Nonuniform Concerted Evolution and Chloroplast Capture: Heterogeneity of Observed Introgression Patterns in Three Molecular Data Partition Phylogenies of Asian *Mitella* (Saxifragaceae)

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Interspecific hybridization is one of the major factors leading to phylogenetic incongruence among loci, but the knowledge is still limited about the potential of each locus to introgress between species. By directly sequencing three DNA regions: chloroplast DNAs (*matK* gene and *trnL-F* noncoding region), the nuclear ribosomal external transcribed spacer (ETS) region, and internal transcribed spacer (ITS) regions, we construct three phylogenetic trees of Asian species of *Mitella* (Saxifragaceae), a genus of perennials in which natural hybrids are commonly observed. Within this genus, there is a significant topological conflict between chloroplast and nuclear phylogenies and also between the ETS and the ITS, which can be attributed to frequent hybridization within the lineage. Chloroplast DNAs show the most extensive introgression pattern, ITS regions show a moderate pattern, and the ETS region shows no evidence of introgression. Nonuniform concerted evolution best explains the difference in the introgression patterns between the ETS region and ITS regions, as the sequence heterogeneity of the ITS region within an individual genome is estimated to be twice that of an ETS in this lineage. Significant gene conversion patterns between two hybridizing taxa were observed in contiguous arrays of cloned ETS-ITS sequences, further confirming that only ITS regions have introgressed bidirectionally. The relatively slow concerted evolution in the ITS regions probably allows the coexistence of multiple alleles within a genome, whereas the strong concerted evolution in the ETS region rapidly eliminates heterogeneous alleles derived from other species, resulting in species delimitations highly concordant with those based on morphology. This finding indicates that the use of multiple molecular tools has the potential to reveal detailed organismal evolution processes involving interspecific hybridization, as an individual locus varies greatly in its potential to introgress between species.

Introduction

As molecular data concerning organismal evolutionary processes accumulates, the transfer of genetic materials from one species to another by hybridization, termed introgression, has been found to be a widespread and important evolutionary process in both animals (Bullini 1994; Hatta et al. 1999; Grant and Grant 1992, 2002; Machado et al. 2002; Machado and Hey 2003; Tosi, Morales, and Melnick 2003) and plants (Arnold, Buckner, and Robinson 1991; Aguilar, Rosselló, and Feliner 1999*a*; Doyle et al. 2003; Choler et al. 2004). Many plant lineages are thought to have experienced hybridization events frequently throughout their genealogical histories. Ellstrand, Whitkus, and Rieseberg (1996) surveyed five major biosystematic flora, finding that 16% to 34% of native plant families have at least one recognized hybrid. Interspecific hybridization followed by repeated backcrossing causes genomic mixing between species, creating ambiguities in phylogenetic inference based on the use of any single locus or linkage group. In extreme instances, interspecific hybridization forms novel species with or without polyploidization (Rieseberg 1991; Rieseberg, Van Fossen, and Desrochers 1995; Sang, Crawford, and Stuessy 1995; Wolfe, Xiang, and Kephart 1998; Cronn, Small, and Wendel 1999; Ferguson and Sang 2001; Doyle et al. 2003; Smedmark et al. 2003), which further complicates the reconstruction of historical relationships of organisms that are based on a limited amount of molecular data.

Recent studies have focused on phylogenetic incongruence between different loci caused by introgressive hybridization: the focus has been, in particular, on incongruence between plant chloroplast and nuclear DNA (Soltis and Kuzoff 1995; Soltis, Johnson, and Looney 1996; Baum, Small, and Wendel 1998; Setoguchi and Watanabe 2000; Yoo, Lowry, and Wen 2002) and between animal mitochondria and nuclear DNA (Sota and Voglar 2001). However, knowledge is still limited about the potential of each locus to introgress between species. Whereas incongruence between cytoplasmic and nuclear DNA can be explained by different modes of inheritance, the likelihood of different nuclear loci intermixing between species and the genetic processes involved in creating this difference are still largely unknown (but see Rieseberg, Linder, and Seiler [1995]). In this study, we show that interspecific hybridization among Asian species of *Mitella* has caused marked incongruence of phylogenetic signals between chloroplast (the *matK* gene and the *trnL-F* noncoding region) and nuclear loci, as well as between closely linked nuclear loci (ribosomal internal transcribed spacer [ITS] and external transcribed spacer [ETS]). We also show that the observed difference in phylogenetic signals between the ETS and ITS regions can be attributed to differential intensities of concerted evolution acting on each locus after introgressive hybridization.

Concerted evolution is the homogenization of sequences among different copies of the same gene family or reiterated sequences across the entire genome through unequal crossover or gene conversion process (Zimmer

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et al. 1980; Elder and Turner 1995; Wendel, Schnabel, and Seelanan 1995). Although there are hundreds to thousands of repeats of the ETS and ITS regions of the 18S-5.8S-26S nuclear ribosomal DNA (rDNA) cistron in the plant genome, these regions are thought to undergo strong concerted evolution that eliminates sequence variation among the different copies. The resultant intragenomic sequence uniformity of rDNAs is a major advantage in phylogenetic reconstruction (Baldwin et al. 1995),and has led to frequent use of ETS-based and ITS-based phylogenetic analyses in plant systematic studies (Álvarez and Wendel 2003). However, very little is known about how concerted evolution maintains and eliminates sequence variation after interspecific hybridization. Clarifying these mechanisms would not only illustrate the ambiguities associated with interpreting phylogenetic results based on rDNAs but also shed light on the process of organismal evolution involving interspecific hybridization.

In the *Mitella* genus of perennials, hybridization events occur frequently in natural populations (Soltis and Kuzoff 1995; Y. Okuyama, unpublished data), and, therefore, the genus is ideal for studying the impact of introgression on phylogenetic inferences. *Mitella* belongs to the *Heuchera* group within the Saxifragaceae *sensu stricto*, but the genus is paraphyletic to several related genera (Soltis and Kuzoff 1995; Soltis et al. 2001). *Mitella* species are found in easternmost Russia, China, Korea, Japan, Taiwan, the United States, and Canada. In this study, we sampled species belonging to a well-defined monophyletic section, *Asimitellaria* (Wakabayashi 2001), which occurs exclusively in Japan and Taiwan. Phylogenetic analysis of the *Asimitellaria* revealed a heterogeneity in the introgression pattern among the three data sets. Because the ETS and ITS regions occur near rDNA arrays, they are thought to contain similar phylogenetic signals (Baldwin and Markos 1998; Andreasen and Baldwin 2001; Álvarez and Wendel 2003). However, our data indicate that there is a significant and previously unappreciated incongruence between these closely linked regions. Here we show that uneven rates of concerted evolution after introgressive hybridization can cause a marked incongruence among what are often considered complementary sources of phylogenetic information in plant phylogenetic studies.

Materials and Methods

Sampling and DNA Extraction

We sampled 66 plants, encompassing all of the described Asian *Mitella* taxa (14 taxa, 12 species; see Wakabayashi [2001] for the morphological delimitation of each taxon), throughout their geographic distribution, as well as individuals from several allied taxa (*Tiarella*, *Tolmiea*, *Peltoboykinia*, and *Rodgersia*). Five Asian *Mitella* taxa of restricted distributions (*M. koshiensis*, *M. stylosa* var. *stylosa*, *M. kiusiana*, *M. yoshinagae*, and *M. formosana*) were sampled from single populations, whereas others were sampled from 2 to 19 populations according to their distribution ranges (fig. 1). All samples were taken from wild populations, with the exception of *Tolmiea*, which was taken from a cultivated plant. The sampling locations and the voucher accessions of the studied plants are listed in the Supplementary Material online.

For the subsequent subcloning of ETS-ITS arrays (see below), 11 plants representing nine *Mitella* species were chosen from the 66 samples. Only *M. furusei* var. *subramosa* was sampled from three geographically distant populations for this purpose, as this taxon was suspected to have hybridized with *M. acerina* in one of the three populations.

Each leaf tissue sample (10 to 30 mg), taken from living or herbarium material, was ground to a fine powder in liquid nitrogen and washed with 1 ml HEPES-HCl buffer (pH 8.0) containing 20 μl of 2-mercaptoethanol, 10 mg of polyvinylpyrrolidone, and 9 mg of L-ascorbic acid. DNA extraction was then conducted by following a standard CTAB protocol (Doyle and Doyle 1987; Kawahara et al. 1995).

Primer Design, Amplification, Cloning, and Sequencing

Initially, we amplified and directly sequenced the *matK, trnL-F*, ETS, and ITS regions individually. However, we found that phylogenetic signal contents of ETS and ITS were markedly incongruent in a subset of the plants analyzed. Because ETS and ITS regions occur in large numbers of copies within a single genome, it is possible that this difference resulted from assortative sampling of copies from different parts of the genome. Therefore, we also amplified and subcloned the contiguous ETS-18S-ITS1-5.8S-ITS2 (ETS-ITS) array to confirm that the studied loci were closely linked.

The locations of the primers used for polymerase chain reaction (PCR) and sequencing of each target locus are shown in figure 2. Ex-*Taq* polymerase (TaKaRa) was used for all PCR experiments. For the ETS reaction, we designed the primer F-ETS1 *Heu* by following the method of Baldwin and Markos (1998). The PCR conditions used were as follows: for cp DNA and ITS, denaturation for 4 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C, and a final extension for 7 min at 72°C; for the ETS reaction, denaturation for 4 min at 94°C, 25 cycles of 30 s at 94°C, and 1.5 min at 72°C; and for the ETS-ITS reaction, denaturation for 4 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 55°C, and 4 min at 72°C, and a final extension for 12 min at 72°C. PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen) or Labo Pass PCR (Cosmo Genetech).

Subcloning was performed using the pGEM-T-easy cloning vector (Promega) and *Escherichia coli* JM109 competent cells according to the manufacturer's protocol. Transformant colonies were collected with a micropipette tip, diluted in TE buffer (pH 8.0), boiled at 98°C, and used as templates for reamplification of the ETS-ITS array. Only colonies resulting in target DNA products of about 3.0 kb were analyzed further by sequencing. Sequencing reactions were performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) according to the manufacturer's instruction. Both strands of uncloned fragments were sequenced, as they often contained base polymorphisms that required verification. Only one strand was sequenced for each cloned

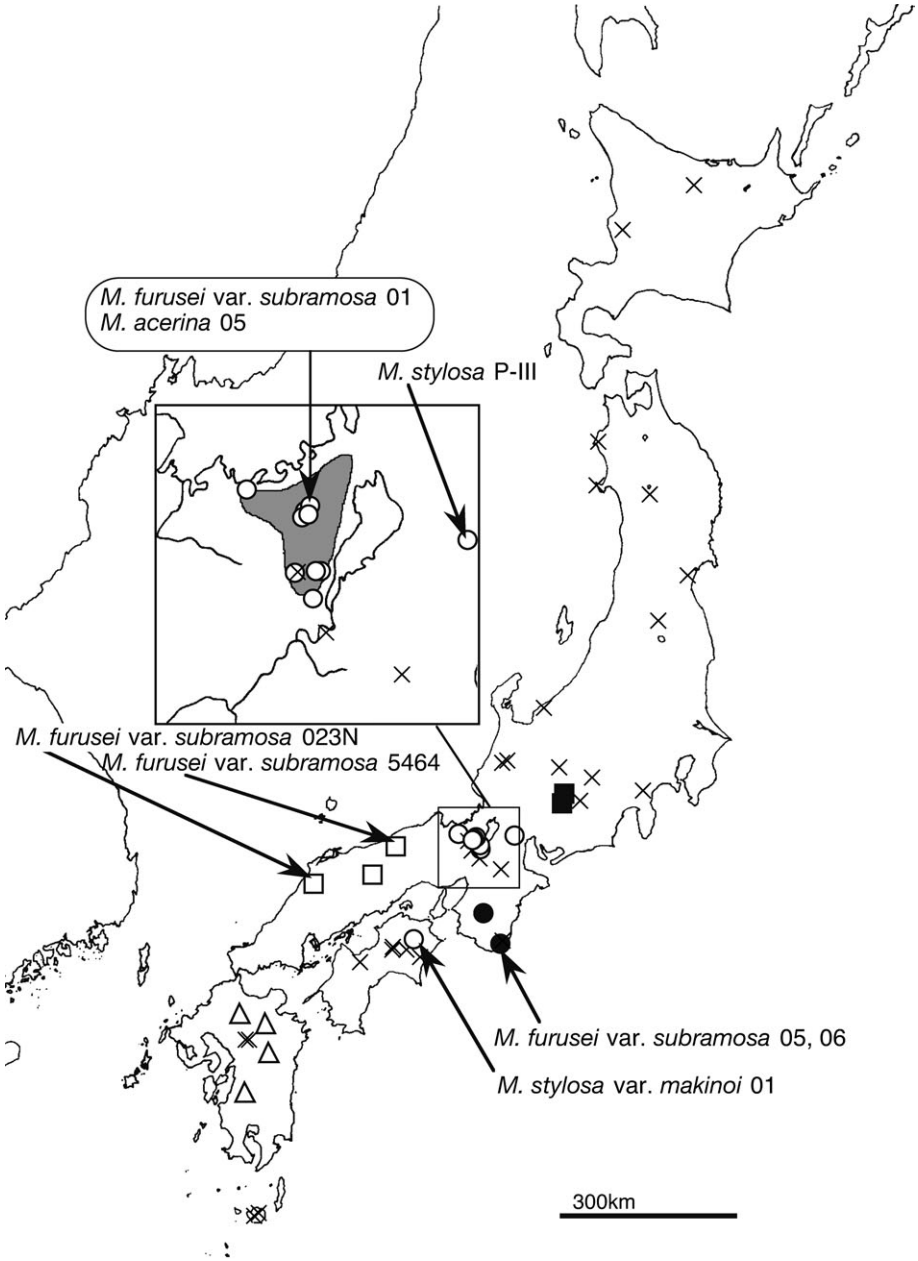

FIG. 1.—Locations from which *Mitella* plants were sampled in Japan. Filled or open circles, squares, and triangles correspond to the individuals of clades C to F in figure 3. The rest of the plants were collected at locations indicated by the symbol ×. The shaded area indicates the distribution range of *M. acerina*, which is equivalent to the hybrid zone between *M. acerina* and *M. furusei* var. *subramosa*.

template, because the electrophoretic patterns obtained for these molecules were clear and simple. Sequencing was performed using ABI 377 or ABI 3100 DNA sequencers (Perkin-Elmer). Sequences have been deposited in the DDBJ database under accession numbers AB116669 to 116706 (cpDNAs), AB161113 to 161174 (cpDNAs), and AB163440 to 163732 (rDNAs).

Phylogenetic Analysis

The length of the *matK*, *trnL-F*, ETS, ITS1, and ITS2 fragments used for phylogenetic analysis were 1299 to 1,305 bp, 736 to 923 bp, 438 to 441 bp, 265 to 269 bp, and 233–238 bp, respectively. All polymorphic sites encountered in the electropherograms of directly sequenced fragments were coded as polymorphic characters. Alignment was performed using Sequence Navigator (Perkin-Elmer) with the default settings, and obvious errors were corrected by hand. Because the ETS and ITS regions of *Peltoboykinia*, *Rodgersia*, and *Tolmiea* were highly heterogeneous with respect to other sequences, they were not used in the analysis, resulting in unambiguous alignment of the remaining sequences, requiring only a few gaps. Because all indels, with the exception of the poly-T site in *trnL-F* (sites 826 to 833 in the aligned data sets), were potentially informative (Kawakita et al. 2003), we coded them as binary (presence/absence) characters using the method of Simmons and Ochoterena (2000).

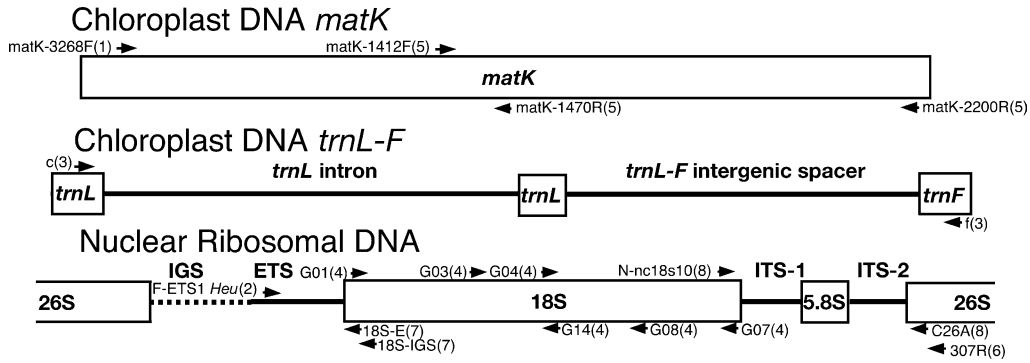

FIG. 2.—Positions of primers in each locus. The primer sequences were 1, 5′-TATTTATGCACTGGCTCATGAT-3′; 2, 5′-GGTGCCTAAAATGCGTGGGTGGACAGG-3′; 3, see Taberlet et al. (1991); 4, see Saunders and Kraft (1993); 5, see Johnson and Soltis (1994); 6, see Soltis and Kuzoff (1995); 7, see Baldwin and Markos (1998); 8, see Kuzoff et al. (1999).

All phylogenetic analyses were performed using PAUP* version 4.0b10 (Swofford 2002). The heterogeneity of phylogenetic signals across data partitions was analyzed using the incongruence length difference (ILD) test (Farris et al. 1994). The ILD test was conducted with parsimony-informative characters only (Cunningham 1997; Lee 2001) using 1,000 replications of heuristic searches with 100 random addition analyses and TBR branch-swapping, using Steepest Descent and disabling the MULTREES option. Because the two chloroplast data sets had little sequence variation and contained highly concordant phylogenetic signals ($P = 0.473$; ILD test), these sets were combined into a single data set. For the same reason, the directly sequenced ITS1 and ITS2 data were combined ($P = 0.144$; ILD test). Because the cloned ITS1 and ITS2 regions showed significant incongruence ($P < 0.01$; ILD test), we initially conducted a separate tree search for each partition and observed some topological conflicts (see Supplementary Material online for the separately analyzed ITS1 and ITS2 MP trees). However, as the incongruence between the two trees did not affect our conclusion, and each partition was poor in informative characters, we subsequently combined them. Because all other combinations of data sets yielded significant incongruence ($P < 0.01$; ILD test), they were analyzed separately. We, therefore, obtained five data sets in this study by directly sequencing cpDNA, ETS, and ITS data sets and cloning ETS and ITS data sets.

Maximum-parsimony (MP) trees were obtained using a heuristic search with 100 random addition sequence replications (saving no more than 300 trees per replication) and TBR branch-swapping algorithm. To assess nodal support for all of the obtained trees, we performed bootstrap analysis with 10,000 replications using the same settings as in the ILD test and Bremer support (Bremer 1994) analysis in combination with the program TreeRot version 2 (Sorenson 1999).

Maximum-likelihood (ML) tree searches were also conducted for the above data sets. For ML searches, multiple accessions of the same taxon with identical sequences were reduced to one, because including identical sequences in the search would require extraordinarily long calculation time. Best-fit substitution models were determined with ModelTest version 3.06 (Posada and Crandall 1998) using the Akaike information criterion (AIC [Akaike 1974]). The best-fit model for each data set was as follows: TVM + G ($\alpha = 0.6129$) for cpDNAs, TrN + I (proportion of invariable sites = 0.7185) for direct ETS, and GTR + G ($\alpha = 0.3837$) for direct ITS. ML heuristic searches were performed with 10 random addition sequence replications and tree-bisection-reconnection (TBR) branch-swapping algorithm.

Statistical Tests for Topological Conflict Among Data Sets

The significance of topological conflict between data sets and the incongruence between the obtained tree topologies and the morphology-based taxonomy were tested using the one-tailed Shimodaira-Hasegawa test (Shimodaira and Hasegawa 1999; Goldman, Anderson, and Rodrigo 2000). Alternative trees were defined by constraining morphologically defined taxa or a node supported by one data set as monophyletic and constructing ML trees under the topological constraint.

Rates of Concerted Evolution in ETS and ITS

If concerted evolution proceeds to completion, the sequences within an individual will be nearly identical. Therefore, we considered that sequence heterogeneity within an individual is a good indicator of the degree of concerted evolution. To estimate the sequence heterogeneity of ETS, ITS1, ITS2, and combined ITS regions within individuals, we calculated the average uncorrected pairwise distances among cloned sequences derived from single individuals (*Di*). However, the sequence heterogeneity within an individual is also correlated with the mutation rate for each locus, as well as the *Taq* error rate. Thus, to estimate the mutation rate roughly, we calculated the average of all pairwise distances of subcloned sequences (*Da*) for each partition. In addition, the *Taq* error rate (*Te*) was calculated by comparing the sequences of six cloned 18S rDNA sequence data (10,271 bp) to the corresponding direct 18S rDNA sequence data, as there were no base polymorphisms within the latter, suggesting that only a single 18S rDNA sequence is present in each individual. Finally, we estimated the sequence heterogeneity within each individual corrected for the mutation

rate (H), which is given by $H=(Di-Te)/(Da-Te)$, because Da and Di always contain the Taq error rate.

Testing Structural Differences between ETS and ITS

Incongruence among data sets can arise from various biological processes, including introgressive hybridization, recombination, and gene conversion. However, incongruence can also be explained merely by the simple process of mutation. Therefore, we tested whether the observed incongruence between the ETS and ITS data sets was a chance consequence of the simple mutation process using the cloned ETS-ITS arrays of *M. acerina* and *M. furusei* var. *subramosa*. Specifically, we determined whether the positions at which the conflicting phylogenetic signals occur are significantly more clustered to either of the ETS or ITS regions than expected at random. The method used here is essentially identical to that of Crandall and Templeton (1999), which is designed for detecting recombination. We first identified the potential recombinants (sequences with conflicting phylogenetic signals between the ETS and ITS regions) and their parental sequences. Because we identified two sequence groups of recombinants (each group with *M. acerina*–type ETS and *M. furusei* var. *subramosa*–type ITS, or vice versa), these groups were analyzed separately. To assess whether each presumed recombinant group arose through a recombination-like process (including gene conversion), we united the group to both of its parental sequence groups with the MP method (using branch-and-bound search with PAUP* software) and mapped the required base changes on the nodes. We then tested whether the changes on each node were significantly clustered by calculating the probability of the observed clustering pattern, which is given by equation 5.3 of Crandall and Templeton (1999).

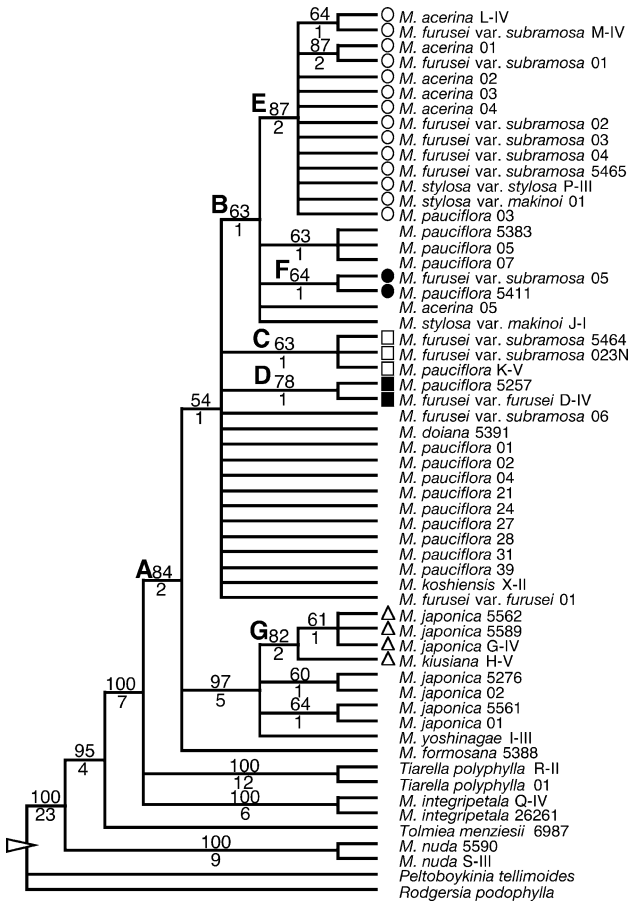

FIG. 3.—A strict consensus of 216 most-parsimonious (MP) trees (length = 239, consistency index excluding uninformative characters = 0.8456, retention index = 0.9346, rooted at the node indicated by a white arrow) based on combined chloroplast *matK* and *trnL-F* sequence data sets. The numbers above the branches indicate bootstrap support with 10,000 replicates (shown only when >50%), and those below branches indicate Bremer support for each node. See text for descriptions of the letters A to G. Filled or open circles, squares, and triangles correspond to those in figure 1.

Results

The Phylogeny of *Mitella* Species and Their Topological Conflicts

The aligned data matrices of directly sequenced cpDNA, ETS, and ITS had 2,287, 451, and 518 nucleotide sites, respectively, including seven indels in each. Of these, 108, 77, and 105 were parsimony informative.

Figures 3, 4*a*, and 4*b* show the strict consensus of equally most-parsimonious trees derived from the directly sequenced cpDNA, ETS, and ITS data sets, respectively. The obtained ML tree topologies were almost identical to those of the MP trees (see Supplementary Material online). The combined cpDNA data set indicated that the genus *Mitella* is not monophyletic (figs. 3), which is consistent with the finding of Soltis and Kuzoff (1995). All three data sets indicated that the section of *Asimitellaria* endemic to Japan and Taiwan is monophyletic, with high bootstrap support (84% to 99% [figs. 3, 4*a*, and 4*b*, clade A]). However, the relationships within the section *Asimitellaria* were highly incongruent among the data sets. In the cpDNA tree, several well-defined taxa, such as *M. acerina*, *M. furusei*, *M. pauciflora*, and *M. stylosa*, were scattered among several nodes with low to moderate bootstrap support (63% to 87% [clades B to F]); forcing three of them to be monophyletic significantly lowered the likelihood scores (table 1). In addition, *M. kiusiana* was unexpectedly placed as the sister of the *M. japonica* individuals of close geographic origin with moderate bootstrap support (clade G), although constraining *M. japonica* to be monophyletic did not significantly lower the likelihood score (table 1).

In contrast, the ETS tree was highly concordant to the morphology-based species delimitations. All of the taxa in clade H were recovered as monophyletic, although the relationship within clade J, which consists exclusively of *M. furusei*, *M. koshiensis*, and *M. pauciflora*, was unresolved. The monophyly of *M. japonica* (clade I) was not recovered, but because an ML tree with *M. japonica* forced to be monophyletic was not significantly different from the unconstrained tree (table 1), the monophyly of *M. japonica* was not rejected. ETS data also corroborated morphologically defined supraspecific taxa. For example, the monophyly of the four species in clade K was recognized based on the unique papillose surface of their seeds. The ITS topology was similar to the ETS topology but differed in the placement of *M. acerina* (fig. 4). In the ITS tree, *M. acerina*

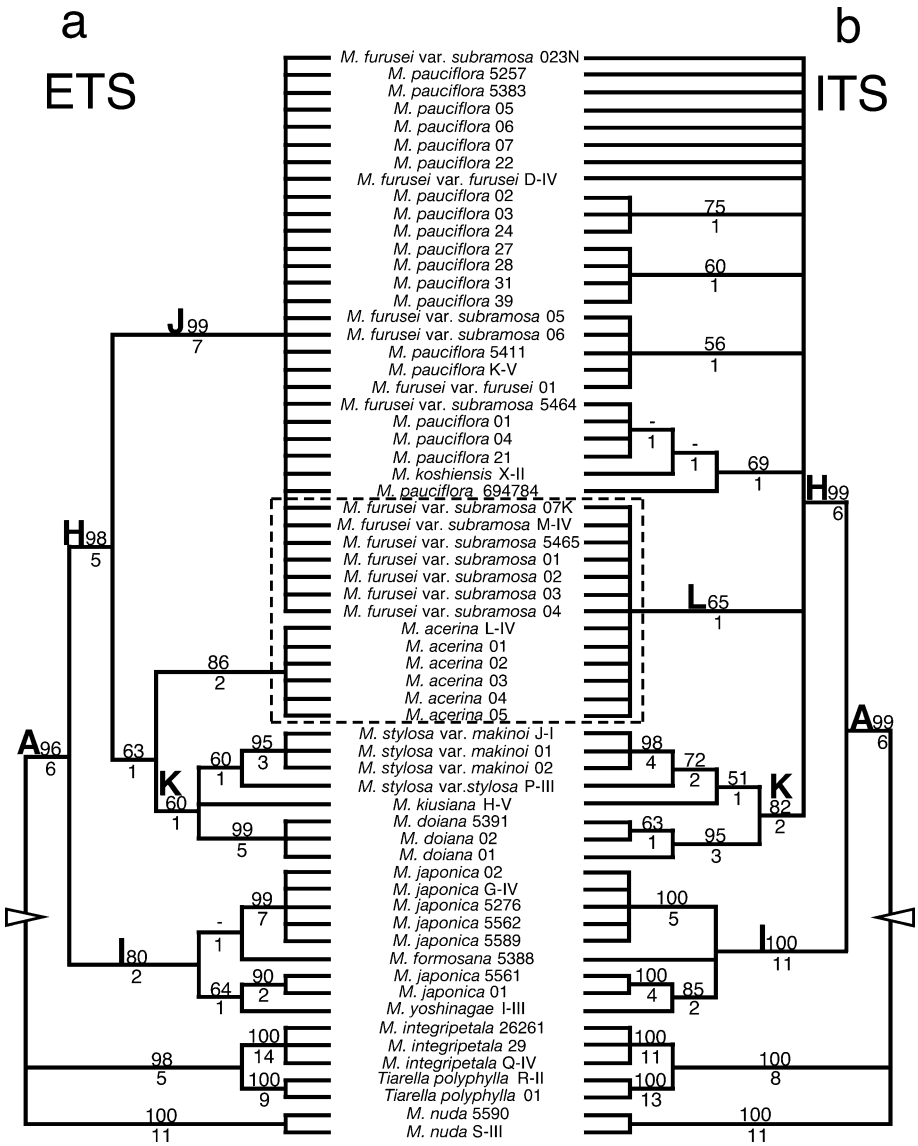

FIG. 4.—A *Mitella* phylogeny based on rDNA sequences. The numbers above the branches indicate bootstrap support with 10,000 replicates (shown only when >50%), and those below the branches indicate Bremer support for each node. Arrows indicate the roots of the trees. (*a*) A strict consensus tree of 16,200 MP trees (length [L] = 157, consistency index excluding uninformative characters [CI] = 0.6408, retention index [RI] = 0.9083) based on the directly sequenced ETS data set. (*b*) A strict consensus tree of 22,500 MP trees (L = 341, CI = 0.4320, RI = 0.7946) based on the directly sequenced ITS data set. The dashed square indicates conflicting topologies between the two data sets involving sympatric individuals of *M. acerina* and *M. furusei* var. *subramosa*. See text for descriptions of the letters A to L.

formed a weakly supported clade L (fig. 4*b*) with individuals of *M. furusei* var. *subramosa* collected from the Kyoto and Fukui populations, where they occur in sympatry with *M. acerina* (fig. 1). Consequently, clade J (fig. 4*a*) was not recovered in the ITS tree (fig. 4*b*), although forcing clade J to be monophyletic did not significantly lower the likelihood score (table 1). In the ETS data, clade L was not recovered (fig. 4*a*), and forcing the monophyly of clade L significantly lowered the likelihood score of the ML tree (table 1).

Estimated *Taq* Error Rate

The estimated *Taq* error rate was 0.088%, calculated on the basis of nine base substitutions thought to be the result of *Taq* error observed in a total of 10,271 bp.

ETS and ITS Sequence Variation Within an Individual

Base polymorphisms within a single nucleotide position were often found in the direct-sequence data of both ETS and ITS regions, indicating the presence of several different alleles in an individual genome.

After alignment, the data sets of the cloned ETS and ITS regions had totals of 427 and 535 characters (each containing seven indels), respectively, of which 73 and 114 were parsimony informative. Figures 5*a* and 5*b* show one of the MP trees derived from 49 ETS/ITS clones sampled from 11 individuals, representing nine species scattered among all of the major clades in figure 4 and including three allopatric *M. furusei* var *subramosa* plants and a *M. acerina* plant (fig. 1). The topologies of the obtained phylogenetic trees were comparable to those of

Table 1
Multiple Comparisons of Log-Likelihood Scores Across Alternative Phylogenetic Hypotheses in Each Data Set

Topological Constraint	Direct cpDNA	Direct ETS	Direct ITS
None (ML)	L = 4562.25819	L = 1294.58666	L = 1768.67258
Forcing "*M. acerina*"	**Δ = 47.32835, *P* = 0.027**	Best	Best
Forcing "*M. furusei*"	**Δ = 94.47585, *P* = 0.002**	Δ = 9.51239, *P* = 0.216	**Δ = 59.74517, *P* = 0.003**
Forcing "*M. japonica*"	Δ = 14.02681, *P* = 0.506	Δ = 4.27551, *P* = 0.513	Δ = 10.06482, *P* = 0.515
Forcing "*M. pauciflora*"	**Δ = 71.25826, *P* = 0.012**	Δ = 6.29579, *P* = 0.321	**Δ = 48.00031, *P* = 0.016**
Forcing "*M. stylosa*"	Δ = 22.96855, *P* = 0.264	Best	Best
Forcing "Clade J"	**Δ = 55.65117, *P* = 0.010**	Best	Δ = 19.69074, *P* = 0.281
Forcing "Clade L"	Δ = 22.96901, *P* = 0.264	**Δ = 27.36183, P = 0.003**	Best

NOTE.—Comparison is by means of the one-tailed Shimodaira-Hasegawa test. Comparisons with significant differences are shown in bold.

the trees based on the direct sequence data. All of the ETS copies within each individual were highly similar (fig. 5*a*), whereas there were two divergent ITS allele groups each in *M. furusei* var. *subramosa* 01, *M. acerina* 05, and *M. pauciflora* 02 (fig. 5*b*). Only *M. furusei* var. *subramosa* 01 and *M. acerina* 05 shared the ITS copies in clade M (fig. 5*b*). As shown in table 2, the degree of sequence heterogeneity within an individual was more than twice as high in the ITS than ETS region. This difference was primarily caused by the ITS1 region being much more heterogeneous within an individual than either of the ETS and ITS2 regions (table 2).

Clustering Patterns of Phylogenetic Signals in ETS-ITS Arrays

We first suspected that the cloned ITS sequences of *M. acerina* that belong to clade N and those of *M. furusei* var. *subramosa* that belong to clade M were derived from introgression in the ITS region only, by recombination or gene conversion. This means that the ETS and ITS regions in these clones give conflicting phylogenetic signals. We treated the rest of the clones are candidates for parental sequences. Figure 6 illustrates the parsimonious network of each putative recombinant and its parental sequences. As shown in figure 6, every node that unites the putative recombinant to each parental sequence group showed base changes in either the ETS or ITS region only. Given that eight to nine changes in the ETS and four changes in the ITS were involved, this physical clustering pattern is highly significant ($P < 0.005$), indicating that both groups are the result of a recombination-like process.

Discussion

Heterogeneity of Observed Introgression Pattern

We found that the chloroplast phylogeny did not agree with morphology-based taxonomy, whereas the nuclear ETS and ITS phylogenies agreed with the morphology to some degree. Although there are many factors that cause discrepancies between gene trees and species trees or among gene trees, including lineage sorting, long-branch attraction, nonhomologous sampling of duplicated gene, and horizontal gene transfer (Hendy and Penny 1989; Doyle 1997; Maddison 1997; Sang and Zhong 2000), our results probably represent introgression. Table 3 summarizes all of the recognized combinations of *Mitella* species in Japan that hybridize in natural populations. The recognized hybridizations explain most of the unexpected topologies in the chloroplast tree that unite different taxa into a monophyletic clade (table 3). For example, the clades C to G in figure 3 are composed of several taxonomically unrelated individuals of close geographic origin (fig. 1) that potentially hybridize (table 3). This pattern indicates that recent introgressive hybridization is sufficient to explain the anomalous tree topologies derived from the chloroplast data.

In contrast, the ETS phylogeny was highly concordant to the morphology-based system, and the ITS phylogeny was only slightly less concordant to this system. The only significant topological conflict between the ETS and ITS trees was the placement of sympatric individuals of *M. acerina* and *M. furusei* var. *subramosa*. In the ETS tree, the two species were placed into two well-isolated clades (fig. 4*a*), whereas in the ITS tree, they formed a clade L with low bootstrap support (65% [fig. 4*b*]). In addition, ITS data indicated that the *M. furusei* var. *subramosa* individuals, sampled apart from the *M. acerina* population, are not related to *M. acerina* (figs. 1 and 4*b*). *Mitella acerina* is a species of very narrow distribution that always grows sympatrically with another taxon of wider distribution, *M. furusei* var. *subramosa*. The two taxa are known to hybridize and produce fertile hybrid in nature, despite differences in principal pollinators (Okuyama, Kato, and Murakami 2004). Therefore, the placement of sympatric individuals of the two species within a moderately supported, monophyletic clade L most likely results from introgression.

It is possible that the observed difference in the level of introgression between ETS and ITS is an artifact of a lack of sufficient nucleotide variability within the ETS region, which would obscure potential introgression events. However, the results of Shimodaira-Hasegawa tests showed that forcing the monophyly of clade L in the ETS phylogeny significantly lowers the likelihood score of the ML tree (table 1), indicating that the ETS data do not support the evidence of introgression between *M. acerina* and *M. furusei* var. *subramosa* as inferred from the ITS data. Whether or not the ETS data support the evidence of introgression within poorly resolved clade J, however, is not clear because of the low nucleotide variability.

Chloroplast Capture

The heterogeneity of the observed introgression pattern in the three data partitions provides valuable insight into the patterns of sequence evolution in a lineage

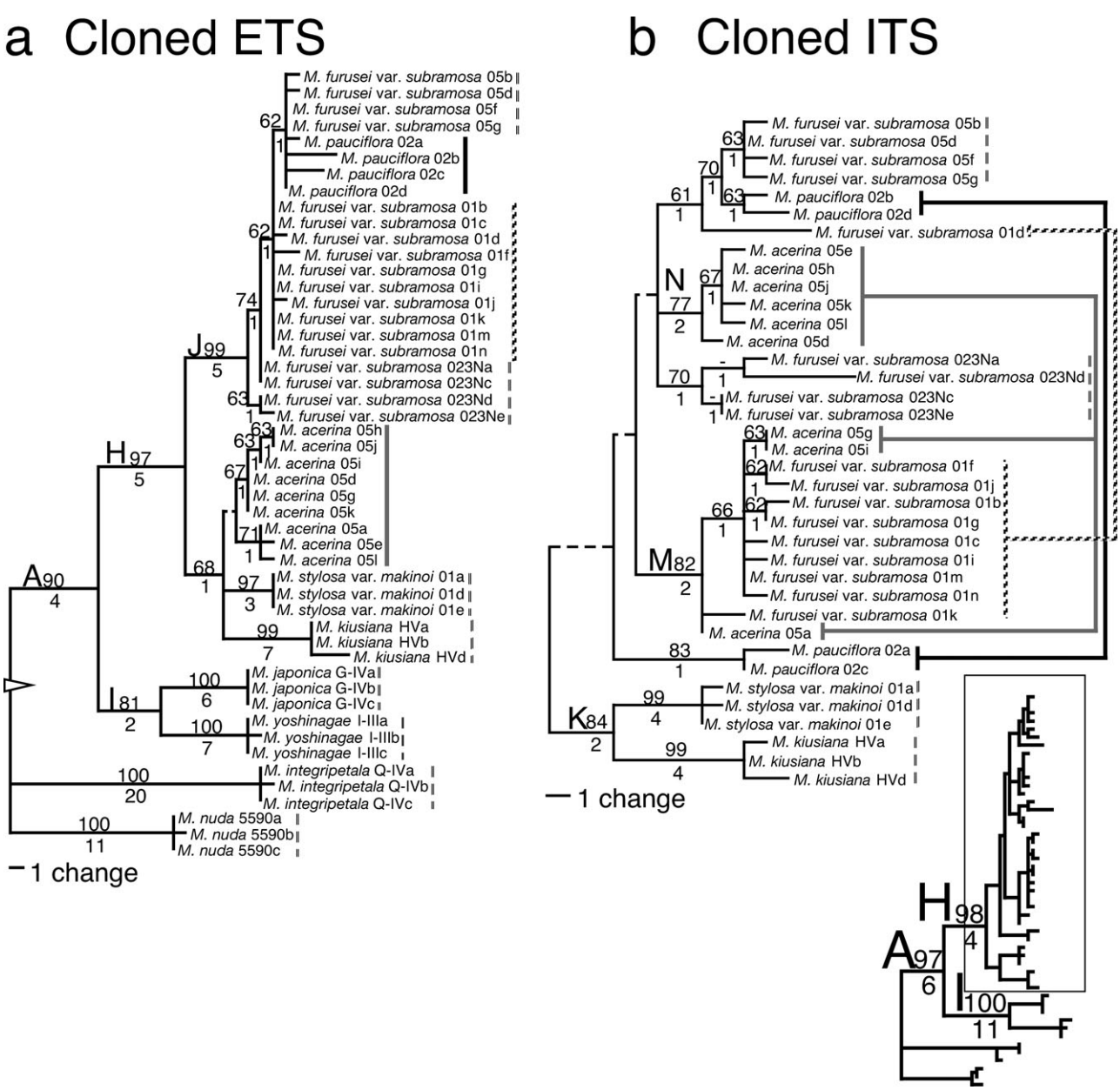

FIG. 5.—Phylogenetic relationships of 49 cloned rDNA sequences sampled from 11 individual plants representing nine *Mitella* species found in Japan. The numbers above the branches indicate bootstrap support with 10,000 replicates (shown only when >50%), and those below the branches indicate Bremer support for each node. The nodes that collapse in the strict consensus trees are indicated by dashed lines. The clades A and H to K correspond to those in figures 3 and 4. Small letters after the individual identity (e.g., *M. acerina* 05"a") refer to the clone identity. (*a*) One of 30,000 MP trees (L = 118, CI = 0.8557, RI = 0.8538) based on the cloned ETS sequences. Note that alleles of each individual, including *M. acerina* 05 (gray line), *M. furusei* var. *subramosa* 01 (shaded line), and *M. pauciflora* 02 (black line) are nearly identical. (*b*) One of 24 MP trees (L = 179, CI = 0.7949, RI = 0.9430) based on the cloned ITS sequences. Note that the ITS copies of *M. acerina* 05 (gray line), *M. furusei* var. *subramosa* 01 (shaded line), and *M. pauciflora* 02 (black line) are isolated in two divergent clades. *M. furusei* var. *subramosa* 01 and *M. acerina* 05 are sympatric (fig. 1) and share the unique alleles that were placed in clade M.

that displays frequent hybridization. Not unexpectedly, the chloroplast tree showed the most extensive introgression pattern of the three, indicating that chloroplast capture is common, as has been suggested previously for many plant taxa, including the Saxifragaceae (Soltis et al. 1991; Rieseberg and Soltis 1992; Soltis and Kuzoff 1995; Soltis, Johnson, and Looney 1996). Tsitrone, Kirkpatrick, and Levin (2003) used theoretical models to show that cyto-nuclear incompatibilities and female fitness gains are the principal factors that promote chloroplast capture. They also showed that partial selfing, as commonly observed in Asian *Mitella* species (Okuyama, Kato, and Murakami 2004; Y. Okuyama, unpublished data), facilitates chloroplast capture under certain conditions.

The effective population size for a maternally inherited organellar gene is less than a quarter of that for a biparentally inherited nuclear gene (Birky, Maruyama, and Fuerst 1983; McCauley 1994; Levy and Neal 1999). This effect is probably acute in *Mitella* and its allies because most of these species depend on gravity or water

Table 2
Averages of Uncorrected Pairwise Distances Among All Cloned Sequences, Within Individual Cloned Sequences, and Degrees of Sequence Heterogeneity Within Individuals for Each rDNA Partition

	ETS	ITS (combined)	ITS1	ITS2
All pairwise (*Da*)	3.83×10^{-2}	4.45×10^{-2}	4.68×10^{-2}	4.21×10^{-2}
Within individual pairwise (*Di*)	3.77×10^{-3}	8.32×10^{-3}	1.15×10^{-2}	4.97×10^{-3}
Degree of sequence heterogeneity within individual (*H*)	7.74×10^{-2}	1.71×10^{-1}	2.31×10^{-1}	9.93×10^{-2}

for seed dispersal (Savile 1975), making long-distance dispersal difficult, although possible (Soltis et al. 2001). Therefore, once locally introgressed, it is expected that a cytoplasmic gene of a *Mitella* population would be more easily fixed to a haplotype derived from other species than to a nuclear gene. This may constitute a secondary explanation for rare hybridization events seeming to affect the chloroplast phylogeny of *Mitella* species.

Nonuniform Concerted Evolution

Although nuclear rDNA may be expected to show fewer introgression patterns than cpDNA, the difference between trees in the observed introgression pattern derived from the ETS and ITS sequences, which are neighboring regions within a single rDNA repeat, was unexpected. Because nuclear rDNA occurs in hundreds to thousands of copies, it is unlikely that this incongruence was caused by recombination, because recombination must have occurred at the same site in all copies to obtain the observed incongruence between ETS and ITS. A more plausible explanation is that this difference was caused by an uneven rate of concerted evolution (gene conversion) within the rDNA array of the *Mitella* lineage. Because *M. acerina* and *M. furusei* var. *subramosa* hybridize frequently, different alleles derived from the two species would be expected to coexist within a genome if concerted evolution of the rDNA after introgression is incomplete. Alternatively, if concerted evolution was completed rapidly after hybridization, within a few backcrossed generations, the alien alleles would now be "overwritten" by the original alleles from each species, keeping alleles derived from each species monophyletic (our assumption requires some directional mode of concerted evolution [Aguilar, Rosselló, and Feliner 1999*b*] in the ETS region such that the introgressed alleles converge into the original ones).

Our cloned ETS and ITS data supported the latter hypothesis (fig. 5). Every individual had only one or a few similar ETS alleles (fig. 5*a*), suggesting that concerted evolution drives sequences within a genome to near identity. In contrast, *M. furusei* var. *subramosa* 01, *M. acerina* 05, and *M. pauciflora* 02 have two very divergent ITS allele groups within their genomes (fig. 5*b*). Importantly, sympatric *M. furusei* var. *subramosa* 01 and *M. acerina* 05 shared the ITS alleles in clade M (figs. 1 and 5*b*), whereas their ETS alleles were not closely related to each other (fig. 5*a*). In addition, the *M. furusei* var. *subramosa* 05 and 023N individuals, which were sampled away from

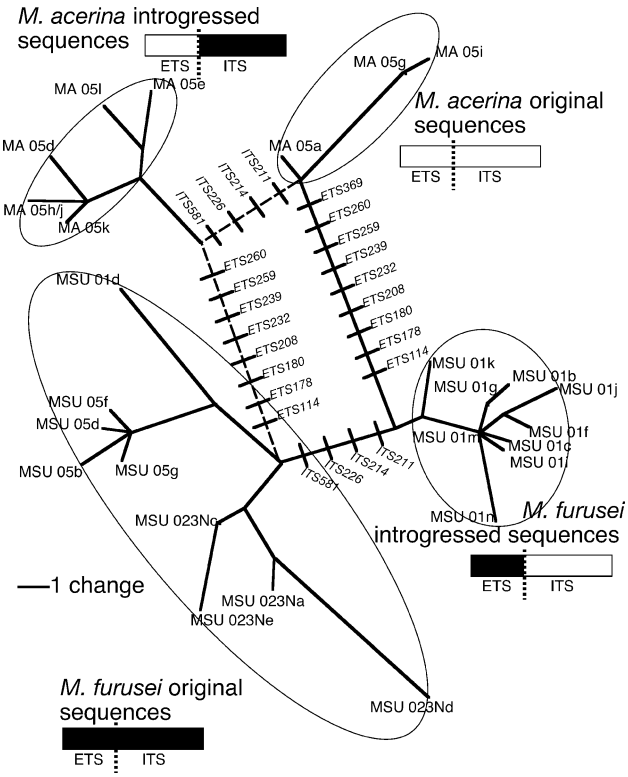

FIG. 6.—A parsimony network of parental 3′-ETS-18S-ITS sequence arrays of *M. acerina* and *M. furusei* var *subramosa* and their putative recombinants. Short lines on the nodes indicate the required parsimonious base changes at the indicated site (e.g., "ETS114" indicates the 114th aligned site of the ETS region) from the parental *M. acerina* and *M. furusei* var *subramosa* sequence group to the introgressed sequence (putative recombinant) group. Bars represent the structure of the ETS-ITS arrays of each sequence group, with bars of the same color indicating the same structure. Note that the putative recombinant sequences are different from both parental sequences in the ETS or ITS regions. MA = *M. acerina*; MSU = *M. furusei* var. *subramosa*.

Table 3
Known Combinations of Hybridizing *Mitella* Taxa in Japan and the Clades to Which the Parental Taxa Belong Based on the Chloroplast Phylogeny

Combination of Taxa	Clades Containing the Parental Taxa
M. acerina × *M. furusei* var. *subramosa*	B,E
M. acerina × *M. pauciflora*	B,E
M. furusei var. *furusei* × *M. pauciflora* (*M.* × *inamii*)	D
M. furusei var. *furusei* × *M. stylosa* var. *stylosa*	—
M. furusei var. *subramosa* × *M. pauciflora*	B,C,E,F
M. japonica × *M. kiusiana*	G

the hybrid zone (fig. 1), did not possess any ITS alleles belonging to clade M (fig. 5*b*). The significance of this pattern was confirmed by analyzing the physical clustering pattern of the two different sets of phylogenetic signals. The parsimonious base changes differentiating the introgressed (putative recombinant) ETS-ITS sequence group of *M. acerina* and *M. furusei* var. *subramosa* from both parental sequence groups were clustered exclusively in either the ETS or the ITS region (fig. 6). This physical clustering pattern is highly significant, and, thus, the significant structural differences between the ETS and ITS point toward a gene conversion process. Our results further suggest that the introgression in the ITS region between *M. acerina* and *M. furusei* var. *subramosa* might be bidirectional or, in other words, the ITS alleles of clade M in *M furusei* var. *subramosa* and clade N in *M. acerina* are derived from *M. acerina* and *M furusei* var. *subramosa*, respectively. Given that the ETS and ITS are closely linked, it is unlikely that only the ITS has introgressed independently. It is much more likely that, after introgression of the entire rDNA, greater concerted evolution in the ETS region eliminated the alien alleles derived from hybridized species. Conversely, the introgressed ITS alleles were retained, probably because sequence elimination by the concerted evolutionary process in the ITS region did not keep up with the recurrent and frequent introgression.

Taking into account the mutation rate in each partition and the *Taq* error rate (0.088%), the ITS sequence heterogeneity within an individual was twice that of the ETS (table 2). Therefore, species-specific ETS sequences may be maintained by strong concerted evolution, rendering the phylogeny of these sequences similar to the morphology-based species delimitation. In other words, the *Mitella* phylogeny based on the ETS sequences may be resistant to introgressive hybridization because the concerted evolutionary force is strong compared with the degree of introgression.

The assumption of nonuniform concerted evolution is very likely because there are some reports of similar patterns in other organisms. For example, Polanco et al. (1998) reported higher intensity of concerted evolution in the intergenic spacer region (IGS; containing ETS) of rDNAs over the ITS region in *Drosophila melanogaster*. They attributed this difference to the presence of recombination "hotspot" within the IGS region, which probably corresponds to the RNA polymerase active promoter sequence (Polanco et al. 1998, and the references therein). It is possible that a similar mechanism is involved in causing nonuniform concerted evolution between ETS and ITS in plants.

An incomplete sampling of multiple rDNA arrays potentially results in misleading phylogenetic inferences (Álvarez and Wendel 2003). However, the PCR condition and the primer set used here successfully amplified the target region in the distantly related *Peltoboykinia* and *Rodgersia*, as well as that of all samples of the *Heuchera* group, suggesting that our sampling covers most of the orthologous copies of a focal rDNA lineage that emerged before the diversification of the *Heuchera* group. Therefore, our data are sufficient for an assessment of nonuniform concerted evolution, although caution should be used when inferring the branching order within the genus *Mitella* based solely on ETS sequences. Provided that the ETS tree is in agreement with the morphology-based taxonomy, there is no reason to reject the phylogenetic hypothesis of *Mitella* proposed here. Nevertheless, we believe that it is necessary to analyze as many loci as possible to gain a better species phylogeny of a target lineage, as individual loci, especially from different three plant genomes, vary greatly in their evolutionary dynamics (Wolfe, Li, and Sharp 1987; Gaut 1998; Muse 2000) and reflect different aspects of organismal evolutionary processes.

The ETS and ITS regions are usually treated as parts of a single molecule that possess the same evolutionary history (Baldwin and Markos 1998; Álvarez and Wendel 2003) In fact, there are many reports that the ETS and ITS have very similar phylogenetic signals (Beardsley and Olmstead 2002; Becerra 2003; Nepokroeff et al. 2003). However, as we have revealed here, these sequences may have different evolutionary histories (see also Clevinger and Panero [2000], Andreasen and Baldwin [2003], and Jousselin, Rasplus, and Kjellberg [2003], who obtained significant ILD scores between the ETS and ITS regions), possibly owing to the different pattern of concerted evolution within the rDNA arrays (Polanco et al. 1998). As in the *Mitella* ETS, a region with a strong concerted evolutionary force will recover a simple history of organismal cladogenesis in the narrowest sense, in which no evidence of introgression is observed (but this can be misleading when species of hybrid origin are included in the analysis; see Sang and Zhong [2000]). Alternatively, the region with slower concerted evolution will exhibit evidences of ancient and/or recent introgression events (Sang, Crawford, and Stuessy 1995). Because nonuniform concerted evolution has been reported in the α-globin gene duplication units of human (Hess, Schmid, and Shen 1984), the chorion multigene family of silkmoth (Eickbush and Burke 1986; Hibner, Burke, and Eickbush 1991), and the rDNAs of peonies, *Drosophila*, and marine algae (Sang, Crawford, and Stuessy 1995; Polanco et al. 1998; Durand et al. 2002), it may not be a rare feature of multicopy gene families, which all undergo concerted evolution. Thus, similar to data from organellar genes and low-copy and single-copy genes, this uneven structure in multicopy genes will prove a valuable source of data for resolving details of organismal speciation processes involving introgressive hybridization, which constitutes one of the most challenging and fascinating subjects in evolutionary biology today.

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