestingly, these diploid accessions have been described based on morphological data exclusively as *A. holboellii* var. *retrofracta*. Here we assume that hybrid speciation on the diploid level of *A. holboellii* with another unknown parental *Arabis* species occurred, resulting in this new variety with multiple ITS types. In summary, an analysis of the several cases (1 to 7) described for *A. divaricarpa* has shown that in *A. holboellii* only 5 accessions carried typical *A. drummondii* ITS types, which might indicate either backcrosses of *A. divaricarpa* with *A. holboellii* or the rare event of introgression of *A. holboellii* into *A. drummondii* (rare because of high levels of pollen sterility in *A. holboellii*). This explanation is supported by plastome type variation characterizing these five accessions with typical *A. drummondii* plastome types (Koch and Dobeš, in preparation).

Important for the understanding of the evolutionary scenario is the mode of reproduction. Apomixis has been shown to play a major role in the evolution of North American *Arabis* (Rollins 1993, Sharbel and Mitchell-Olds 2001). This finding fits with our analysis of pollen quality (fig. 6). In correlation with the findings described above, *A. drummondii* is a mostly diploid species that produces well-developed pollen, whereas *A. divaricarpa* and, to a lesser extent, *A. holboellii* mostly produce infertile pollen. We can assume that apomixis is widely distributed in *A. holboellii* and most likely in *A. drummondii* as well. However, pollen fertility in these taxa cannot be excluded totally, as shown above. These finding are strongly supported by analysis of plastome type variation and distribution of aneuploidy in *A. holboellii* (Sharbel and Mitchell-Olds 2001). From our ITS data it can be assumed that *A. drummondii* regularly served as a paternal hybridization partner. Triploids and aneuploids predominate in *A. holboellii* and *A. divaricarpa*, which favors the hypothesis that the hybrids reproduce by apomixis. However, subsequent reticulate evolution is also apparent. In these cases, the hybrid *A. divaricarpa* most likely served as the maternal plant because of high levels of pollen sterility.

In summary, this hybrid complex shows strongly directed gene flow from *A. drummondii* into *A. holboellii* and *A. divaricarpa*. This scenario is supported by morphological data indicating a clearly defined *A. drummondii* but more variable *A. holboellii* and *A. divaricarpa*.

Phylogeography and Multiple Origin

In the context of this article we do not discuss phylogeographic aspects in detail. A comprehensive screening of maternally inherited plastidic genetic variation with more than 1,000 accessions has been finished and the published study will focus on speciation and phylogeography (Koch and Dobeš, in preparation). Here, we will concentrate on some aspects of the general spatial distribution of ITS types. If we compare the present-day distribution of those accessions with multiple ITS types found in *A. holboellii* (fig. 1), it is obvious that more complex hybrid ITS types are distributed outside the range of the last maximum glaciation in North America (Wisconsin glaciation 18,000 years ago). Only a few accessions with multiple ITS types are distributed within this range; moreover, all those accessions showed ITS types differing by a single mutation only. In contrast, in *A. divaricarpa* numerous accessions within the formerly glaciated area carry multiple, hybrid ITS types. This scenario is best explained by the assumption that hybrids formed postglacially in relic areas southwest of the maximum ice shield and followed the retreating glaciers in a north and north-east direction.

Many other accessions of *A. divaricarpa* within the formerly glaciated area have only a single ITS type (either

Table 2
Number of Variable Nucleotide Positions per 10 bp Among (1) the Ingroup Taxa Only, and (2) Among Ingroup Plus Outgroup Taxa and the Corresponding Quotient (i/o)

	Region	(1) Ingroup	(2) Outgroup	i/o
ITS1	I	0.7	1.8	2.7
	II	2.4	2.7	1.1
	III	2.0	2.0	1.0
	IV	1.1	2.5	2.3
ITS2	I	2.1	4.9	2.3
	II	0.9	0.9	1.0
	III	1.2	2.0	1.7
	IV	0.05	0.0	~1.0

NOTE.—Refer to figure 7*a* and figure 8*a* and *b* for the positions of the different regions.

a fixed parental type or a fixed mosaic structured type), which indicates ongoing hybridization and reticulation. A few accessions distributed within the formerly glaciated area have ITS types that suggest a single recombination event between two parental copies from *A. drummondii* and *A. holboellii* from the same area. These findings suggest that, from a glacial refugium southwest of the maximum ice shield of the Wisconsin glaciation, *Arabis* started the recolonization of northern parts of its current range. Both, *A. drummondii* and *A. holboellii* recolonized the formerly glaciated areas successfully. Sequence data from the plastome indicating maternal seed dispersal and migration agree with this hypothesis (Koch and Dobeš, in preparation), and for *A. drummondii* they show a two-way migration of two different plastome types of *A. drummondii*, resulting in a western clade distributed along the mountain regions in the western United States and an eastern clade following the Great Lakes.

For *A. holboellii*, such a simple migration scenario was not observed. Instead, different plastome types are distributed randomly in this taxon, and plastome type diversity decreased with increasing distance from the assumed relic area in the southwestern United States. However, overall present-day distribution and the occurrence of several diverse ITS types in Alaska might indicate that for this taxon a second glacial refugium existed in unglaciated areas of Alaska and the Great Lakes. However, this hypothesis has to be substantiated by analysis of plastome type variation and microsatellite variation (Koch and Dobeš, in preparation).

Disjunct populations of *A. holboellii* have been described from the St. Lawrence River Valley in Québec (Böcher 1951), which may have been a glacial refugium (Marie-Victorin and Rolland-Germain 1964). Typical refugia south of the glacial maximum have been recognized, such as the Klamath-Siskiyou Mountains in California (Whittaker 1961; Smith and Sawyer 1988). Several other possible northern refugia have been reviewed in Soltis et al. (1997) and comprise coastal refugia such as northwestern Vancouver Island or the Queen Charlotte Islands.

North American *Arabis* (*Boechera*) as a Study System

The North American *Arabis* are an ideal model system in which to study historical and ongoing evolutionary processes. In this genus many different species have been described (Rollins 1993), providing a suitable source for morphological, ecological, and physiological variation. Some of this variation has been used recently to study evolutionary aspects such as phenotypic plasticity and adaptation (McKay et al. 2001), apomixis (Roy 1995; Roy and Rieseberg 1989; Sharbel and Mitchell-Olds 2001), flower biology and host-pathogen interaction and evolution (Roy 2001), or the evolution of genes and gene families (Bishop, Dean, and Mitchell-Olds 2000; Koch, Haubold, and Mitchell-Olds 2000). The distribution pattern of the species indicates postglacial range extension and recolonization. However, the existence of a center of species diversity in the southwestern United States, which is also a center of molecular diversity (within and between species) suggests multiple range fluctuations during the Pleistocene. Preliminary plastid data showed that within-species variation is comparable with between-species variation (Koch and Dobeš, in preparation), and, moreover, many plastome types are distributed across species. Two explanations for this are possible. First, extensive hybridization and reticulation may have occurred. Second, all of the genetic variation was present before pleistocenic speciation of the genus started, and plastome types have been distributed randomly among the several lineages. We believe that both explanations need to be invoked to describe the distribution of molecular variation and the complex evolutionary scenario. Reticulation and hybridization have been demonstrated herein for the *A. divaricarpa* example. However, the existence of a diverse "ancient" gene pool is supported by cpDNA sequence distances. ITS sequences of the different *Arabis* species used for outgroup analysis (fig. 2) revealed pairwise DNA sequence differences ranging from 0.3% to 1.3%. These are values that have been expected for Pleistocene differentiation. Although molecular clock hypotheses are still under debate, for ITS a substitution rate of approximately 0.5% to 2.5% nucleotide divergence per 1 million years can be assumed (for comparison: highest substitution rates with 5.3×10^{-9} substitutions/site/year, Wendel, Schnabel, and Seelanan 1995*a*; lowest rates with 0.35×10^{-9} substitutions/site/year, Suh et al. 1993). In the case of the North American *Arabis* these literature-based comparisons of ITS substitution rates would account for Pleistocene speciation. In strong contrast, plastidic sequence variation of the *trn*L intron and the *trn*L-F spacer region from the different populations exceed those of the ITS region by far (up to 2.1% sequence differences), although these sequences evolve with much lower substitution rates than the ITS region.

The closest relatives that can serve as outgroups from the genera *Halimolobus*, *Cusickiella*, or *Polyctenium* are also restricted to the North American continent. Phylogenetic hypothesis among these taxa are available and robust (Koch, Bishop, and Mitchell-Olds 1999; Koch, Haubold, and Mitchell-Olds 2000; 2001). In contrast to numerous other species selected for fundamental research in plant sciences, the North American *Arabis* are mostly restricted to pristine habitats. This enables us to study evolutionary processes in nature not biased by direct human activities.

Acknowledgments

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