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The phylogenetic position of the endemic flat-needle pine *Pinus krempfii* (Pinaceae) from Vietnam, based on PCR-RFLP analysis of chloroplast DNA

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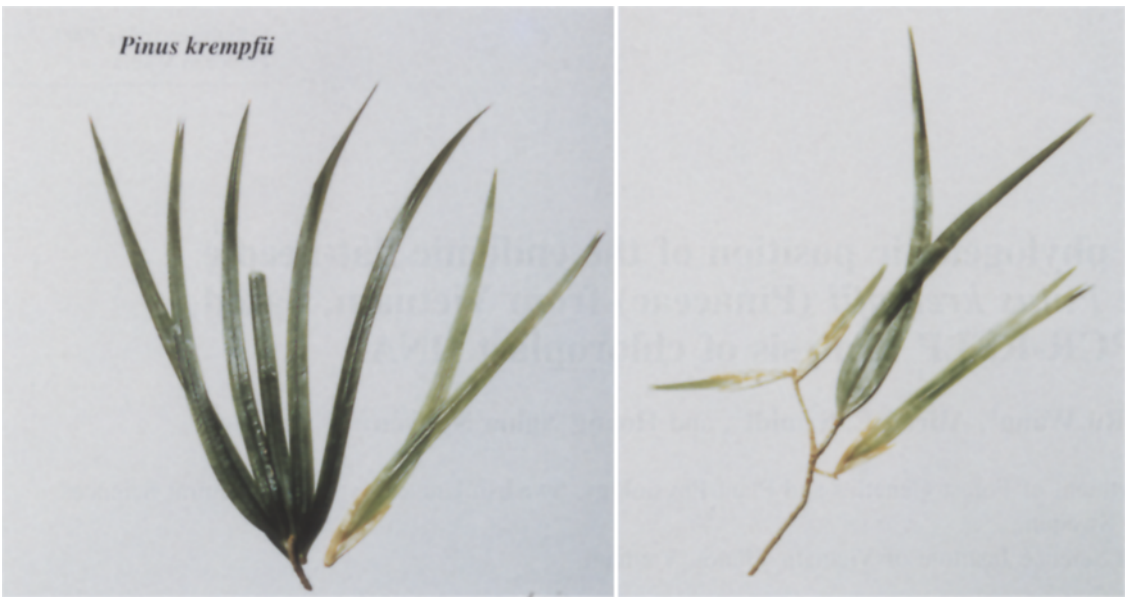
Abstract. *Pinus krempfii* is morphologically very unique as compared to other *Pinus* species by having flat leaf-like needles. Its taxonomic position has been problematic ever since its discovery. In this study, an attempt was made to infer the taxonomic status of *P. krempfii* through restriction fragment length polymorphism analysis of 12 PCR amplified chloroplast (cp) DNA regions. Phylogenetic analysis was conducted using 10 representatives of the two *Pinus* subgenera: *Strobus* and *Pinus*. In addition, to infer the position of *P. krempfii* in Pinaceae in relation with other genera, 14 representatives of eight additional genera were included in the analysis. Our cpDNA-based results indicate that: 1) *P. krempfii* clearly belongs to the genus *Pinus*. This result does not favour the creation of a new genus *Ducampopinus* in Pinaceae for this taxon. 2) Within the genus *Pinus*, *P. krempfii* is more allied with species in subgenus *Strobus* and differs distinctly from species in subgenus *Pinus*. 3) Despite the similarity in certain morphological and anatomical leaf and wood characters to *Keteleeria* and *Pseudolarix*, the cpDNA data do not support the hypothesis for close relationship between *P. krempfii* and these two genera.

Key words: Gymnosperm, Pinaceae, *Pinus*, *P. krempfii*, cpDNA, PCR-RFLP, phylogeny.

The genus *Pinus* is widely distributed in the Northern Hemisphere, from the tree limit in sub-arctic lowlands to the tree limit in high mountains of subtropical and tropical regions (Critchfield and Little 1966, Mirov 1967). The genus is usually divided into two subgenera *Strobus* (=*Haploxylon*) and *Pinus* (=*Diploxylon*), and these further into sections and subsections (Little and Critchfield 1969, Mirov 1967). Classification of the genus, especially the number of *Pinus* species recognized from Asia, varies among the authors (Farjon 1984, Little and Critchfield 1969, Mirov 1967, Shaw 1914). The taxonomic status of many Asian pines is poorly understood to date. Perhaps the most prominent unsolved issue in this genus is the taxonomic position of *Pinus krempfii* Lecomte endemic to Vietnam.

In 1921, a pine-like evergreen conifer was discovered in the mountains of southern



Vietnam and was described by Lecomte (1921) as *P. krempfii*. *Pinus krempfii* is a large tree, up to 30 m in height, with a trunk diameter up to 80 cm (Nguyên and Vidal 1996). Unlike any other pine, *P. krempfii* is characterized by having two flat leaf-like needles rather than typical pine-like needles (Lecomte 1921, 1924). The needles of *P. krempfii* are narrowly lanceolate, arranged in fascicles of two, concentrating at tips of twigs (Fig. 1). Young trees have longer needles (10–15 cm long and up to 6 mm wide) than mature trees (3–7 cm long and 2–5 mm wide) (Nguyên 1993, Nguyên and Vidal 1996). The seed cones are sub-pendulous, ovate when closed, 4–9 cm long and 3–8 cm in diameter. The seeds have well-developed articulate wings (Nguyên and Vidal 1996, Anonymus 1996b). The distribution of *P. krempfii* in Vietnam is very limited. It is found only in some localities of Khanh Hoa and Lam Dong provinces at elevations of 1200–2000 m (Nguyên 1993). It occurs naturally in tropical mixed broadleaf forest, occasionally together with *P. dalatensis*, *P. merkusii* and *P. kesiya* (Mirov 1967, Anonymus 1996b). *Pinus krempfii* occurs in small groups of 10–30 trees and grows together with species of Fagaceae, Magnoliaceae, Lauraceae, *Cryptocarya* sp., *Illicium* sp., *Rhodoleia* sp., and *Podocarpus* sp., which form very dense forests (Nguyên 1993, Anonymus 1996a).

Pinus krempfii has several morphological and wood anatomical features giving unusual combinations of characters (Buchholz 1951, De Ferré 1948, Ickert-Bond 1997). It has been suggested that the taxon represents a link between the genus *Pinus* and other genera such as *Keteleeria* and *Pseudolarix* of the family Pinaceae (De Ferré 1948, 1953). Chevalier (1944) has elevated this taxon to the rank of a monospecific genus in the family Pinaceae and renamed the taxon *Ducampopinus krempfii*. Other authors, however, only recognized a subgenus *Ducampopinus* in the genus *Pinus* to accommodate this taxon (De Ferré 1953, Gaussen 1960, Little and Critchfield 1969). In Pilger's (1926) classification, on the other hand, *P. krempfii* was placed in the same section *Paracembra* as *P. bungeana* and *P. gerardiana*, but in a different subsection together with *P. balfouriana* and *P. aristata*.

Farjon (1984) following the subdivision of Van der Burgh (1973) placed *P. krempfii* in section *Parrya,* monospecific subsection *Krempfianae.* Florin (1931) considered *P. krempfii* to belong to the subgenus *Strobus* (*Haploxylon*). Price (1989) was also of the opinion that this species fits well into subgenus *Strobus*, supported by the presence of a single vascular bundle in the needle and the heartwood phenolic compounds characteristic of subgenus *Strobus* (Erdtman et al. 1966, Ickert-Bond 1997). Due to the lack of more detailed studies, the taxonomic position of this unique, flat-needle pine remains uncertain.

Phylogenetic inferences based on chloroplast (cp) DNA markers may provide additional insights into relationship and evolution of plants. cpDNA has proven to be a useful source of data for phylogenetic reconstruction at different taxonomic levels (e.g. Brunsfeld et al. 1994, Chase et al. 1993, Lavin et al. 1991, Plunkett et al. 1997). Although with some limitations, established methods in cpDNA-based molecular systematics are regarded as an additional useful approach, complementing morphological characters, in inferring phylogenies in plants. Recent research on *Pinus* evolution has provided much new information on the relationships among groups of species from different geographic regions (e.g. Farjon 1996, Karalamangala and Nickrent 1989, Klaus 1989, Millar 1993, Wang 1992). Many of the studies have utilized DNA characters in their phylogenetic analysis (Govindaraju et al. 1992, Krupkin et al. 1996, Moran et al. 1992, Pérez de la Rosa et al. 1995, Strauss and Doerksen 1990, Wang and Szmidt 1993). Unfortunately, only a limited number of reports focused on (Wang and Szmidt 1993) or included (Govindaraju et al. 1992, Liston et al. 1999, Piovesan et al. 1993, Strauss and Doerksen 1990, Tsumura et al. 1995) Asian species. More regrettably, almost all these studies omitted *P. krempfii*, mainly due to the difficulties in obtaining the plant material owing to the rarity of this species in Vietnam. To date, only in one recent phylogenetic study on *Pinus* by Liston et al. (1999), based on nuclear ribosomal (r) DNA internal transcribed spacer (ITS) sequences, was *P. krempfii* included. The analysis of rDNA ITS regions placed *P. krempfii* in a clade consisting members of subsections *Strobi* and *Cembrae* of the subgenus *Strobus* (Liston et al. 1999). Considering its unique morphology and its still unclear phylogenetic status, this unusual pine clearly deserves more research to assist its classification.

In the present study, we used restriction fragment length polymorphism (RFLP) analysis of 12 polymerase chain reaction (PCR) amplified cpDNA regions to examine the position of *P. krempfii* in Pinaceae and in the genus *Pinus*. For comparison, we selected several pines from the two subgenera *Strobus* and *Pinus* occurring in Asia. In a previous study by Tsumura et al. (1995), phylogenetic relationships among 45 conifer species from five families were examined by PCR-RFLP analysis of six cpDNA regions. We used these six cpDNA regions in the present analysis to infer the relationship of *P. krempfii* with other genera in Pinaceae. The present paper represents the first report from our ongoing research investigating the phylogeny of *P. krempfii*. Our main aims are to reexamine some suggestions raised by previous students on this unique species and to establish the generic affinity of *P. krempfii* in Pinaceae and its position within the genus *Pinus*.

Material and methods

Species sampled. Needle samples of *P. krempfii* were collected from six individual trees in Lac Duong, Lamdong, Vietnam (12°N, 108°–109° E). To infer relationship of *P. krempfii* with the neighbouring Asian pines, we selected three species from subgenus *Strobus*: *P. bungeana*, *P. gerardiana* and *P. wallichiana* and two species of subgenus *Pinus*: *P. merkusii* and *P. kesiya*. Our choice of species was determined by their geographical distribution and the prior knowledge of cpDNA variation in individual *Pinus* species

Table 1. List of species analyzed in this study

Family	Genus	Species	Geographic distribution
Pinaceae	*Pinus*[1]		
	Subgenus *Ducampopinus*	*P. krempfii* Lecomte	Vietnam
	Subgenus *Strobus*		
	Section *Strobus*		
	Subsect. *Cembrae*	*P. koraiensis* Sieb. et Zucc.[2]	Northeastern Asia
	Subsect. *Strobi*	*P. parviflora* Sieb. et Zucc.[2]	Japan
		P. strobus L.[2]	Eastern America
		P. Wallichiana A. B. Jackson	Himalayas
	Section *Parrya*		
	Subsect. *Gerardianae*	*P. bungeana* Zucc.- Endl.	China
		P. gerardiana Wall.	Himalayas
	Subgenus *Pinus*		
	Section *Pinus*		
	Subsect. *Sylvestres*	*P. merkusii* Jungh. et De Vriese	Southeastern Asia
		P. kesiya Royle	Southeastern Asia
		P. densiflora Sieb. et Zucc.[2]	Eastern Asia
		P. thunbergii Parl.[2]	Eastern Asia
	Picea	*P. abies* (L.) Karst.	Eurasia
		P. jezoensis (Sieb. et Zucc.) Carr.[2]	Eastern Asia
	Abies	*A. homolepis* Sieb. et Zucc.[2]	Japan
		A. mariesii Masters [2]	Japan
		A. sachalinensis (Fr. Schmidt) Masters [2]	Japan
		A. veitchii Lindl.[2]	Japan
	Cedrus	*C. deodara* (Roxb.) G. Don in Loud.[2]	Himalayas
	Keteleeria	*K. davidiana* (Bertr.) Beissn.[2]	Southeastern Asia
	Pseudotsuga	*P. japonica* (Shirasawa) Beissn.[2]	Japan
		P. wilsoniana Hayata [2]	Southeastern Asia
	Tsuga	*T. diversifolia* (Maxim.) Masters [2]	Japan
		T. sieboldii Carr.[2]	Japan
	Larix	*L. kaempferi* (Lamb.) Carr.[2]	Japan
	Pseudolarix	*P. amabilis* (Nelson) Rehder [2]	China
Taxodiaceae	*Sequoia*	*S. sempervirens* (D. Don) Endl.[2]	North America
Cupressaceae	*Thuja*	*T. standishii* Carr.[2]	Japan

[1] *Pinus* classification following Little and Critchfield (1969). [2] cpDNA data retrieved from Tsumura et al. (1995).

from Asia (Wang and Szmidt 1993). *Picea abies* was included in the analysis as an outgroup species (Table 1). Needle samples of *Picea abies* were collected from one documented individual in Umeå, Sweden. The origin of samples for *P. kesiya*, *P. merkusii*, *P. wallichiana*, *P. gerardiana* and *P. bungeana* was as described in Wang and Szmidt (1993).

To facilitate the placement of *P. krempfii* in relation to other genera of Pinaceae, the seven species included in our study were combined with 20 additional taxa analyzed by Tsumura et al.

(1995). Two of these 20 species, *Sequoia sempervirens* and *Thuja standishii* from two different families: Taxodiaceae and Cupressaceae, respectively, were included as outgroup species. The conifers are traditionally divided into seven families: Pinaceae, Podocarpaceae, Araucariaceae, Taxaceae, Cephalotaxaceae, Taxodiaceae and Cupressaceae (Pilger 1926). We are aware of the current discussion on the merger of Taxodiaceae into Cupressaceae (e.g. Brunsfeld et al. 1994, Eckenwalder 1976, Hart 1987, Stefanović et al. 1998). However, since so far the taxonomists have not reached final decision on the issue, we kept the status of Taxodiaceae in the present paper. Morphological (Hart 1987) and molecular data (Stefanović et al. 1998) have shown that within the conifers, Pinaceae appear to be the sister group of the rest of families. Thus, possibly any conifer family outside Pinaceae could be used as outgroup to Pinaceae in cladistic analysis. It is possible that Podocarpaceae and Araucariaceae could be better for rooting Pinaceae because of the closer position of Podocarpaceae to Pinaceae revealed by a morphological cladistic analysis (Hart 1987), as well as the sister group relationship of Podocarpaceae and Araucariaceae revealed by 28S rRNA sequence data (Stefanović et al. 1998). We did not include these two families due to the lack of compatible RFLP data. However, in the initial analysis, we included *Torreya nucifera* (Taxaceae) and *Cephalotaxus harringtonia* (Cephalotaxaceae) as outgroups. Inclusion of these two taxa did not affect either the monophyly of Pinaceae or the topology of the ingroup species, but the most parsimonious trees were 53 steps longer than the trees found with only *S. sempervirens* and *T. standishii* as outgroups. Thus, in the following analysis we kept only *S. sempervirens* and *T. standishii* as outgroups.

DNA isolation, PCR amplification and digestion. Genomic DNA was isolated from needles of individual trees according to Szmidt et al. (1986). The $CsCl_2$ purified DNA samples were used for PCR amplification of specific cpDNA regions. The PCR reaction mix contained 15–20 ng DNA template, 200 µM each of dNTP (Pharmacia), 0.4 µM each of the primers, and 0.75U of Taq DNA polymerase (Pharmacia) in a total volume of 25 µl. PCR amplification was carried out at 94 °C, 3 min for initial denaturation, followed by 35 cycles of denaturation at 94 °C for 1 min, primer annealing for 1 min, extension at 72 °C for 2 min, and termination at 72 °C for 5 min. The annealing temperature varied among primer pairs for different cpDNA regions as specified in Table 2.

To confirm successful amplification and to determine the size of the amplified cpDNA fragments, the PCR products generated by each pair of the primers were first examined by 1% agarose gel electrophoresis of 3 µl of the PCR products. Subsequently, the PCR products were digested separately with 6–14 restriction enzymes (Table 2) and used for RFLP analysis. The digested cpDNA fragments were separated by electrophoresis in 2% agarose gel in 1X TAE buffer. The gels were stained with EtBr and the restriction fragment patterns were visualized under UV light.

The selected cpDNA regions and data scoring. Twelve regions (six genic and six intergenic spacer regions, Table 2) of the chloroplast genome were analyzed. The six genic regions, *frx*C, *rbc*L, *psb*A, *psb*D, *trn*K and 16S, have been used by Tsumura et al. (1995). To make our data set comparable to that study, we amplified these regions with the same primers and used the same set of restriction enzymes to digest each of the amplified cpDNA fragments (Table 2). The restriction fragment patterns generated by each enzyme were defined for restriction site gain/loss changes. The presence/absence of a site was coded as the 1/0 character state respectively. This data set (hereafter referred to as data set I) included site data for the seven species from this study and for the 20 species reported by Tsumura et al. (1995). The site changes that were found in our seven taxa, but were not reported by Tsumura et al. (1995) were not included.

Furthermore, to obtain better coverage of the chloroplast genome we amplified six additional intergenic spacer regions: *psb*D-16S, *trn*V-H, *rpl*20-*trn*W, *trn*C-D, *trn*L-V, and *trn*T-F. These six spacer regions are located on different parts of the chloroplast genome (Parducci and Szmidt 1999, Wakasugi et al. 1994). The primer sequences, size of the PCR products and the restriction enzymes used to digest each cpDNA fragment are listed in Table 2. Due to the complex digestion patterns of the six intergenic spacer regions, precise assignment of the mutation types was not always possible. Thus, in this data set each

Table 2. List of primers and the restriction enzymes used in the present study

cpDNA region	Primer sequence (F- forward; R- reverse)	Annealing Tm	Size (bp)	Restriction enzyme
*frx*C[1]	F: 5′- ATAGCAGTTTACGGGAAAGG -3′			
	R: 5′- TGAATAATTCCCGATCTGGA -3′	56 °C	779	*Bgl*II, *Dra*I, *Eco*RV, *Hae*III, *Hinf*I, *Msp*I, *Rsa*I, *Sau*3AI, *Sca*I, *Sty*I, *Taq*I, *Xba*I
*rbc*L[1]	F: 5′- TGTCACCAAAAACAGAGACT -3′			
	R: 5′- TTCCATACTTCACAAGCAGC -3′	56 °C	1387	*Bam*HI, *Bgl*II, *Cfo*I, *Hae*III, *Msp*I, *Pst*I, *Rsa*I, *Sau*3AI, *Sty*I, *Taq*I, *Xba*I
*psb*A[1]	F: 5′- TACGTTCGTGCCATAACTTCC -3′			
	R: 5′- CTAGCACTGAAAACCGTCTT -3′	56 °C	939	*Hae*III, *Hinf*I, *Hind*III, *Msp*I, *Rsa*I, *Sau*3AI, *Sty*I, *Taq*I
*psb*D[1]	F: 5′- TATGACTATAGCCCTTGGTA -3′			
	R: 5′- TAGAACCTCCTCAGGGAATA -3′	54 °C	1042	*Alu*I, *Bam*HI, *Bgl*II, *Bsm*I, *Cfo*I, *Dra*I, *Hae*III, *Hinf*I, *Msp*I, *Mva*I, *Rsa*I, *Sau*3AI, *Sty*I, *Taq*I
*trn*K[1]	F: 5′-AACCCGGAACTAGTCGGATG -3′			
	R: 5′-TCAATGGTAGAGTACTCGGC -3′	57 °C	2569	*Bam*HI, *Bsm*I, *Dra*I, *Eco*RV, *Msp*I, *Rsa*I, *Sca*I, *Sty*I
16S[1]	F: 5′- ACGGGTGAGTAACGCGTAAG -3′			
	R: 5′- CTTCCAGTACGGCTACCTTG -3′	57 °C	1375	*Cfo*I, *Eco*RI, *Hinf*I, *Sau*3AI, *Sma*I, *Taq*I, *Rsa*I, *Msp*I
*psb*D-16S[2]	F: 5′- CCACAAAAACGAAACGGTCT -3′			
	R: 5′- ACTAACTAATCAGACGCGAGCC -3′	55 °C	2084	*Cfo*I, *Dra*I, *Hae*III, *Hin*fI, *Msp*I, *Rsa*I, *Taq*I
*trn*V-H[2]	F: 5′- GCTCAGCAAGGTAGAGCACC -3′			
	R: 5′- CTTGGTCCACTTGGCTACGT -3′	58 °C	2538	*Bcl*I, *Bsm*I, *Cfo*I, *Dra*I, *Eco*RV, *Hae*III, *Hinf*I, *Msp*I, *Rsa*I, *Sty*I, *Taq*I
*rpl*20-*trn*W[2]	F: 5′- TTTTCGAACTGCTAACCAAGC -3′			
	R: 5′- ACCTACGGCATCAGGTTTTG -3′	57 °C	2191	*Alu*I, *Hae*III, *Hinf*I, *Msp*I, *Rsa*I, *Taq*I
*trn*C-D[2]	F: 5′- ACTGCAAATCCTCCATCCC -3′			
	R: 5′- CTCAATTGGTTAGAGTACCGCC -3′	55 °C	2051	*Alu*I, *Hae*III, *Hinf*I, *Msp*I, *Rsa*I, *Taq*I
*trn*L-V[2]	F: 5′- CTGCTTCCTAAGAGCAGCGT -3′			
	R: 5′- TTGACATGGTGGAAGTCATCA -3′	57 °C	2214	*Bcl*I, *Bsm*I, *Cfo*I, *Dra*I, *Eco*RV, *Hae*III, *Hinf*I, *Msp*I, *Rsa*I, *Taq*I
*trn*T-F[3]	F: 5′- CATTACAAATGCGATGCTCT -3′			
	R: 5′- ATTTGAACTGGTGACACGAG -3′	54 °C	1500	*Alu*I, *Cfo*I, *Hae*III, *Hinf*I, *Msp*I, *Rsa*I, *Taq*I

[1] Primers presented in Tsumura et al. (1995). [2] Primers presented in Parducci and Szmidt (1999). [3] Primers presented in Taberlet et al. (1991).

individual cpDNA fragment revealed on the restriction fragment patterns was scored as a discrete phenotypic character of the species in question. This data set (hereafter referred to as data set II) included only the seven taxa from our experiment on all the 12 cpDNA regions.

In theory, restriction-fragment data are not recommended for phylogenetic analysis primarily because they violate the assumption of homology and independence among characters (Dowling et al. 1996). If used, they should be restricted to closely related sequences. Fragment data set II comprised only six pines and one spruce. This data set was included in the present analysis to compare with the results from data set I. Furthermore, several studies have demonstrated that exclusion of putative length mutations from the data matrix subjected to cladistic analysis has usually little effect on the topology of the constructed phylogenetic trees (Bremer 1991, Sandbrink and Van Brederode 1991).

Data analysis. Wagner parsimony analyses were performed using the computer program PAUP 3.1.1 (Swofford 1993). Data set I was analyzed using heuristic searches with 1000 random stepwise taxon addition. The Tree Bisection Reconnection (TBR), with MULTIPARS option and ACCTRAN branch length optimization were used during the searches. *Thuja standishii* and *S. sempervirens* were used as outgroup taxa. Data set II was analyzed using the Branch and Bound algorithm with furthest taxon addition, MULTIPARS option, and ACCTRAN optimization. *Picea abies* was used as an outgroup for this data set. To examine the confidence for the clades obtained for both data sets, bootstrap values (Felsenstein 1985) were calculated. The bootstrap analysis involved 1000 replicates of simple taxon addition with TBR branch swapping. To evaluate the strength of the parsimony result, the consistency index (CI) (Kluge and Farris 1969), retention index (RI) (Farris 1989) and decay index (Bremer 1988, Donoghue et al. 1992) were calculated. Decay indices were calculated using the Auto-Decay program v. 3.03 (T. Eriksson and N. Wikström, Stockholm University, Sweden).

Results

Restriction cpDNA variation. The total size of the cpDNA region amplified by the 12 pairs of primers was 20669 bp (ca. 17% of the genome). In data set I (27 OTUs and six cpDNA regions), a total of 168 restriction sites was scored, of which 147 were variable, 21 monomorphic, and 113 were classified as phylogenetically informative. In data set II (seven OTUs, 12 cpDNA regions), a total of 638 restriction fragments was scored, of which 490 were variable, 148 monomorphic, and 275 were informative (Table 3). Both data sets can be obtained upon request from the authors. The partitioning of these sites/fragments among the 12 cpDNA regions is listed in Table 3. For data set II, the number of polymorphic fragments scored for the seven OTUs (six pines and one spruce) varied significantly among the 12 cpDNA regions. For example, no informative character was scored in the 16S region, while as many as 70 informative characters were scored for the *trn*V-H region. The ratio of informative/total scored fragments ranged from 0.00 to 0.68. In general, for this data set, the spacer regions provided more informative characters than the genic regions. For data set I, however, the informativeness of the six cpDNA regions for the 27 OTUs varied only slightly. The ratio of informative/total scored sites ranged from 0.56 to 0.77 (Table 3).

Phylogenetic analysis. For data set I the Wagner algorithm produced 30 most parsimonious trees with 194 character state changes, CI = 0.582, RI = 0.785 (CI = 0.645, RI = 0.785 including uninformative characters). The strict consensus tree of these 30 trees is shown in Fig. 2A. This tree has a strong bifurcation into two clades one comprising all analysed *Pinus* species and the other comprising all the remaining taxa. The two clades appeared in 100% of the bootstrap samples. The *Pinus* clade is further split into two groups corresponding to the subgenera *Strobus* and *Pinus* with bootstrap values of 86% and 100% respectively. The node with 86% bootstrap value has a decay index of at least two steps. *Pinus krempfii* was placed in the *Strobus* clade in all of the 30 shortest trees. Further division

Table 3. RFLP information content of the 12 cpDNA regions

	*frx*C	*rbc*L	*psb*A	*psb*D	*trn*K	16S	*psb*D-16S	*trn*V-H	*rpl*20-*trn*W	*trn*C-D	*trn*L-V	*trn*T-F	Total
Fragment size (bp)	779	1387	939	1042	2569	1375	2084	2538	2191	2051	2214	1500	
Data set I (27 OTUs)													
No. sites scored	18	42	20	31	39	18							168
Polymorphic	16	34	16	27	39	15							147
Monomorphic	2	8	4	4	0	3							21
Informative	12	26	15	20	30	10							113
Informative/total sites	0.67	0.62	0.75	0.65	0.77	0.56							
Data set II (7 OTUs)													
No. fragments	34	46	28	38	42	26	54	103	58	64	97	48	638
Polymorphic	11	20	13	17	31	3	47	101	52	60	96	39	490
Monomorphic	23	26	15	21	11	23	7	2	6	4	1	9	148
Informative	2	11	6	9	25	0	23	70	28	25	58	18	275
Informative/total fragments	0.06	0.24	0.21	0.24	0.60	0.00	0.43	0.68	0.48	0.39	0.60	0.38	

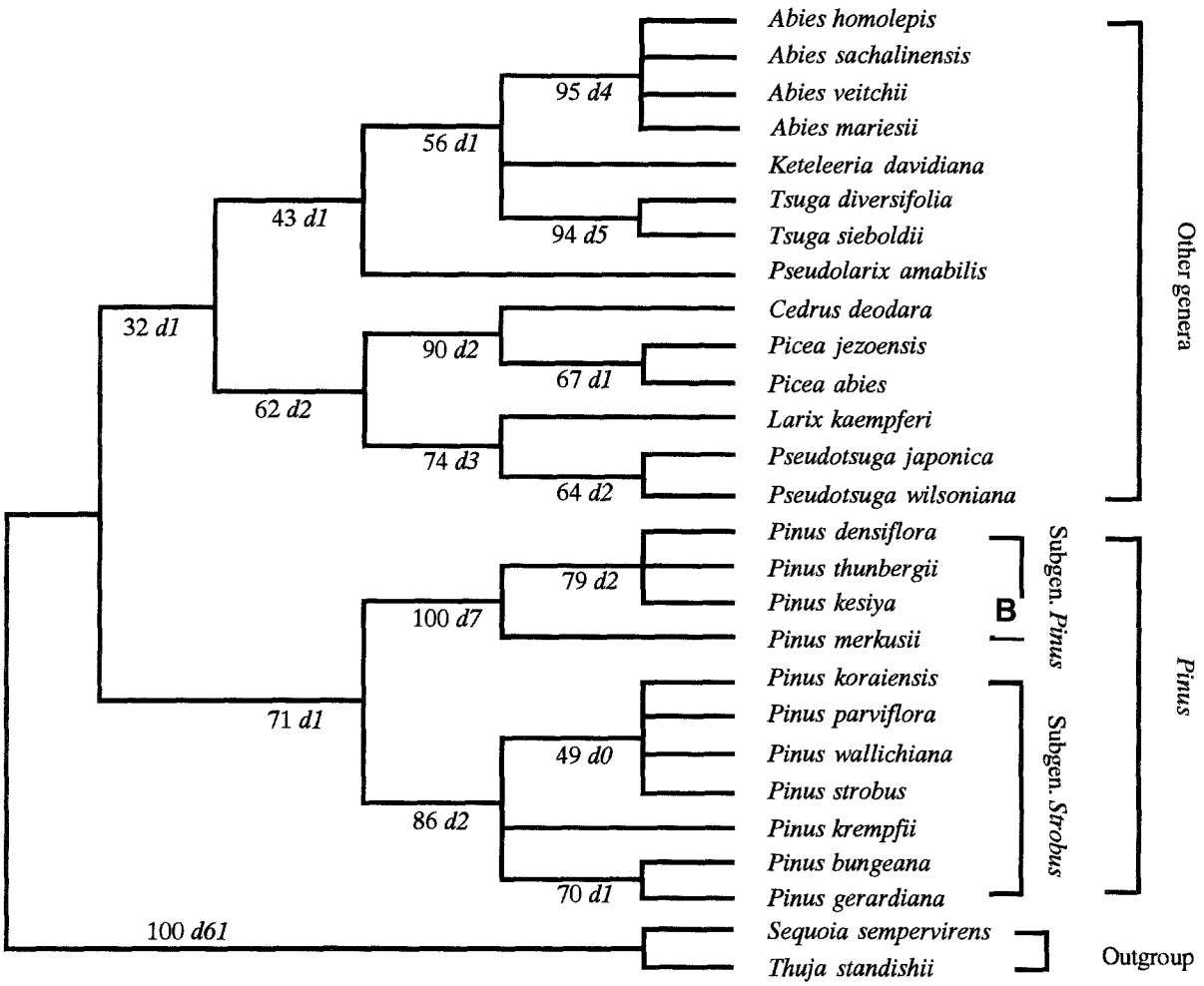


Fig. 2A. Strict consensus tree of the 30 most parsimonious trees produced *via* Wagner parsimony analysis of six cpDNA regions in 27 conifers; numbers associated with internal branches indicate the percentage of times that the branch was recovered in 1000 bootstrap samples; numbers preceded by "d" indicate decay indices. The tree has a length of 194 character state changes and CI = 0.582, RI = 0.785 (CI = 0.645, RI = 0.785 including uninformative characters)

within this group appeared to be weak with our data. The position of *P. krempfii* within the *Strobus* clade varied among individual most parsimonious trees. In six of the 30 shortest trees, it occupied a basal position in subgenus *Strobus*, but derived from within the subgenus in the rest of the trees. Within the subgenus *Pinus* group, *P. merkusii* formed a separate branch sister to the rest of the members. The clades containing other Pinaceae species are similar to those found by Tsumura et al. (1995). A phylogram for one of the 30 most parsimonious trees is presented in Fig. 2B.

For data set II, the Branch and Bound search produced a single most parsimonious tree, requiring 367 steps, CI = 0.749; RI = 0.753 (CI = 0.842; RI = 0.753 including uninformative characters) (Fig. 3). High bootstrap and decay indices characterize all nodes. The node with bootstrap value 91% has a decay index of at least 14 steps. As with data set I, the genus *Pinus* divides into two clades

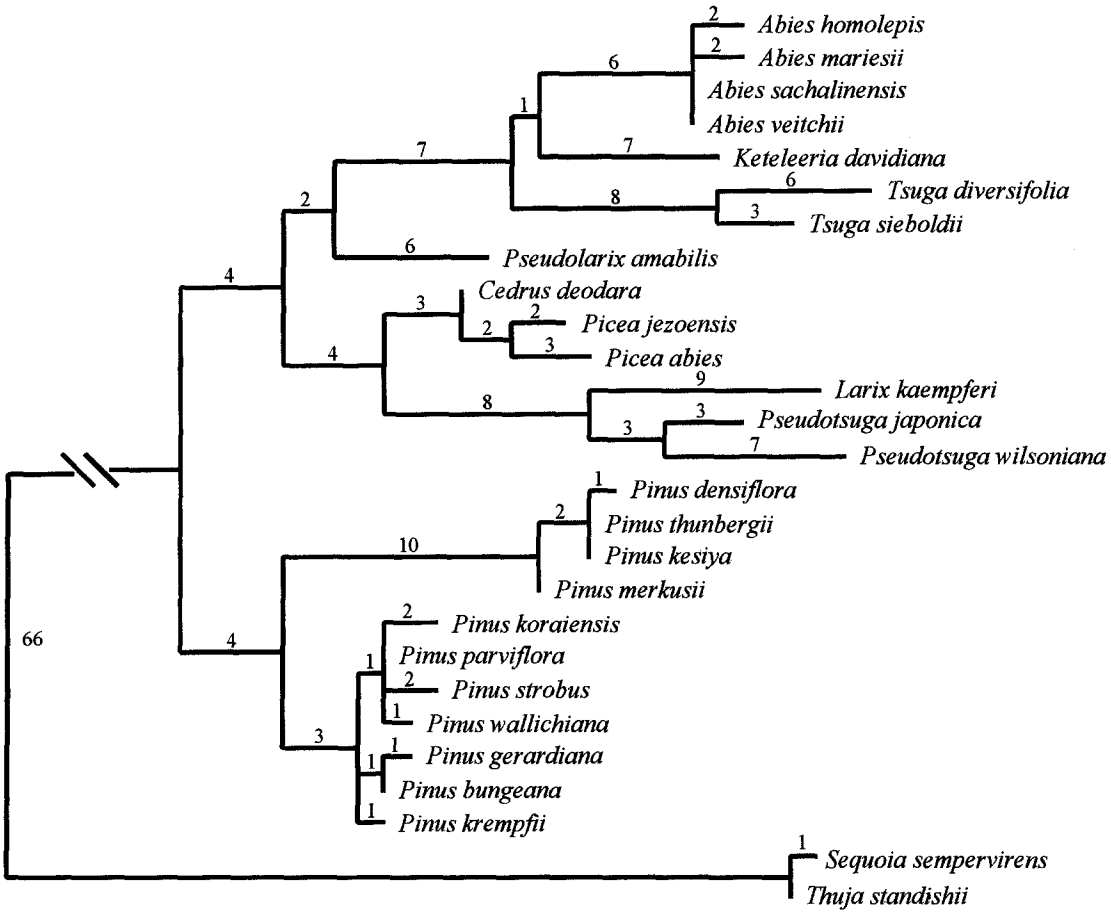


Fig. 2B. One of the 30 most parsimonious trees shown as phylogram. Branch lengths are indicated above each branch

corresponding to the subgenera *Strobus* and *Pinus*, respectively. Similar to the trees obtained with data set I, *P. krempfii* is clearly placed in the *Strobus* clade.

Discussion

The affinity and classification of *P. krempfii* are poorly understood and problematic ever since its discovery. *Pinus krempfii* is a pine-like tree but with two highly flattened needles and early caducous fascicle sheath (Lecomte 1921, 1924). On the basis of leaf anatomy, De Ferré (1948) suggested a close relationship of *P. krempfii* to *Pseudolarix* and *Keteleeria*. Gaussen (1960) even thought that it was a hybrid of either of the two with *Pinus*. Examining its wood anatomy, Buchholz (1951) found that *P. krempfii* shared more characters with *Keteleeria* and *Pseudolarix* than with *Pinus* and, thus, favoured the proposal by Chevalier (1944) to place the taxon in an independent genus *Ducampopinus* Chevalier. Further investigations have shown, however, that many wood anatomy characters of *Pinus*, especially of the subgenus *Strobus*, such as ray tracheids and horizontal resin canals which were reported absent in *P. krempfii* by Buchholz (1951), are in fact present in *P. krempfii* (Mirov 1967, and references therein). However, in a recent abstract, Ickert-Bond (1997) reported the absence of ray tracheids in *P. krempfii*. This further shows that some unresolved characters

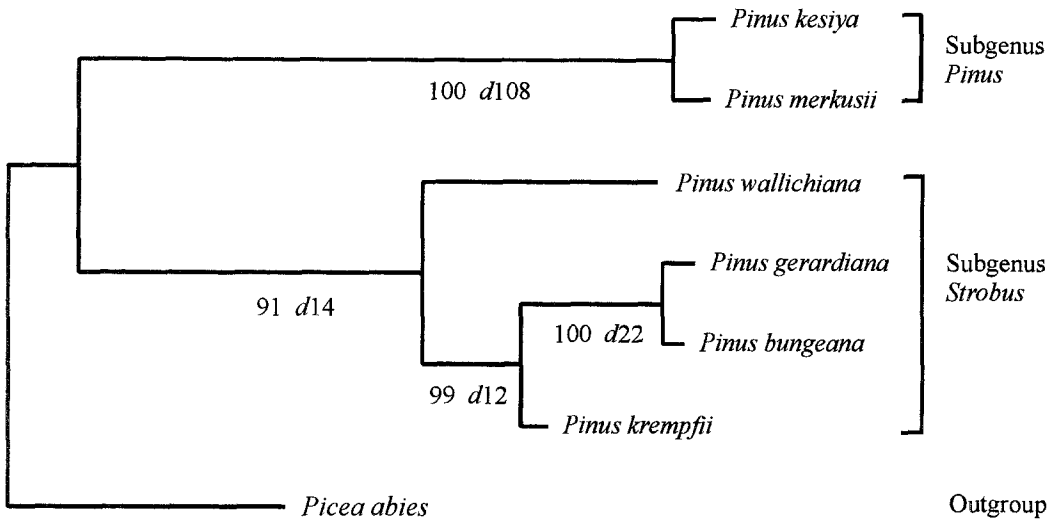


Fig. 3. Single most parsimonious tree produced *via* Wagner parsimony analysis of 12 cpDNA regions in six pines and one spruce; numbers associated with internal branches indicate the percentage of times that the branch was recovered in 1000 bootstrap samples; numbers preceded by "d" indicate decay indices. The tree has a length of 367 character state changes and CI = 0.749, RI = 0.753 (CI = 0.842, RI = 0.753 including uninformative characters). Branch lengths are proportional to the number of character changes between taxa

in the species remain uncertain till today. Nevertheless, based on wood, leaves, cuticle micromorphology and ovulate-cone morphology, Ickert-Bond (1997) concluded that *P. krempfii* should be included in the genus *Pinus*, and more likely in the subgenus *Strobus*.

Based on cpDNA data, the nine different Pinaceae genera included in the analysis were divided into two main clades, with the genus *Pinus* forming one clade, and all the other genera forming the other. This dichotomy was found in all our shortest trees. A similar grouping pattern of the Pinaceae genera was obtained by Tsumura et al. (1995). These results add additional support to the monophyly of the large genus *Pinus*, including *P. krempfii* (Mirov 1967, Farjon 1990). Based on the observation on early fossil cones of Pinaceae, Miller (1976) suggested that early evolution of the family was *Pinus* centered. The seed cones known at the Lower-most Cretaceous show considerable diversity but have more features characteristic of *Pinus* than of any other modern genus (Miller 1977). The relationships among the genera in Pinaceae have been the focus of several previous studies (e.g. Farjon 1990, Frankis 1989, Prager et al. 1976, Price et al. 1987), and are not discussed in the present paper. On all the most parsimonious trees obtained in the present study, *P. krempfii* was invariably placed in the *Pinus* clade and not in any of the clades containing *Keteleeria* and *Pseudolarix*. Therefore, our present results do not support the hypothesis for close association of *P. krempfii* with *Keteleeria davidiana* and *Pseudolarix amabilis*. On the contrary, from the present results, we favour the classification of *P. krempfii* in the genus *Pinus* rather than in a separate genus.

Within the genus *Pinus*, a separate subgenus was created to accommodate *P. krempfii* (De Ferré 1953, Gaussen 1960, Little and Critchfield 1969). However, other authors considered *P. krempfii* to belong to the subgenus *Strobus* (Farjon 1984, Florin 1931, Pilger 1926, Price 1989). The presence of a

single vascular bundle in the needles, the occurrence of calcium oxalate crystals in the phloem and cortex of the stem and cuticular features indicated characters of the subgenus *Strobus* (Buchholz 1951, Ickert-Bond 1997, Mirov 1967). In addition, a chemical investigation of the wood of *P. krempfii* very clearly showed that this species is chemically closely related to the haploxyl pines and differs distinctly from diploxyl pines (Erdtman et al. 1966). As in some pines of the sections and subsections of subgenus *Strobus*, the heartwood of *P. krempfii* contains a series of carbon-methylated flavanones (Erdtman et al. 1966). Our present results are in accordance with the proposal for the association of *P. krempfii* with subgenus *Strobus*. On the obtained trees the genus *Pinus* was clearly divided into two distinct clades supported by high bootstrap values: one for subgenus *Strobus* and the other for subgenus *Pinus*. Analysis of data set II, which contained information from 12 cpDNA regions, also placed *P. krempfii* in the subgenus *Strobus*. The application of fragment data in phylogeny has been viewed critically (Dowling et al. 1996). We restricted this type of data to a smaller group of closely related taxa (six pines and one spruce) but on a larger number of cpDNA segments. The result from this data set confirmed the strong bifurcation of genus *Pinus* into two subgenus clades, as well as the association of *P. krempfii* with subgenus *Strobus* as revealed from data set I. Thus, when obtaining restriction site data is difficult, fragment data on highly specific cpDNA segments may still be useful for analyzing relationships among closely related taxa.

Within subgenus *Strobus*, the position of *P. krempfii* is poorly resolved with our data sets. Its position in this clade varied among the equally most parsimonious trees. Based on the analysis of rDNA ITS region, Liston et al. (1999) reported a sister relationship of *P. krempfii* with the members of subsections *Strobi* and *Cembrae* of the subgenus *Strobus*. However, the position of *P. krempfii* within the *Strobi-Cembrae* clade was unresolved. Our cpDNA-based results agree with their nuclear rDNA-based report and add further support to the close association of *P. krempfii* with the *Strobus* subgenus. However, the placement of *P. krempfii* in the *Strobi-Cembrae* clade is not supported by our data. As the further divisions in this subgenus were poorly resolved, phylogenetic analysis including additional species of the subgenus *Strobus* from a wider geographic range is necessary to better ascertain the position and the closest allies of *P. krempfii* within this subgenus. Considering several existing classification schemes, it is obvious that more species from section *Parrya* should be included in future analyses. If high divergence of *P. krempfii* from other species in this clade is further confirmed, it might justify its classification as a separate subgenus, as suggested by De Ferré (1953). However, the consideration of a separate subgenus for this still very little known taxon would require more detailed morphological and molecular studies. Based on the results from the present analysis alone, we feel reluctant to express a strong opinion about the justification of raising *P. krempfii* to the rank of a subgenus.

Results from the present study further support the previous studies on phylogeny of Asian pines based on cpDNA polymorphisms (Wang and Szmidt 1993). Evolutionary distinctiveness of some *Pinus* species, e.g. *P. gerardiana* and *P. bungeana*, was also revealed by previous cpDNA RFLP analysis (Wang and Szmidt 1993). These two species are endemic to Asia and both are three-needle pines, a rather unusual phenomenon among pines of subgenus *Strobus*, and differ distinctly from the rest of Asian species (all five-needle pines) also in their bark morphology (Kwei and Lee 1963). According to Farjon (1984) and Farjon and Styles (1997), these two species are presumably related to *P. pinceana* and *P. nelsonii* in Mexico. Finally, as in our previous analyses (Szmidt et al. 1996, Wang and Szmidt 1993), *P. merkusii* appeared as a very strongly diverging taxon of the subgenus

Pinus clade, suggesting its relatively remote relationship to other Asian members. Based on cone morphology, Frankis (1993) suggested a closer affinity of *P. merkusii* to *P. brutia* and *P. pinaster*. Future analyses including at least some Mediterranean species of subgenus *Pinus* could shed more light on the evolutionary history of *P. merkusii*.

Techniques using PCR and RFLP offer an additional method for gathering DNA-based data useful for phylogenetic analysis (Wolfe and Liston 1997). The entire chloroplast genome sequence of *P. thunbergii* provided the basis for sequence and restriction map comparisons among *Pinus* species. It also provided the template for designing PCR primers to cover different regions of the genome. The easy use of the PCR-RFLP approach has facilitated the examination of phylogenetic relationships of large numbers of taxa (Tsumura et al. 1995, Wolfe et al. 1997). As pointed out by Wolfe et al. (1997), this approach can also be viewed as a pre-sequencing selection for the informative regions that will be subject to detailed sequencing analysis. Indeed, the present analysis identified several cpDNA regions that proved to be particularly useful for phylogenetic analysis in *Pinus*, such as *trn*K and *rpl*20-*trn*W regions. We include these regions in our ongoing sequence analysis of a larger set of *Pinus* species. We expect that these sequence data will provide much new information to assist the classification of *P. krempfii*.

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Note added in proof

In this paper we referred to a study by Liston et al. (1999) which included *P. krempfii*. After submitting the final version of our paper, we were informed that the *P. krempfii* sample used by Liston et al. (1999) was apparently from *P. armandii*. Thus, the parts of our paper comparing our findings about *P. krempfii* with those presented by Liston et al. (1999) should be ignored.

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