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Internal transcribed spacer region evolution in *Larix* and *Pseudotsuga* (Pinaceae)[1]

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The nuclear ribosomal DNA (nrDNA) internal transcribed spacer (ITS) region has been characterized in the sister genera *Larix* and *Pseudotsuga* (Pinaceae). Complete sequences were obtained for seven species of *Larix* from North America and Eurasia and five species of *Pseudotsuga* from western North America and eastern Asia. ITS region lengths ranged from 1759 to 1770 bp in *Larix* and from 1564 to 1571 bp in *Pseudotsuga.* In both genera, ITS1 is three times as long as the 5.8S plus ITS2 and contains subrepeats as observed in other genera of Pinaceae. Secondary structure models predicted that the subrepeats fold into terminal stem and loop domains. ITS polymorphism detected within individuals of *Larix* and *Pseudotsuga* suggests a slow rate of concerted evolution among nrDNA loci. Except for the placement of *L. sibirica,* phylogenetic analyses of the ITS region agreed with previously reported restriction site analyses of *Larix* and *Pseudotsuga.* The data were not consistent with phylogenetic hypotheses for *Larix* based primarily upon ovulate cone characters, failing to support a derivation of the North American *L. laricina* from a short-bracted Eurasian lineage. The phylogenetic hypothesis did not conflict with a stepping stone model of evolution for *Pseudotsuga,* but a basal lineage could not be inferred for either genus.

Key words: concerted evolution; dispersed subrepeats; internal transcribed spacer region; *Larix*; nuclear ribosomal DNA; Pinaceae; *Pinus pinea*; *Pseudotsuga.*

Morphological and molecular systematic treatments of Pinaceae (Coniferales) have been extensive at the generic level (Prager, Fowler, and Wilson, 1976; Miller, 1977; Hart, 1987; Price, Olsen-Stojkovich, and Lowenstein, 1987; Chase et al., 1993; Tsumura et al., 1995; Chaw et al., 1997). The main source of nucleotide sequence data for the family has been the plastid-encoded *rbcL* (ribulose 1,5-bisphosphate carboxylase/oxygenase) gene, but its slow rate of substitution limits its utility for studies among recently derived species. The internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) appears to have fewer functional constraints on substitution and has been used widely in reconstructing generic and infrageneric plant phylogenies (Baldwin et al., 1995). Although the ITS region is ~565–700 bp in angiosperms, it is longer in nonflowering seed plants, particularly in the Podocarpaceae and Pinaceae (Bobola, Smith, and Klein, 1992; Liston et al., 1996). In Pinaceae, published lengths vary from 1550 bp in *Pseudotsuga menziesii* (Mirb.) Franco to 3125 bp in *Picea abies* (L.) H. Karsten. This substantial length variation suggests that alignment of ITS1 may be difficult across genera of Pinaceae and therefore unsuitable for phylogenetic studies above the generic level. A complete 3061-bp nucleotide sequence of the ITS region has been reported for *Pinus pinea* (Marrocco, Gelati, and Maggini, 1996), but detailed comparisons of full-length ITS region sequences between species or genera in Pinaceae have not been published.

To determine the utility of the ITS region for reconstructing phylogenies in Pinaceae we undertook an analysis of two genera, *Larix* Miller (larch) and *Pseudotsuga* Carrière (Douglas-fir). The sister relationship between these two genera has been well established on the basis of fertilization and pollen development (Christiansen, 1972; Takaso, 1996), immunoassays (Prager, Fowler, and Wilson, 1976; Price, Olsen-Stojkovich, and Lowenstein, 1987), anatomical and chemical characters (Hart, 1987), sequence comparison of *rbcL* (Chase et al., 1993), chloroplast DNA RFLPs (restriction fragment length polymorphisms; Tsumura et al., 1995; Xiao-Quan et al., 1997), and 18S rRNA sequences (Chaw et al., 1997).

Larix is composed of ten species (Table 1) occurring throughout the Northern Hemisphere in cooler environments at higher altitudes or more northerly latitudes than *Pseudotsuga* (Farjon, 1990). Morphological (Farjon, 1990; LePage and Basinger, 1991, 1995; Schorn, 1994) and molecular (Qian, Ennos, and Helgason, 1995) treatments of systematic relationships within *Larix* are in conflict (Table 2). Patschke (1913) was the first to divide the genus into two sections based upon the morphology of the female cone. Section *Larix* (or *Pauciserialis*) included species with bracts on the ovulate cone that did not extend well beyond the cone scales, and section *Multiserialis* included species with bracts extending far beyond the cone scales. Several classifications based on ovulate cone morphology have been proposed. Schorn (1994) placed the two western North America species, *L. occidentalis* and *L. lyallii,* both of which have bracts on the ovulate cone that are extended well beyond the cone scales (exserted), into group I "Aristatus." *Larix griffi-*

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TABLE 1. Synopsis of *Larix* and *Pseudotsuga* species. Unless indicated otherwise, the ITS region was sequenced directly from PCR products.

Taxon	Source	Voucher no./GenBank accession number[a]
Eurasian *Larix*		
L. decidua Mill.[b]	Hoyt Arboretum, Portland, Oregon, USA	DSG001/GBAN-AF041343
L. gmelinii (Rupr.) Kuzen.[c]	Hoyt Arboretum, Portland, Oregon, USA	DSG002/none
L. griffithiana (Lindl. et Gord.) Carr.	Lachung, Sikkim, India	DSG003/GBAN-AF041349
L. kaempferi (Lamb.) Carr.	Mt. Yatsugatake, Nagono Prefecture, Japan	DSG004/GBAN-AF041344
L. mastersiana Rehder et Wilson	not sampled	
L. potaninii Batalin	not sampled	
L. sibirica Ledeb.[b]	Forestfarm Nursery, Williams, Oregon, USA	DSG016/GBAN-AF041345
North American *Larix*		
L. laricina (Du Roi) K. Koch	19951411, Edinburgh, United Kingdom	DSG005/GBAN-AF041348
L. lyallii Parl.	Anthony Lakes, Malheur Co., Oregon, USA	DSG006/GBAN-AF041346
L. occidentalis Nutt.	J. H. Stone Nursery, Jackson Co., Oregon, USA	DSG007/GBANAF041347
Asian *Pseudotsuga*		
P. sinensis Dode	Ps871, USDA Forest Service Arboretum, Benton Co., Oregon, USA	DSG014/GBAN-AF041350
P. wilsoniana Hayata	19934044, Edinburgh, United Kingdom	DSG015/GBAN-AF041351
North American *Pseudotsuga*		
P. flahaultii Flous[c,d]	19890267, Edinburgh, United Kingdom	DSG008/none
	Sierra de Arteaga, Cerro El Coahuilón, Coahuila, Mexico	DSG017/none
P. guinieri Flous[c,d]	19922183, Edinburgh, United Kingdom	DSG009/none
P. japonica (Shiras.) Beissn.	Botanical Garden, Kukizaki, Ibaraki, Japan	DSG010/GBAN-AF041352
P. macrocarpa (Vasey) Mayr[b]	Palomar Mt., California, USA	DSG011/GBAN-AF041354
P. menziesii (Mirb.) Franco[b]	McDonald Forest, Benton Co., Oregon, USA	DSG012/GBAN-AF041353
P. rehderi Flous[c,d]	19870763 Edinburgh, United Kingdom	DSG013/none

[a] The prefix GBAN has been added for linking the on-line version of *American Journal of Botany* to GenBank and is not part of the actual GenBank accession number.

[b] The ITS region was cloned and partially sequenced, in addition to direct sequencing.

[c] The ITS region was only restriction site mapped.

[d] Mexican species generally synonymized with *P. menziesii* var. *glauca* (Beissn.) Franco. A fourth species, *P. macrolepis* Flous, has also been described from Mexico.

thiana, L. mastersiana, L. potaninii, and *L. kaempferi,* all of which have bract scales that extend only slightly beyond the cone scale, were placed in group IIa ''Laminatus.'' *Larix decidua, L. siberica, L. gmelinii,* and *L. laricina* all possess bracts that are shorter than the cone scales and were placed in group IIb ''Laminatus.'' Using RFLP analysis of cpDNA, Qian, Ennos, and Helgason (1995) contributed the first molecular evidence that classification based on exserted or inserted bract scales was inconsistent with the evolutionary history of *Larix.*

Two unsettled questions regarding the systematics of *Pseudotsuga* are the species limits within its two most wide-ranging members, *P. menziesii* and *P. sinensis* sensu lato (s.l.), and whether the two North American species, *P. menziesii* and *P. macrocarpa,* are monophyletic or paraphyletic. Recent classifications of *Pseudotsuga* have recognized from four (Farjon, 1990) to eight (Hermann, 1982) species (Table 1). *Pseudotsuga menziesii* s.l. includes var. *menziesii,* a coastal form occurring from British Columbia south to California and var. *glauca* (Beissn.) Franco, an interior form occurring from Canada to Mexico (Farjon, 1990). Populations of *P. menziesii* var. *glauca* become increasingly isolated in the southwestern United States and Mexico, where they form isolated populations confined to high altitudes. Four Mexican species (Table 1) accepted by Martínez (1949) are considered synonyms of *P. menziesii* var. *glauca* by Farjon (1990) based on a lack of consistent morphological differences. Whether or not morphological differences deserving of species rank exist in Mexican populations, there is evidence of marked genetic differences. In a UPGMA analysis using allozyme data collected broadly in the United States but from only two populations from Mexico (Li and Adams, 1989), one of two Mexican populations sampled was too highly differentiated to occur within a cluster of either variety of *P. menziesii.* The taxonomy of *Pseudotsuga* from mainland China and Taiwan is also unsettled. At least eight species of *Pseudotsuga* have been described from China and Taiwan, but they have generally been synonymized with the mainland *P. sinensis* and the Taiwanese *P. wilsoniana* (Hermann, 1982). Farjon (1990) argued that variation was insufficient for many segregate species of *Pseudotsuga* in China and, therefore, recognized only a single species, *P. sinensis.* Farjon included both mainland and Taiwanese populations within *P. sinensis* var. *sinensis,* and proposed new combinations, var. *brevifolia* (Cheng et Fu) Farjon and var. *gaussenii* (Flous) Farjon, for two taxa with more restricted ranges in mainland China that have more commonly been given species rank. Nevertheless, geneticists have been able to discriminate many of the segregate species of *Pseudotsuga* from China. El-Kassaby, Colangeli, and Sziklai (1983) used discriminant function analysis to show that karyotypes of three putative species from mainland China and *P. wilsoniana* could each be readily distinguished from one another.

While the unresolved question of whether genetic and morphological differences are significant enough to warrant the segregation of *P. menziesii* and *P. sinensis* into more varieties or species results in taxonomic inconsistency in the literature, the second controversy surrounding *Pseudotsuga* concerns the evolutionary sequence that gave rise to extant species. Strauss, Doerksen, and Byrne (1990) used RFLP data to hypothesize a stepping stone model of evolution, with a North American lineage migrating across the Bering Land Bridge, giving rise to *P.*

japonica, which in turn gave rise to *P. sinensis* and *P. wilsoniana* (a stepping stone model of evolution; Table 2). This proposed North American origin of *Pseudotsuga* is consistent with its older fossil record in North America (Hermann, 1985; Axelrod, 1990), but of the two most parsimonious trees obtained in the analysis by Strauss, Doerksen, and Byrne (1990), only one topology should be interpreted as positive evidence of a North American origin of extant species of *Pseudotsuga,* while the other neither supports nor conflicts with a North American origin.

The current study provides structural information about the ITS region of *Larix* and *Pseudotsuga,* both of which have ITS1 subrepeats like those reported in other genera of Pinaceae (Marrocco, Gelati, and Maggini, 1996; Vining and Campbell, 1997). We report that ITS region divergence is low in closely related species of *Larix* and *Pseudotsuga,* revealing few fixed differences between closely related species using a direct sequencing approach. Complete ITS region sequences for seven species of *Larix* and five species of *Pseudotsuga* provide an opportunity to reevaluate conflicting phylogenetic hypotheses for both genera. These phylogenetic conclusions are used to reconcile the evolutionary histories of both genera with their fossil records.

MATERIALS AND METHODS

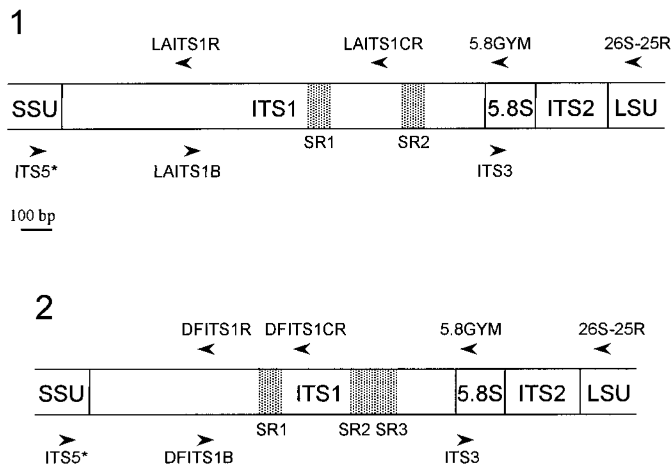
Taxa included—The ITS region was sequenced for a total of 12 species of *Larix* and *Pseudotsuga* (Table 1). The *Larix* sample included representatives from all three bract length types and from each of Qian, Ennos, and Helgason's (1995) cpDNA types. From *Pseudotsuga,* the four species recognized by Farjon (1990) and *P. wilsoniana* (treated by Farjon as a synonym of *P. sinensis*) were sequenced. Molecular and morphological phylogenetic analyses of Pinaceae suggest that *Pinus* or *Picea* are appropriate outgroups for *Pseudotsuga* and *Larix* (Prager, Fowler, and Wilson, 1976; Price, Olsen-Stojkovich, and Lowenstein, 1987; Hart, 1987; Chase et al., 1993; Tsumura et al., 1995; Xiao-Quan et al., 1997; Chaw et al., 1997). The full-length ITS region sequence of *Pinus pinea* (GenBank accession number X87936) was included for comparison. A restriction site study was performed on the ITS region that also included another Asian species of *Larix, L. gmelinii.* Restriction site data were also collected for *P. flahaultii, P. guinieri,* and *P. rehderi,* three of four named Mexican taxa of *Pseudotsuga* usually synonymized with *P. menziesii* var. *glauca* (Hermann, 1982; Farjon, 1990).

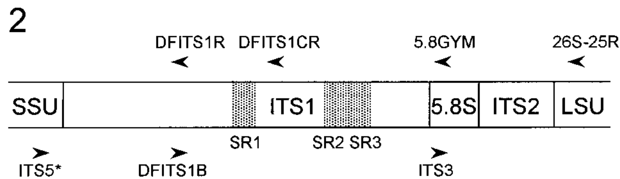
DNA isolation—DNA was extracted using the method of Doyle and Doyle (1987). Samples were ground in 65°C 2X CTAB (cetyltrimethylammonium bromide; United States Biochemical, Cleveland, Ohio) buffer supplemented with 2% sodium bisulfite and 2% PVP (polyvinylpyrrolidone), extracted twice in chloroform : isoamyl alcohol 24:1, precipitated in isopropanol and 7.5 mol/L ammonium acetate for 60 min at −20°C, spun, washed in 70% ethanol, and resuspended in Tris-EDTA (10 mmol/L Tris, 1 mmol/L EDTA, pH 8.0).

PCR amplification and DNA sequencing—PCR (polymerase chain reaction) was performed in 100-μL volumes with 2.5 units Replitherm™ DNA polymerase (Epicentre Technologies, Madison, Wisconsin), Replitherm™ buffer, 1.5 mmol/L $MgCl_2$, 1 μmol/L dNTP (pH 7.0, Epicentre Technologies, Madison, Wisconsin), 1 μmol/L ITS5* (Liston et al., 1996), 1 μmol/L 26S-25R (Nickrent, Schuette, and Starr, 1994), 5% DMSO, 1% BSA, and ~100 ng sample DNA. PCR amplification reaction conditions were 35 cycles of 60 s at 94°C denaturing, 60 s at 55°C annealing, and 3 min at 72°C extension. Reactions were terminated following a final extension for 7 min at 72°C. Products were

TABLE 2. Phylogenetic hypotheses proposed for extant *Larix* and *Pseudotsuga.* Hybrids, varieties, and fossil taxa have been excluded. Key to abbreviations: anc = hypothetical ancestor, *Lde* = *L. decidua, Lgm* = *L. gmelinii, Lgr* = *L. griffithiana, Lka* = *L. kaempferi, Lla* = *L. laricina, Lly* = *L. lyallii, Lma* = *L. mastersiana, Loc* = *L. occidentalis, Lpo* = *L. potaninii, Lsi* = *L. sibirica, Pja* = *P. japonica, Pma* = *P. macrocarpa, Pme* = *P. menziesii, Psi* = *P. sinensis, Pwi* = *P. wilsoniana.*

Source	Phylogenetic hypothesis	Data source	Analysis
LePage and Basinger (1991)	((((*Lgm, Lsi*) *Lde*) *Lla*)(((*Lma, Lgr*) *Lpo*) *Lka*)(*Loc, Lya*))anc)	cones, geographical distribution, fossil record	unspecified
Schorn (1994)	(((((*Lgm, Lla*)(*Lsi, Lde*))(*Lka*(*Lgr*(*Lma, Lpo*))))(*Loc, Lya*))anc)	female cone anatomy	unspecified
Qian, Ennos, and Helgason (1995)	(*Lla* (*Loc* (*Lsi* (*Lgr* (*Lka, Lpo, Lde, Lgm*)))))	cpDNA RFLPs	neighbor-joining
Strauss, Doerksen, and Byrne (1990)	1. ((((*Psi, Pwi*) *Pja*) *Pme*) *Pma*) *Loc*) 2. (((*Psi, Pwi*) *Pja*)(*Pme, Pma*)) *Loc*)	nuclear, cpDNA, mtDNA	parsimony





Figs. 1–2. Diagrams for the ITS region of representative members of *Larix* and *Pseudotsuga.* **1.** *Larix occidentalis.* **2.** *Pseudotsuga macrocarpa.* Arrows above and below figures represent the reverse and forward primers, respectively. Primers designed for this study are LAITS1R (5′-CATCCGAGTTGGTACACGC-3′), LAITS1B (5′-CCAAGGGCCTTGCATCAT-3′), LAITS1CR (5′-AGCGACAACAAGCAATGC-3′), DFITS1R (5′-GTATGCAAAGGCAGGCGG-3′), DFITS1B (5′-CCGCCTGCCTTTGCATAC-3′), and DFITS1CR (5′-GGCCAACAGCAAACAATGC-3′).

prepared for sequencing by gel purification (Qiagen, Chatsworth, California). Additional primers used for sequencing were 5.8GYM (Liston et al., 1996), ITS-3 (White et al., 1990), and six genus-specific primers designed for sites along ITS1 of *Larix* and *Pseudotsuga* (Figs. 1–2). Cycle sequencing with dye-terminator chemistry was performed using an ABI model 377 fluorescent sequencer (PE Applied Biosystems, Inc., Foster City, California). Products were sequenced in both 5′ to 3′ and 3′ to 5′ directions.

Full-length PCR products were digested with the restriction enzymes *Hae*III (GGCC), *Hha*I (GCGC), and *Hin*fI (GANTC) according to manufacturer's instructions. Digested products and a 100-bp ladder size standard (Life Technologies, Gaithersburg, Maryland) were electrophoresed on a 2% agarose gel in Tris-Borate-EDTA buffer (pH 8.6). Gels were visualized with ethidium bromide under UV light. Restriction site maps determined from the available full-length ITS region sequences indicated that some restriction enzymes would produce multiple bands of similar size (within 10 bp) in the 50–200 bp range. Since these similar-sized bands would be difficult to resolve using agarose gel electrophoresis, no bands smaller than 50 bp were interpreted, nor were some digestion products in the 50–200 bp range.

Direct sequencing of PCR products revealed several species with potential site and length polymorphisms at the 5′ end of ITS1. PCR products from four of these species (*P. macrocarpa, P. menziesii, L. decidua,* and *L. sibirica*) were ligated into a pCR®2.1 vector using a TOPO TA Cloning Kit (Invitrogen, San Diego, California). Seven to eight cloned inserts per species were reamplified with ITS5* and 26S-25R and digested with *Hae*III, *Hha*I, and *Hin*fI. Restriction profiles were compared to that of the original PCR product. For each of the four species, a single clone matching the restriction profile of the original PCR product was sequenced with ITS5*. An additional 400 bp were sequenced from the *P. menziesii* clone using the forward primer DFITS1B. A single clone from *L. sibirica* with a restriction site loss relative to the original PCR product was also sequenced with ITS5*.

Sequence analysis—Sequences were assembled and edited using Genetic Data Environment (GDE) (Smith et al., 1994). Ambiguous sites determined by the presence of multiple peaks at a single position were designated by IUBMB symbols. Sequence data for the ITS region were aligned using the PILEUP option in Wisconsin Package Version 9.0 (1996) (gapweight = 1.0, gaplengthweight = 0.2). Further manual adjustments were made for the alignment used in an analysis of ITS1 subrepeats. Alignments are available from the authors upon request. Secondary structures were generated using mfold version 2.3 (Jaeger, Turner, and Zuker, 1990) and displayed with RnaViz (De Rijk and De Wachter, 1997). BLAST (Altschul et al., 1990) and Gapped BLAST (Altschul et al., 1997) were used to search DNA sequence databases for high similarity with other accessions. Parsimony and distance analyses were performed using PAUP* 4.0.0d60 for UNIX (Swofford, 1998). Gaps were treated as missing characters. Parsimony and distance trees for the ITS region were obtained using the branch-and-bound and neighbor-joining methods, respectively. Neighbor-joining analyses were performed with a Kimura two-parameter correction. For the full-length ITS region and the 5.8S plus ITS2 parsimony analyses, bootstrap values were obtained from 500 replicates each using 50 random addition sequences and a maxtrees limit of 3000. For the subrepeat analysis, a random sequence addition heuristic search with 40 replicates was employed using tree bisection and reconnection branch swapping. The ITS region sequence from *Pinus pinea* was used to root the phylogenetic analyses of ITS1 subrepeats and the 5.8S plus ITS2. The full-length ITS region analysis was midpoint rooted based on the assumption of a sister-group relationship between *Larix* and *Pseudotsuga.* Phylogenetic trees were displayed using TREEVIEW (Page, 1996).

RESULTS

Sequence heterogeneity—Direct sequencing of gel-purified PCR products resulted in sequence reads of fair to poor quality at the 5′ end of ITS1 in *Larix decidua, L. sibirica,* and *L. kaempferi* and in all five *Pseudotsuga* species, but was not observed in *L. lyallii, L. occidentalis, L. laricina, L. kaempferi,* or *L. griffithiana.* A prominent feature of the signal degradation was the presence of multiple peaks that could be observed in both the 5′ to 3′ and 3′ to 5′ direction. In most instances, one peak was stronger than the others. This strong peak was scored by the automated sequencer or by manual editing.

Sequences of *Larix decidua, L. sibirica, Pseudotsuga menziesii,* and *P. macrocarpa* had ~400-bp stretches of degraded signal characterized by multiple peaks at most positions. These sequence reads were interpreted as having peaks from a sequencing template with small-length polymorphisms. Restriction digests of cloned PCR products from these four species made it possible to confirm the presence of restriction site variation. RFLP patterns differing from the original PCR product were found in six of eight *L. sibirica* clones, three of eight *L. decidua* clones, two of eight *P. menziesii* clones, and three of seven *P. macrocarpa* clones. In *L. sibirica,* the *Hha*I RFLP pattern of the original PCR product included bands at 209 and 150 bp (precise sizes determined from sequence). Clones with RFLP patterns that lacked one of these bands apparently had gained *Hha*I sites. The 209-bp and 150-bp bands in the original PCR product from *L. sibirica* were of low intensity relative to larger and smaller bands, suggesting that only a fraction of the ITS region copies possessed these patterns and therefore polymorphisms were also present in the original PCR product. In clones from the other three species, restriction site differences resulted in large bands that were not visible in restriction digests of the original PCR product. The polymorphic 209-bp product in *L. sibirica* mapped to ITS2, but there was no evidence of polymorphism in the direct

sequence reads from that region. One clone had another *Hha*I site in ITS2 that gave two bands of ~97 and 112 bp. Two sites differing by 1 bp from an *Hha*I site (GGGC and GCGG) were identified in the sequence reads 90–115 bp downstream of the invariant ITS2 site.

Sequence reads using the ITS5* primer were obtained for each of the four species that were cloned. The ITS5* read from a *Larix decidua* clone matching the RFLP pattern of the original PCR product differed at eight of the first 565 sites in ITS1. Four of the site differences could be seen as weak or multiple peaks in the direct sequence reads, and the remaining four were not. This ITS type was interpreted as not matching the main type present in the *Larix decidua* exemplar. An ITS5* sequence was determined for two RFLP profiles of *L. sibirica.* Discrepancies among the two clones and the direct sequence read were traced to eight polymorphic positions. Five of those positions were also polymorphic in either *L. kaempferi* or *L. decidua.* In the *P. menziesii* clone, the ITS5* sequence differed at only one of the first 600 bp from the sequence determined from the original PCR product. The ITS5* read for *P. macrocarpa* differed at seven of the first 600 bp. In *L. sibirica, L. decidua,* and *P. macrocarpa,* the first discrepancy between the sequence of the clone and the sequence of the original PCR product occurred at the onset of signal degradation. Sequences from clones lacked a nucleotide present in the original PCR products at ITS1 position 4 of *L. sibirica* and *L. decidua,* and ITS1 position 62 of *P. macrocarpa.* In *L. sibirica,* an additional gap relative to the direct sequence read occurred at position 422, and this gap corresponded to the onset of signal degradation in the reverse read from the LAITS1R primer site. Additional ambiguity symbols were placed into polymorphic positions in these four species to account for the discrepancies between sequences from the clones and the direct PCR products. The final alignment had a total of 20 ITS1 positions that were ambiguous in at least one species. This represented 5.1% of all variable positions. Fifteen ambiguity symbols were placed at ten positions in one or more species of *Larix,* and each of the five species of *Pseudotsuga* had a single ambiguity symbol placed at a unique position.

Conserved sequence motifs, subrepeats, and secondary structure—The 16-bp conserved motif observed in ITS1 of angiosperms (Liu and Schardl, 1994) is not present in *Larix, Pseudotsuga,* or *Pinus pinea.* Yet ITS1 subrepeats with sequence homology to five found in *Pinus pinea* (Marrocco, Gelati, and Maggini, 1996) were observed in the 3′ 620 bp of ITS1 in *Larix* and *Pseudotsuga* (Figs. 1–2). Although the ITS1 length of *Larix* was observed to exceed that of *Pseudotsuga* by ~200 bp, *Pseudotsuga* had three copies of the subrepeat and *Larix* only two. A 230-bp region separated the first subrepeat from the second subrepeat in both *Larix* and *Pseudotsuga.* The final two subrepeats in *Pseudotsuga* were in tandem. All subrepeats had a highly conserved, central core of GGCCACCCTAGTC. By searching for this core sequence, a sixth, unreported subrepeat was found in *Pinus pinea.* The sixth subrepeat, hereafter referred to as subrepeat zero (SR0), was more divergent than the five previously reported. In *Pinus pinea,* similarities among subrepeats one through five (SR1–SR5) ranged from 81.9 to 95.5%, compared to a similarity range of 48.3–57.5% with SR0. The core started at position 572, 883 bp upstream of the conserved region of the first of the five tandem subrepeats in *Pinus pinea. Larix* and *Pseudotsuga* subrepeats were more similar to each other regardless of position in ITS1 than they were to those reported in *Pinus pinea.* Similarities between the first and second subrepeats in *Larix* ranged from 66.6% in *L. decidua* to 71.3% in *L. griffithiana* (mean ± SD for all species: 68.4 ± 1.7%). In *Pseudotsuga,* similarities between the second and third subrepeats ranged from 83.1% in all three Asian taxa to 89.6% in *P. macrocarpa* (mean ± SD: 84.9 ± 2.8%). Similarities between the first and second subrepeats in *Pseudotsuga* ranged from 73.9% in *P. menziesii* to 79.7% in *P. macrocarpa* (mean ± SD: 78.3 ± 2.5%). Similarities between the first and third subrepeats in *Pseudotsuga* ranged from 70.4% in *P. menziesii* to 78.3% in *P. macrocarpa* (mean ± SD: 74.8 ± 2.8%).

The lengths of the *Larix* and *Pseudotsuga* subrepeats on either side of the core were judged by visual examination of an alignment. The first subrepeat ranged from 68 to 72 bp in species of *Larix* and *Pseudotsuga* compared to 215–237 bp reported for the five subrepeats in *P. pinea.* The second subrepeat in *Larix* and the second and third subrepeats in *Pseudotsuga* ranged from 77 to 78 bp. Secondary structure analysis of ITS1 revealed that the first *Pseudotsuga* subrepeat (Fig. 3) and both *Larix* subrepeats (data not shown) formed short terminal helices with two hairpin loops. In *Pseudotsuga,* nucleotides in SR2 and SR3 paired together to form longer terminal helices with three hairpin loops. The secondary structure of the subrepeats did not change at 20°, 25°, or 37°C or in less optimal folds. Other portions of ITS1 were sensitive to the inclusion or exclusion of flanking small subunit and 5.8S nucleotides (data not shown). Structures reported here included the final 8 bp of the small subunit and the first 32 bp of the 5.8S. These regions have been shown to interact with ITS1 using yeast as an experimental system (Yeh, Thweatt, and Lee, 1990; van Neus et al., 1994). In preliminary folds, portions of the 5′ 100 bp and 3′ 100 bp of ITS1 interacted with one another. This interaction was prohibited in the final folds by constraining nucleotides to base pair only with neighbors within 400 positions, which is more consistent with studies on yeast.

A heuristic search was performed in PAUP* to determine the relationships among the subrepeats (Fig. 4). The topology of *Larix* and *Pseudotsuga* subrepeats in the resulting strict consensus of 70 most parsimonious trees (not shown) suggested that concerted evolution is occurring too slowly to homogenize subrepeats in these species. A clade composed of the first *Larix* subrepeat from each species of *Larix* was sister to a clade composed of the first subrepeat from each species of *Pseudotsuga.* A second clade composed of the second *Larix* subrepeats was sister to a clade composed of the third *Pseudotsuga* subrepeats, but bootstrap support was only 48%. The six *Pinus pinea* subrepeats formed a clade, a pattern different from that seen in *Larix* and *Pseudotsuga* but suggestive that concerted evolution is homogenizing subrepeats over a longer evolutionary time span.

BLAST searches with the two subrepeats in *L. occi-*

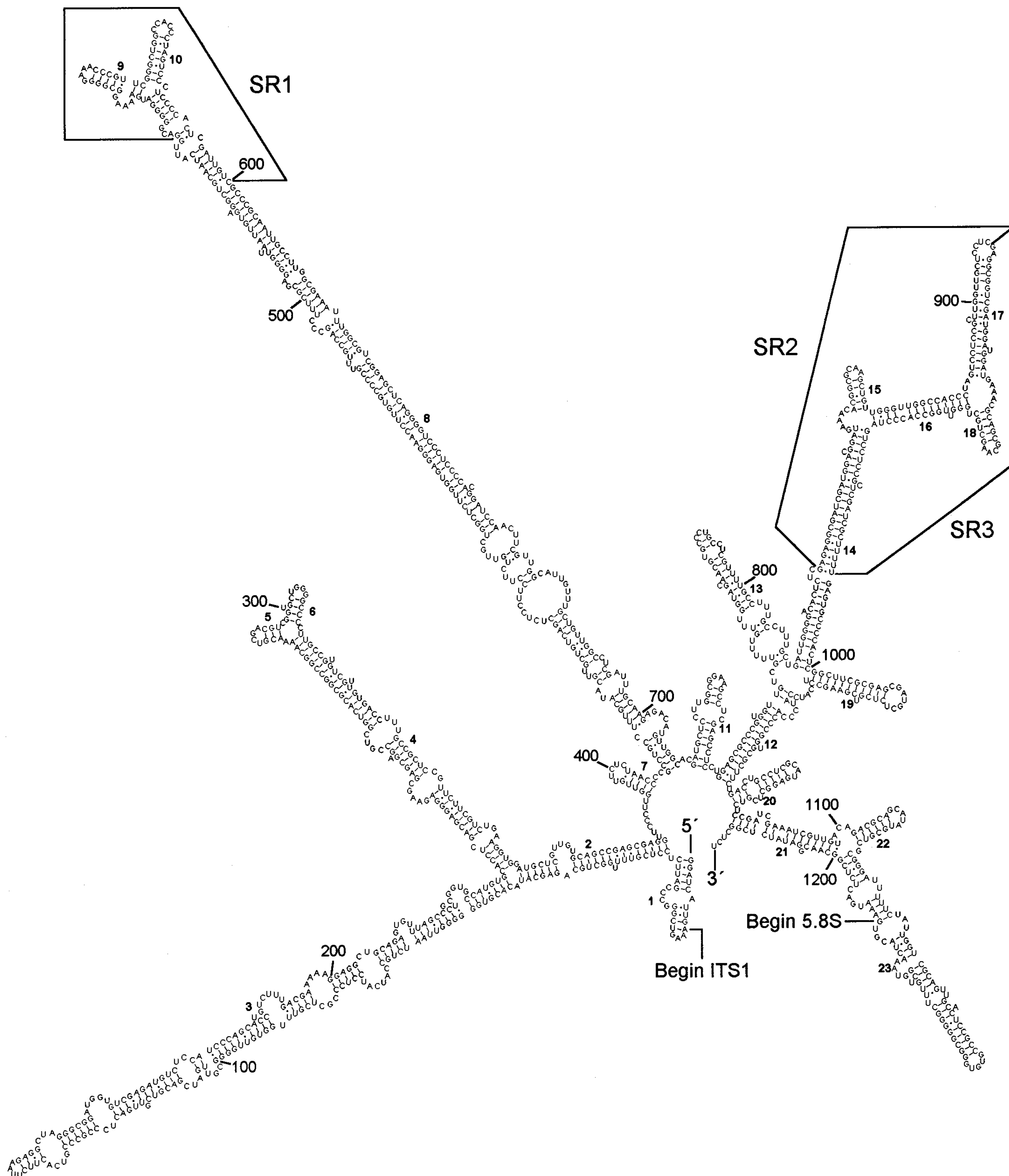


Fig. 3. Secondary structure of ITS1 for *Pseudotsuga menziesii* (energy = −2431 kJ/mol). Nucleotides are constrained to pair only within 400 bp. The free energy of the unconstrained fold for *P. menziesii* (not shown) is −2474 kJ/mol. *Pseudotsuga menziesii* has a 3-bp autapomorphic insertion in SR1. The interaction between SR2 and SR3 is predicted in folds for all species of *Pseudotsuga.* Helices are numbered 1–23.

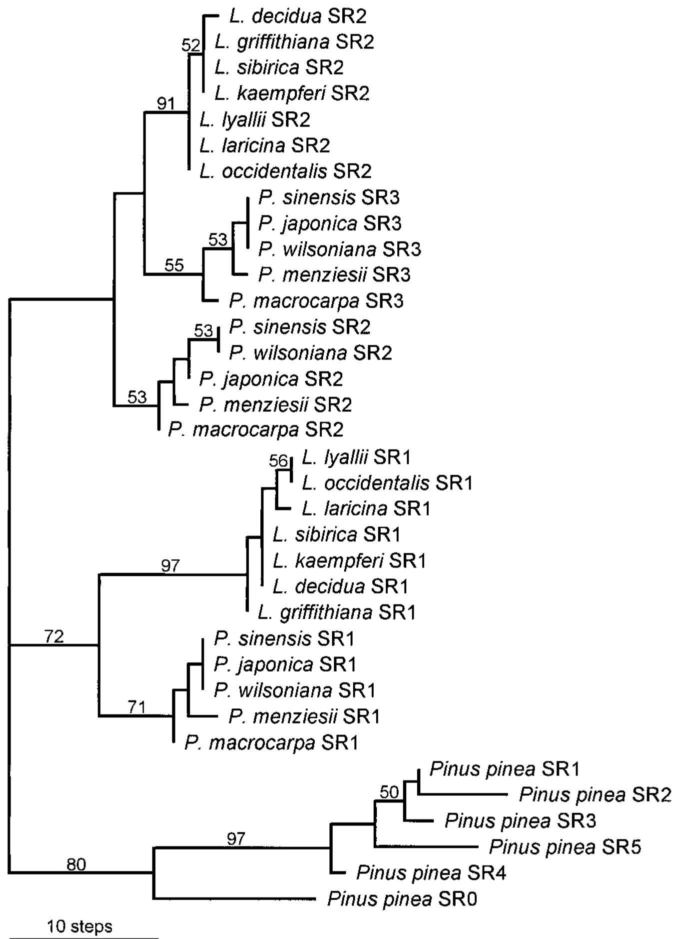


Fig. 4. One of 70 most parsimonious trees found in a heuristic search of ITS1 subrepeats (116 steps, CI = 0.651 excluding uninformative characters, RI = 0.896, and RC = 0.610). In the bootstrap analysis, the maxtrees limit of 3000 trees was reached 27 times. Bootstrap values (100 replicates) ≥ 50% are displayed on the branches. The scale bar represents the number of steps.

dentalis and *L. griffithiana* and the three subrepeats in *P. macrocarpa* and *P. wilsoniana* found high similarity to the *Pinus pinea* ITS region sequence (GenBank accession number X87936). BLAST also found matches in nonhomologous molecules (results not shown). These matches had a low degree of similarity that could have been explained by chance alone.

Phylogenetic analyses—ITS region lengths of the species sampled were close to those reported based on restriction site mapping (Liston et al., 1996). Excluding the flanking small and large subunits, the length of the region in *Larix* ranged from 1759 to 1770 bp and in *Pseudotsuga* from 1564 to 1571 bp (Table 3). Most of the site variation among species (88%) occurred in ITS1. Large indels in ITS1 explained most of the size differences between the two genera. Most of the ITS1 variability occurred at the 5′ end.

The alignment of the 5.8S and ITS2 of *Larix* and *Pseudotsuga* species with *Pinus pinea* was 426 positions in length. The *P. pinea* 5.8S sequence is 161 nucleotides in length, one nucleotide short of the number found in *Larix, Pseudotsuga,* and other species of *Pinus* (Liston et al., in press). The shorter length is attributable to a missing guanine residue 62 bp from the 5′ end that is invariant in all other species sequenced. After insertion of the guanine residue into the *P. pinea* sequence prior to the analysis, there were no gaps in the 5.8S portion of the alignment. The alignment included seven variable sites in the 5.8S, only two of which were phylogenetically informative. The ITS2 alignment included 11 gaps and had 89 variable sites, 42 of which were phylogenetically informative. Maximum parsimony analysis of the ITS2 and 5.8S using branch-and-bound searching found two most parsimonious trees that differed only in the placement of *L. decidua* relative to *L. griffithiana* (Fig. 5). Both *Larix* and *Pseudotsuga* were monophyletic, and there was limited resolution of some species relationships. The North American species of *Larix* formed an unresolved trichotomy separate from the Eurasian species. Of the Eurasian species of *Larix, L. kaempferi* and *L. sibirica* formed a clade that was a component of a polytomy involving *L. decidua* and *L. griffithiana.* The North American species of *Pseudotsuga* were sister taxa, and the three East Asian species sampled formed a clade.

The *Larix* and *Pseudotsuga* full-length ITS region alignment was 1876 characters in length. Sixty-three gaps were placed in ITS1, 51 of which were exclusively for alignment between the two genera. The mean maximum length of the gaps was 6.7 bp (range 1–43 bp). One gap was placed near the 3′ end of ITS2 to account for a nucleotide present in *L. decidua, L. kaempferi,* and *L. sibirica,* but absent in the other nine species. Along the entire ITS region, 329 out of 395 variable sites were phylogenetically informative.

Maximum parsimony analysis of the entire ITS region for *Pseudotsuga* and *Larix* gave a single, fully resolved tree of high consistency with bootstrap values exceeding 70% at all nodes (Fig. 6). The neighbor-joining topology was identical (result not shown). For both genera, the deepest branch separated species according to continent. *Pseudotsuga* was divided into a North American and an Asian clade, and *Larix* was divided into a North American and Eurasian clade. *Pseudotsuga sinensis* and *P. wilsoniana,* the species from mainland China and Taiwan, were identical in sequence (although *P. sinensis* had one ambiguous site). The Eurasian species *L. kaempferi* and *L. sibirica* were the most closely related pair sampled in *Larix,* and *L. griffithiana* was the most divergent. *Pseudotsuga macrocarpa* and *P. menziesii* each had a high number of autapomorphies (unique nucleotide substitutions) relative to other taxa. Maximum parsimony analysis of ITS1 alone found a single most parsimonious tree identical in topology to that of the full data set, although analysis of ITS2 alone failed to resolve the three species of *Pseudotsuga* from Asia or the three species of *Larix* from North America (results not shown).

Additional branch-and-bound analyses of the full-length ITS region were run in PAUP* constraining the topology to conform to previously proposed phylogenetic hypotheses for *Larix* and *Pseudotsuga* (Table 1). Constraining *P. macrocarpa* to occupy a basal position in the *Pseudotsuga* clade (tree 1 in Strauss, Doerksen, and Byrne, 1990) required 433 steps, six steps more than the most parsimonious tree. Enforcing the phylogenetic hy-

TABLE 3. Nuclear ribosomal DNA ITS region sequence statistics for *Larix* and *Pseudotsuga*.

Parameter	ITS1	5.8S	ITS2	Total
Aligned length	1481	162	233	1876
Length range				
Larix	1365–1375	162	232–233	1759–1770
Pseudotsuga	1170–1177	162	232	1564–1571
Mean length ± SD (bp)				
Larix	1369.7 ± 5.1	162 ± 0.0	232.4 ± 0.5	1764.1 ± 5.6
Pseudotsuga	1171.8 ± 2.9	162 ± 0.0	232 ± 0.0	1565.8 ± 2.9
G + C content mean ± SD				
Larix	0.575 ± 0.004	0.512 ± 0.0	0.600 ± 0.004	0.572 ± 0.004
Pseudotsuga	0.589 ± 0.003	0.525 ± 0.0	0.644 ± 0.002	0.590 ± 0.002
Number of variable sites	348	2	45	395
Larix	73	0	5	78
Pseudotsuga	77	0	8	85
Number of informative sites (%)	285 (19.2)	2 (1.2)	42 (18.0)	329 (17.5)
Larix	35 (2.4)	0 (0.0)	3 (1.2)	38 (2.0)
Pseudotsuga	31 (2.1)	0 (0.0)	5 (2.1)	36 (1.9)
Sequence divergence mean ± SD	0.136 ± 0.107	0.0066 ± 0.006	0.094 ± 0.078	0.115 ± 0.009
Larix	0.021 ± 0.011	0	0.009 ± 0.006	0.018 ± 0.009
Pseudotsuga	0.031 ± 0.018	0	0.02 ± 0.01	0.026 ± 0.016
Transitions (minimum)	197	2	27	226
Transversions (minimum)	159	0	20	179
Transitions/Transversions	1.24	undefined	1.35	1.26

potheses proposed for *Larix* by either Schorn (1994) or LePage and Basinger (1991, 1995) both required an additional 30 steps. Constraining *L. sibirica* to a position outside of a clade with *L. decidua, L. kaempferi,* and *L. griffithiana* required an additional 13 steps. Only a single additional step was required to force *L. decidua* and *L. sibirica* to be sister species instead of *L. kaempferi* and *L. sibirica.*

Restriction site analysis of PCR products allowed for the examination of four additional taxa whose ITS region sequences were not determined. The digests yielded a total of 27 scoreable sites, 11 *Hae*III sites, eight *Hha*I sites, and eight *Hin*fI sites. *Larix gmelinii* had a restric-

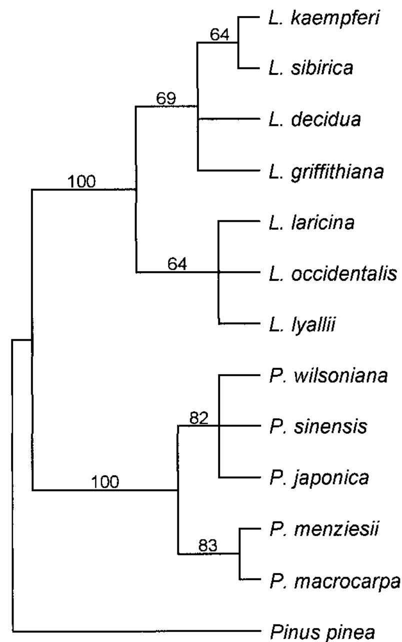


Fig. 5. Strict consensus of two most parsimonious trees generated with a branch-and-bound search of the 5.8S and ITS2 only (112 steps, CI = 0.967 excluding uinformative characters, RI = 0.988, and RC = 0.971). *Pinus pinea* is included as an outgroup. Bootstrap values (500 replicates) ≥ 50% are displayed on the branches.

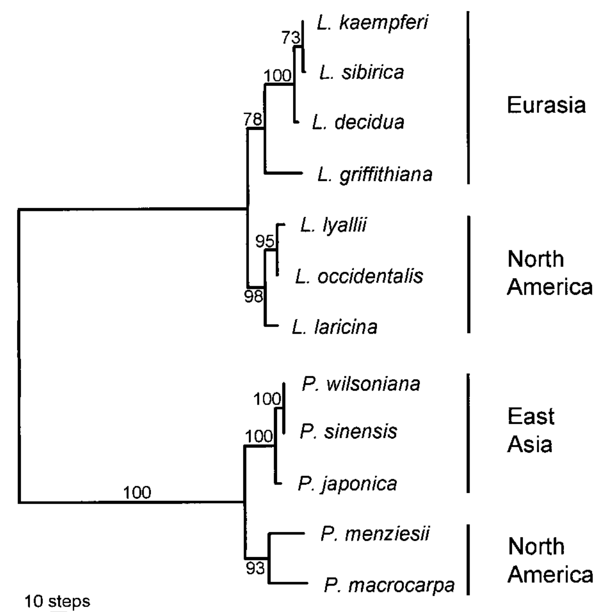


Fig. 6. The single most parsimonious tree generated using a branch-and-bound search of the entire ITS region (427 steps, CI = 0.948 excluding informative characters, RI = 0.984, and RC = 0.940). Bootstrap values (500 replicates) ≥ 50% are displayed on the branches. The scale bar represents the number of steps.

tion profile identical to those of the other three Eurasian larches, *L. decidua, L. kaempferi,* and *L. sibirica.* No synapomorphic restriction sites were found uniting the Eurasian larches, but all Eurasian larches lacked an *Hae*III site in ITS2 and two *Hha*I sites in ITS1 that were shared by the three North American larches. *Larix griffithiana* had a unique *Hae*III site in ITS1 that was absent in all other *Larix* examined and a unique 11-bp insertion in ITS1 that is apparent as a larger fragment when digested with *Hha*I. Restriction digests of the *Pseudotsuga* species allowed for the separation of three groups: *P. macrocarpa, P. menziesii,* and the Asian species of *Pseudotsuga.* The endemic Mexican taxa sampled (*P. guinieri, P. rehderi,* and both exemplars of *P. flahaultii*) had a potential ITS2 length polymorphism visible as a double banding pattern in the fragment mapping to ITS2 for all three restriction enzymes. The additional fragment was ~5–10 bp longer than the fragment present in all species of *Pseudotsuga.* Otherwise, the Mexican taxa had RFLP profiles identical to *P. menziesii.*

DISCUSSION

Evaluating systematic and evolutionary hypotheses for Larix and Pseudotsuga—Including multiple outgroup taxa has been shown to lead to a more robust rooting of the ingroup (Smith, 1994). Both outgroup rooting with *Pinus pinea* and midpoint rooting allowed the inference of a dichotomy between North American and Eurasian *Larix* and North American and East Asian *Pseudotsuga* (Figs. 5–6). Low sequence divergence between western North American species of *Larix,* northern Eurasian species of *Larix,* and east Asian species of *Pseudotsuga* were adequate to resolve relationships among species included in the sequence analysis, but appeared too low to address relationships within *P. menziesii* s.l. or *P. sinensis* s.l. Restriction digests of *P. menziesii* (var. *menziesii*) and exemplars of three segregate species for Mexican taxa otherwise placed in *P. menziesii* var. *glauca* were indistinguishable with the exception of an extra fragment interpreted as an ITS2 polymorphism. Sequences of *Pseudotsuga sinensis* and *P. wilsoniana* were identical with the exception of an ambiguous position scored for *P. sinensis.* Although segregate species in Mexico and China were incompletely sampled here, the results do not contradict the broad species concept for *P. menziesii* and *P. sinensis* used by Farjon (1990).

The sampling of *Larix* species in this study does not correspond completely with the cpDNA study of Qian, Ennos, and Helgason (1995), but the ITS region phylogeny corroborates their results, suggesting that previous systematic arrangements of *Larix* based on ovulate cone characters or fossil evidence (LePage and Basinger, 1991, 1995; Schorn, 1994) do not correspond with the evolutionary history of the group (Figs. 5–6). Instead, species phylogenies inferred from cpDNA and the ITS region data indicate that if exserted bracts are a symplesiomorphic character of *Larix* and *Pseudotsuga,* then reduction in bract length occurred independently in the North American and Eurasian lineages of *Larix.* The phylogenetic position of *L. sibirica* inferred from cpDNA restriction site analysis differs from its position inferred from ITS region sequences. Considering the morphological similarities, geographic proximity, and ITS region similarity among *L. sibirica, L. decidua, L. gmelinii,* and *L. kaempferi,* we suggest that the cpDNA placement of *L. sibirica* as a species removed from this seemingly monophyletic group is inconsistent with the phylogeny of *Larix.* Furthermore, a recent allozyme study documented a close genetic relationship between *L. decidua* and *L. sibirica* (Lewandowski, 1997). The unique cpDNA restriction fragments scored by Qian, Ennos, and Helgason (1995) for *L. sibirica* could be the result of a plastid rearrangement, hybridization, and subsequent chloroplast capture (Rieseberg and Soltis, 1991; Liston and Kadereit, 1995), or an artifact of their study. The position of *L. sibirica* within the northern Eurasian clade (*L. decidua, L. gmelinii, L. kaempferi,* and *L. sibirica*) in the ITS region study is more consistent with morphological and geographical evidence, but its sister relationship with *L. kaempferi* is only one step more parsimonious than a sister relationship with *L. decidua.* Considering the high ITS region and cpDNA sequence similarity among the northern Eurasian species of *Larix,* and the evidence of ITS region polymorphism in the group, further investigation is necessary before robust conclusions may be made regarding the relationships among members of the northern Eurasian clade.

North American *Larix* includes two long-bracted species (*L. occidentalis* and *L. lyallii*) and a short-bracted species (*L. laricina*). The Eurasian clade is composed of two lineages, one leading to the Himalayan *L. griffithiana* and the other to the more widely and northerly distributed *L. decidua, L. sibirica,* and *L. kaempferi.* Although *L. gmelinii* was not sequenced, ITS region restriction site profiles (reported here), cpDNA restriction fragment analysis (Qian, Ennos, and Helgason, 1995), cone anatomy, and geographical distribution place it in the northern Eurasian clade. Two species of *Larix* were not sampled. Based on cpDNA results (Qian, Ennos, and Helgason, 1995), *Larix potaninii* seems best placed with the northern Eurasian clade, but *L. mastersiana,* a rare species reported from high altitudes in the West Sichuan Province of China, awaits inclusion in a molecular phylogenetic analysis.

Strauss, Doerksen, and Byrne (1990) addressed the systematics of *Pseudotsuga* using 30 phylogenetically informative RFLP characters from nuclear, mitochondrial, and chloroplast-encoded genes. The study included five species representing all the main lineages of the genus and *L. occidentalis* as an outgroup. Only six *L. occidentalis* character states were shared with a subset of the *Pseudotsuga* species (symplesiomorphic), resulting in two equally most parsimonious roots connecting *Pseudotsuga* to *Larix.* One topology placed *P. macrocarpa* in the basal position of *Pseudotsuga,* with *P. menziesii* sister to an east Asian clade. The east Asian clade included *P. japonica* in a sister relationship with *P. sinensis* and *P. wilsoniana* (Table 2). In the second topology, *P. macrocarpa* and *P. menziesii* were sister species. In the ITS region study reported here, the single most parsimonious root between *Larix* and *Pseudotsuga* resulted from inclusion of a greater number of informative characters together with a greater number of *Larix* species. The ITS region topology (Fig. 6) agrees with Strauss, Doerksen, and Byrne's second tree, favoring the hypothesis that *P.*

macrocarpa is sister to *P. menziesii* rather than the basal lineage of the genus. Constraining the ITS region tree to agree with the first tree of Strauss, Doerksen, and Byrne (1990) requires six additional steps.

The ITS region results make it worthwhile to reevaluate the evolutionary history of *Larix* and *Pseudotsuga* based on paleontologic, biogeographic, and phylogenetic evidence. The concentration of Tertiary fossils near the present ranges of several extant species suggests a western North American or eastern Asian origin for *Larix* and *Pseudotsuga* (Schorn, 1994). Both genera have an older fossil record in North America than in Asia. Fossil evidence of North American *Pseudotsuga* dates to the Early Oligocene for a *macrocarpa*-like form occupying the lowland Willamette Valley Oregon and west Central Oregon (~32 mya) (Schorn, 1994), but the fossil history of *Pseudotsuga* in Asia extends only to the Pliocene (Hermann, 1985). The oldest fossil evidence of *Larix* dates to a putatively long-bracted *lyallii*-like form from the Thunder Mountain and Coal Creek, Idaho floras (45 mya) (Axelrod, 1990; Schorn, 1994), and a short-bracted form from the Canadian High Arctic found in sediments dating to the Middle to Late Eocene (LePage and Basinger, 1991). Fossil vegetation and cones have also been collected from Oligocene sites in Russia (LePage and Basinger, 1995).

Strauss, Doerksen, and Byrne (1990) hypothesized that the basal lineage of *Pseudotsuga* was North American, probably *P. macrocarpa.* The remaining species evolved in a stepping stone fashion. The ancestral form migrated north, probably giving rise to a *P. menziesii* lineage before extending its range across the Bering land bridge and later reaching Japan. A Japanese lineage spread south to found one or more species in mainland China and Taiwan. The direction of dispersal was supported by only one of their most parsimonious trees. The alternate RFLP topology agrees with the the single most parsimonious ITS region tree (Fig. 6). A continent of origin may not be inferred from this topology because the most recent common ancestor of *Pseudotsuga* is at a node that bifurcates into a North American and an Asian clade.

Attempts to identify centers of origin have been criticized on several grounds (Nelson and Platnick, 1981). One historical weakness has been the inability to determine whether vicariance or dispersal is responsible for the current distribution of a taxon. In the case of *Pseudotsuga,* this criticism is not serious because dispersal across the Bering land bridge is consistent with the occurrence of *Pseudotsuga* microfossils in Homer, Alaska, near the putative dispersal route (Hermann, 1985; Strauss, Doerksen, and Byrne, 1990), the absence of fossil evidence suggesting other dispersal routes, and the unlikelihood of vicariance since the genus is confined to the Pacific Rim but western North America and eastern Asia were never directly connected. The most serious weakness of a North American origin of *Pseudotsuga* is the fragmentary and regionally biased nature of the fossil record. It is less parsimonious but not unreasonable to hypothesize that there were older lineages in Asia or elsewhere that went extinct without leaving fossils or with a fossil record that remains undiscovered.

In contrast to the limited Pacific rim distribution of *Pseudotsuga, Larix* has a wider, more circumboreal distribution. In this case, vicariance events must also be considered because the distribution of *Larix* may be interpreted as resulting from range fragmentation following the breakup of Laurasia and the expansion of polar ice caps. In this study and in the cpDNA study of Qian, Ennos, and Helgason (1995), confinement of North American *Larix* to a single clade eliminates the need to postulate a recent North Atlantic or polar migration (Schorn, 1994) or vicariance as a consequence of Pleistocene glaciation (LePage and Basinger, 1995) to explain the presence of short-bracted species in both North America (*L. laricina*) and Eurasia (*L. decidua, L. gmelinii,* and *L. sibirica*).

Use of the ITS region in systematic studies of Pinaceae—There is a growing awareness of ITS region polymorphism in plants. Buckler, Ippolito, and Holtsford (1997) review evidence for recombination among divergent paralogues and the presence of pseudogenes and functional ITS regions in several studies. In Pinaceae, variability within the nrDNA repeats has been documented previously in *Picea* and *Pinus* (Karvonen and Savolainen, 1993; Karvonen, Szmidt, and Savolainen, 1994; Quijada et al., 1998). Multiple ITS types have been found within individuals of *Pinus rzedowskii* Madrigal & M. Caball (Quijada et al., 1998), and both IGS (intergenic spacer) and ITS polymorphisms have been found within individuals of *Pinus sylvestris* L. (Karvonen and Savolainen, 1993). The Mendelian inheritance pattern of one ITS region size variant of *P. sylvestris* was consistent with the hypothesis that rare variants are found at a single locus and that concerted evolution is occurring more rapidly within chromosomes than among sister chromatids or nonhomologous chromosomes.

In the present study, restriction mapping and DNA sequencing of direct and cloned PCR products were used to verify ITS region polymorphism in two species of *Larix* and two species of *Pseudotsuga.* Length variations and substitutions among ITS region paralogues within an individual were observed in direct sequence reads of PCR products. Analysis of clones showed that several paralogues had RFLP fragments that were not visible in digests of the original PCR product. Comparison of sequences from the direct PCR product to those of the cloned paralogues also revealed that some discrepancies could be traced to weak, subordinate peaks in the sequence reads from the original PCR product. These observations suggest that even when DMSO is used as a denaturing agent to increase the proportion of putatively functional nrDNA paralogues relative to nonfunctional ones during PCR (Buckler and Holtsford, 1996), divergent paralogues present in low abundance may be preferentially cloned. Another possibility is that independent mutational events result in substitutions that accumulate among paralogues throughout the genome. Because of a slow rate of concerted evolution they may be preserved at levels usually low enough to avoid detection by direct sequencing. Although ITS region polymorphism was confirmed in only four individuals representing as many species, it may be present to a varying extent in all species of *Larix* and *Pseudotsuga.*

In angiosperms, ITS region polymorphism has been attributed to hybridization, polyploidy, slow rates of con-

certed evolution across nrDNA loci on nonhomologous chromosomes, and agamospermy (see Buckler, Ippolito, and Holtsford, 1997; Campbell et al., 1997). The polymorphism in Pinaceae may be a result of a slow rate of concerted evolution among nrDNA loci. Species in Pinaceae, which typically have a diploid chromosome number of $2n = 24$, have more nrDNA loci than angiosperms. The number of chromosomes pairs with 18S-5.8S-26S loci has been shown to range from 5 to 8 in *Pinus* and *Picea* (Doudrick et al., 1995; Brown and Carlson, 1997). Recent studies of nrDNA organization in *Pseudotsuga* found 18S-5.8S-26S loci on three or four chromosome pairs (J. E. Carlson, Department of Forest Science and Biotechnology Laboratory, University of British Columbia, personal communication). The number of nrDNA loci and extent of ITS region polymorphism in other families of conifers remain to be explored.

Because of the great effort it would have required to sequence several full-length ITS region paralogues for one or more species of *Larix* or *Pseudotsuga,* the nature, distribution, and extent of the polymorphism were not examined thoroughly. Most importantly from a phylogenetic perspective, concerted evolution among paralogues may not be homogenizing the sequences rapidly enough for them to behave as a single orthologue, thus confounding the ability to infer a species phylogeny from a gene phylogeny (Sanderson and Doyle, 1992). Also, synapomorphic character states could not be distinguished from biased detection of ancestral polymorphism. Despite these limitations, the result of the phylogenetic analysis was a topology that was mostly congruent with other molecular studies performed on the genera (Fig. 6). Furthermore, the result of the full-length ITS region analysis was a single most parsimonious tree with high bootstrap values. We attribute this success to the long length of the ITS region in *Larix* and *Pseudotsuga,* the accompanying large number of informative sites available, and the small percentage of ambiguous sites detected. The presence of polymorphism should most seriously affect the placement of closely related species with low sequence divergence, and this may have occurred in the northern Eurasian *Larix* clade.

This study documents the presence of two ITS1 subrepeats in *Larix* and three in *Pseudotsuga.* In both genera, the subrepeats consistently form terminal helices in secondary structure models (Fig. 3). In *Pseudotsuga,* the second and third subrepeats are in tandem and they fold together. ITS1 subrepeats have also been found in other genera of Pinaceae (Marrocco, Gelati, and Maggini, 1996; Vining and Campbell, 1997). If ITS1 subrepeats in Pinaceae are evolving in concert, they could violate assumptions of character independence. We observed that within the sister genera *Larix* and *Pseudotsuga* there is no evidence of concerted evolution homogenizing subrepeats within a species. All ITS1 subrepeats of *Larix* and *Pseudotsuga* form a single clade, with monophyletic subclades composed of a single subrepeat from each species (Fig. 3). This is in contrast to other examined genera of Pinaceae, where all ITS1 subrepeats of a genus form a separate monophyletic clade (Vining et al., 1998). This pattern in Pinaceae is consistent with a process of concerted evolution that is too slow to be detected in recently diverged lineages (e.g., at the intrageneric level and between recently diverged genera), but becomes apparent over longer periods of time (Vining et al., 1998).

Parasitic flukes also have ITS1 subrepeats (Luton, Walker, and Blair, 1992; Kane and Rollinson, 1994). As in Pinaceae, the number of subrepeats varies among species in the fluke genus *Schistosoma* (Kane and Rollinson, 1994). The length of the *Schistosoma* subrepeats ranges from 72 to 80 bp, which is comparable to the 69–74 bp range in *Pseudotsuga* and *Larix.* Although the function of the subrepeats in Pinaceae is unknown, their conserved nature in Pinaceae and robust behavior in secondary folding models suggest that they may be involved in rRNA processing. No conclusive homologous matches were detected in DNA sequence databases using BLAST or gapped BLAST, except to *Pinus pinea.*

This study demonstrates the utility of nucleotide sequence data from the ITS region for reconstructing a phylogeny for two genera in Pinaceae. Using the entire region made it possible to identify a highly consistent, completely resolved tree. ITS2 alone, though easy to align and consistent with the full-length ITS region phylogeny, was inadequate for resolving relationships among closely related species of *Larix* and *Pseudotsuga.* ITS1 in these genera is longer, and the number of substitutions per site within a genus is roughly twice that for ITS2. These observations are consistent with recent analyses demonstrating the potential for obtaining deep-level phylogenetic signal from plant ITS2 (Hershkovitz and Lewis, 1996). If rates of substitution are comparable across other genera of Pinaceae, the ITS region could be expected to resolve generic and infrageneric relationships, but alignment difficulties may diminish the utility of ITS1 for a family-wide phylogeny.

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