

PHYLOGENY OF *DIPLOXYLON* PINES (SUBGENUS *PINUS*)¹

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ABSTRACT

The plastid DNA sequences of *rbcL*, *matK*, the *trnV* intron and the *rpl20-rps18* spacer were analyzed among 39 species of subgenus *Pinus*. A total of 3932 bp were used to assess relationships using MP, NJ and ML algorithms. Subgenus *Pinus* splits into two distinct lineages, corresponding to Eurasia and North America ("New World hard pines"). The Eurasian lineage was differentiated into two clades; the Mediterranean pines including the Himalayan pine, *P. roxburghii* (subsections *Canarienses*, *Pinea*, *Halepenses*, and *Pinaster*), and subsection *Pinus*. Two North American pines, *P. tropicalis* and *P. resinosa*, are typical members of subsection *Pinus* but did not cluster together. Subsection *Contortae* occupied the basal position in the "New World hard pines" followed by subsection *Ponderosae*. The members of subsection *Australes* from south of U.S. formed a strongly supported clade sister to the remaining species. Of remaining "New World hard pines" subsections, *Attenuatae* was a monophyletic group, and *Oocarpae*, *Leiophyllae* and *Australes* (Florida/Caribbean species) were poorly resolved. *Australes* was paraphyletic in our phylogeny. The divergence times for each subsection were estimated from the *rbcL* sequence data.

Key words: *Diploxylon* pines, phylogeny, chloroplast sequences

INTRODUCTION

The genus *Pinus* is the largest genus of conifers and the most widespread genus of trees in the North Hemisphere. The genus has been divided into two monophyletic subgenera: *Haploxylon* (subgenus *Strobus*) and *Diploxylon* (subgenus *Pinus*), which are further divided into sections and subsections (PRICE *et al.* 1998).

Despite the long and complex taxonomic history, the nomenclature in the North American pines is still not well settled. Discrepancies below the subgeneric level exist among the classifications schemes proposed for this genus (See review in PRICE *et al.* 1998). These large differences in opinions are due not only to the large number of species, but also to the relative lack of morphological characters available to define groups of species. Several morphological characters, especially seed-cone structures have been emphasized in taxonomic studies, nevertheless it is well known that those aspects of seed-cone structure such as size, serotiny and armature can be strongly subject to natural selection or potentially resulting in convergent evolution (STRAUSS *et al.* 1992).

Molecular studies conducted on conifers have included representatives of both subgenera. These

studies have acknowledged a large genetic distance between the two subgenera as well as a lower level of genetic variation in subgenus *Pinus* (WANG & SZMIDT 1993, KRUPKIN *et al.* 1996, WANG *et al.* 1999). However, these studies have been limited in terms of taxonomic sampling and/or geographic scope, particularly in subgenus *Pinus*. The study by KRUPKIN *et al.* (1996) using cpDNA restriction analysis of 18 *Diploxylon* pines showed that the distinctive division within the subgenus was between the North American species (except *P. resinosa*) and the Eurasian species. Subsection *Contortae* was placed on the basal position of North American subsections followed by *Ponderosae*, *Attenuatae* and the complex of Central American species. The analysis of ITS sequences by LISTON *et al.* (1999) involved a broad sampling of *Pinus* subsections. Although the very low sequences divergence detected in the subgenus *Pinus*, the data strongly supported a distinctive group of North American pines where two well-supported subgroups were found, subsection *Ponderosae* and a clade of the remaining subsections. However, the Eurasian species of subsections: *Pinus*, *Pinaster* and *Pinea* were a paraphyletic clade, in which a monophyletic subsection *Pinus* was moderately supported. LISTON *et al.* (1999) also found the Himalayan *P. roxburghii*

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had a sister relationship to the American subsections and that it was paraphyletic to the Asian and Mediterranean pines. On the other hand, WANG *et al.* (1999) assessed the relationships of Eurasian pines using cpDNA sequences, and their results showed that the Mediterranean *Diploxylon* pines formed one clade and the Asian members of subsection *Pinus* formed another. The Himalayan *P. roxburghii* was found to be a strongly divergent taxon from all the remaining Eurasian pines, suggesting its association with North American pines. However, the sister relationship between the North American species and the Himalayan pine still requires confirmation by cpDNA sequence analysis (LISTON *et al.* 1999, WANG *et al.* 1999). Consequently, evolutionary relationships within subgenus *Pinus* remain unclear.

It is expected that there will be further changes in the numbers and delimitation of species, sectional groups and subsectional as more molecular phylogenetic data become available (FARJON & STYLE 1997, PRICE *et al.* 1998). In this paper, we inspected sequences from *rbcL*, *matK*, the *trnV* intron and the *rpl20-rps18* spacer of 39 *Diploxylon* pines. Our main objectives were: (1) provide additional information for the assessment of relationships at sectional and subsectional levels within the subgenus *Pinus*; (2) use a molecular clock together with paleobotanical information to estimate the timing of speciation for the major subsections.

MATERIAL AND METHODS

Sample Species

We followed the classification scheme of Price *et al.* (1998). Taxa sampled, geographic origin and GenBank accession numbers are listed in Table 1. *Pinus parviflora* (subgenus *Strobus*) were chosen as outgroup. Total DNAs were extracted from dried needles using the CTAB procedure (DOYLE & DOYLE 1990).

Amplification and Sequencing

The regions in cpDNA (*rbcL*, *matK*, *trnV* intron and *rpl20-rps18* region) were sequenced. The double-stranded DNA of *rbcL*, the *trnV* intron and the *rpl20-rps18* region were amplified using the primers of WANG *et al.* (1999). Primers amplification of *matK* (WANG *et al.* 1999), one new additional external and six internal primers were designed (primers are available by request). The sequencing was carried out using an ABI 310 Genetic Analyzer (Applied Biosystems, Inc) with an ABI BigDye Terminator Cycle Reaction Kit following the manufacturer's instructions.

Data Analysis

Sequences were aligned with Clustal X software (THOMPSON *et al.* 1997) and further modified manually. The phylogenetic analyses were performed with PAUP* 4.0.0b8 (SWOFFORD 1999) using maximum parsimony (MP), neighbour-joining (NJ; SAITOU & NEI, 1987) and maximum likelihood (ML) analyses, and indels were treated as missing data. Heuristic searches with tree-bisection-reconnection (TBR) and ACCTRAN branch length optimisation were used for MP analysis. To evaluate the robustness of the clades found in the parsimony analysis, 250 bootstrap (BS) replicates (FELSENSTEIN 1985) were calculated. The NJ tree was constructed based on Juke-Cantor model with 1000 bootstrap replicates. The ML heuristic search used HKY85 model with ASIS addition sequence.

The average numbers of nucleotide substitutions with their standard deviations were calculated for each region with MEGA 2.1 (KUMAR *et al.* 2001) based on the Jukes-Cantor model (JUKES & CANTOR 1969). The number of synonymous and nonsynonymous substitutions in *rbcL* and *matK* genes was estimated using the method of NEI and GOJOBORI (1986).

Estimation of Evolutionary Rate and Divergence Time

The constancy in the rate of evolution was assessed through the relative-rate test (TAJIMA 1993) for *rbcL* using *P. parviflora* as outgroup. The test was performed using MEGA 2.1 (KUMAR *et al.* 2001). The rate of substitution per site per year (r) is a function of the time of divergence (T) and the number of nucleotide substitutions per site or sequence divergence value (d_{xy}): $r = d_{xy} / 2T$ (NEI 1987). The molecular clock was calibrated by dating the earliest known fossil possessing unequivocal *Diploxylon* structures to an age of 130 million years (MY) (AXELROD 1986), the mean divergence time and 95% confidence intervals between clades were estimated for *rbcL* following the method of HAUBOLD and WIEHE (2001).

RESULTS

Sequence Characterization

Our *rbcL* sequence included 1256 bp for all OTUs. 28 of 40 polymorphic sites were informative. The 1554 bp of the *matK* gene and 113 bp of the *trnK* intron were detected. The numbers of variable and informative sites were 74 and 42, respectively. One indels of six bp in the *trnK* intron was detected among all the member of subsection *Pinus*. Two indels of six bp were found in *matK* gene, one

Table 1. Sources of *Pinus* samples used in this study, their geographic distribution and GenBank Accession Numbers.

Species	Accession numbers	Geographic region	Source
Section <i>Pinus</i>	<i>rbcL</i> , <i>matK</i> , <i>trnV</i> intron, <i>rpl20-rps18</i> region		
Subsection <i>Pinus</i>			
<i>P. thunbergii</i> Parlatores	D17510	E Asia	5
<i>P. resinosa</i> Aiton	AB063384, AB080945, AB063600, AB064255	NE N America	3
<i>P. tropicalis</i> Morelet	AB063378, AB080920, AB063594, AB064249	Cuba	1
<i>P. nigra</i> Arnold	AB063378, AB084498, AB019891, AB019928	Europe, Mediterranean	3
<i>P. mugo</i> Turra	AB063372, AB081087, AB063588, AB064243	Europe	3
<i>P. sylvestris</i>	AB019809, AB084492, AB019883, AB019920	N Eurasia	3
<i>P. densiflora</i> Siebold & Zuccarini	AB019814, AB084497, AB019888, AB019925	E Eurasia	4
<i>P. massoniana</i> Lambert	AB019815, AB081088, AB019889, AB019926	C-E China, Taiwan	4
<i>P. pinaster</i> Aiton	AB019818, AB084493, AB019892, AB019929	Mediterranean	4
Subsection <i>Canarienses</i>			
<i>P. canariensis</i> C.Smith	AB019823, AB084494, AB019897, AB019934	Canary Islands	1
<i>P. roxburghii</i> Sargent	AB064339, AB084495, AB064341, AB064342	Himalayas	3
Subsection <i>Pinea</i>			
<i>P. pinea</i> Linnaeus	AB019822, AB084496, AB019896, AB019933	Mediterranean	1
Subsection <i>Halepenses</i>			
<i>P. halepensis</i> Miller	AB019819, AB081089, AB019893, AB019930	Mediterranean	1
Section "New World hard pines"			
Subsection <i>Contortae</i>			
<i>P. banksiana</i> Lambert	AB063367, AB080922, AB063583, AB064238	N America	3
<i>P. contorta</i> Loudon	AB063369, AB080821, AB063585, AB064240	W N America	2
<i>P. virginiana</i> Miller	AB063379, AB080923, AB063595, AB064250	S-E N America	3
Subsection <i>Ponderosae</i>			
<i>P. ponderosa</i> P.&C. Lawson	AB063371, AB080924, AB063587, AB064242	W-N America	3
<i>P. douglasiana</i> Martínez	AB063388, AB080925, AB063604, AB064259	W Mexico	3

between *P. nigra*, *P. resinosa* and another in *P. canariensis*. For the non-coding regions, a total of 492 bp of the *trnV* was examined, there were no indels in the subgenus *Pinus*; and seven of 11 polymorphic sites were informative sites. The *rpl20-rps18* region had 10 informative out of 19 polymorphic sites. The aligned sequence length was 521 bp when outgroup was included.

Phylogenetic Relationships

A total of 3932 bp were sequenced for *rbcL*, *matK*, the *trnV* and the *rpl20-rps18* region. MP analysis yielded eight most parsimonious trees of 283 steps (CI = 0.816; RI = 0.903). The topologies of the MP trees (Fig. 1) were essentially identical to those of the NJ and ML trees (Fig. 2). They only differed in the relative position of *P. massoniana* within the clade of subsection *Pinus*. All the topologies showed that species in subgenus *Pinus* split into two distinct lineages, corresponding to Eurasia and North America. The Eurasian lineage was differentiated further into two clades; one strongly supported (BS > 85%) clade included all the members of subsection *Pinus*, except for *P. pinaster*. This clade included two North American pines, *P. resinosa* and *P. tropicalis*.

Pinus resinosa (American red pine) was the sister to *P. nigra*, but was separated from *P. tropicalis* (Cuban pine). *Pinus mugo*, *P. densiflora* and *P. sylvestris* formed a moderately supported monophyletic group (50% < BS < 85%). The second clade of the Eurasian lineage comprised the Mediterranean pines and the Himalayan pine, *P. roxburghii*. Within this clade, *P. halepensis* was a sister taxon of the other Mediterranean pines. *P. pinea*, *P. pinaster*, *P. roxburghii*, *P. canariensis* were monophyletic but received moderately bootstrap support (BS > 50%). Within the North American lineage there were: a monophyletic subsection *Contortae* (*P. banksiana*, *P. contorta* and *P. virginiana*) (BS > 85%); a monophyletic subsection *Ponderosae* (*P. ponderosa*, *P. douglasiana*, *P. jeffreyi* and *P. engelmannii*) (BS > 85%); a clade of *Australes* from south of U.S. (*P. elliotii*, *P. pungens*, *P. serotina*, *P. rigida* and *P. taeda*) (50% < BS > 85%) as a sister position to the remaining species. Relationships among the species and subsections within the last clade were not well resolved. Despite this, *Attenuatae* (Californian closed-cone pines) was strongly supported monophyletic clade, and separated from the subsections *Australes*, *Leiophyllae* and *Oocarpae*. Although

Table 1. (continued)

Species	Accession numbers	Geographic region	Source
<i>P. jeffreyi</i> Balfour	AB080914, AB080926, AB080916, AB080918	W Mexico	3
<i>P. engelmannii</i> Carrière	AB080915, AB080927, AB080917, AB080919	W-C Mexico	3
Subsection <i>Attenuatae</i>			
<i>P. attenuata</i> Lemmon	AB063365, AB080933, AB063581, AB064236	W-N America	2
<i>P. muricata</i> D. Don	AB063387, AB080935, AB063603, AB064258	W-N America	3
<i>P. radiata</i> D. Don	AB063383, AB080934, AB063599, AB064254	W-N America	3
Subsection <i>Australes</i>			
<i>P. caribaea</i> var. <i>caribaea</i> Barret & Golfari	AB063368, AB080940, AB063584, AB064239	Cuba	1
<i>P. caribaea</i> var. <i>hondurensis</i> Barret & Golfari	AB063385, AB080942, AB063601, AB064256	C America	3
<i>P. caribaea</i> var. <i>bahamensis</i> Barret & Golfari	AB063366, AB080941, AB063582, AB064237	Bahamas	1
<i>P. cubensis</i> Grisebach	AB063370, AB080938, AB063586, AB064241	Cuba	1
<i>P. palustris</i> Miller	AB063373, AB080937, AB063589, AB064244	S-E America	2
<i>P. maestrensis</i> Bisse	AB063371, AB080939, AB063587, AB064242	Cuba	1
<i>P. taeda</i> Linnaeus	AB063377, AB080928, AB064248, AB063593	S-E America	2
<i>P. rigida</i> Miller	AB063376, AB080929, AB064247, AB063592	S-E America	3
<i>P. pungens</i> Lamber	AB063375, AB080932, AB064246, AB063591	S-E America	3
<i>P. serotina</i> Michaux	AB081076, AB080930, AB081079, AB081082	S-E America	3
<i>P. elliotii</i> Engelmänn	AB081075, AB080931, AB081078, AB081081	S-E America	3
<i>P. echinata</i> Miller	AB081077, AB080936, AB081080, AB081083	S-E America	3
Subsection <i>Oocarpae</i>			
<i>P. herrerae</i> Martínez	AB063386, AB080943, AB063602, AB064257	C Mexico	3
<i>P. oocarpa</i> Schiede & Schlechtendal	AB063382, AB081084, AB063598, AB064253	C America	3
<i>P. patula</i> Schlechtendal & Chamisso	AB063381, AB080944, AB063597, AB064252	E Mexico	3
Subsection <i>Leiophyllae</i>			
<i>P. leiophylla</i> Sciede & Deppe	AB019825, AB081085, AB019899, AB019936	Mexico	3
Outgroups			
<i>P. parviflora</i> Siebold & Zuccarini	AB019800, AB81086, AB019874, AB019911	E. Asia	4

Note: 1 – Pinar del Río University, Cuba; 2 – Forest Product Research Institute, Japan; 3 – Kamigamo Experimental Station of Kyoto, Japan; 4 – WANG *et al.* (1999); 5 – WAKASUGI *et al.* (1994).

we could not distinguish these subsections from each other, the eastern Cuban pines (*P. cubensis* and *P. maestrensis*) were sister groups.

Substitution Rates and Divergence Times

The average number of nucleotide substitutions per site was calculated for each region (Table 2). The average number of overall nucleotide substitutions (K_0) in *matK* was 1.3-times higher than that in *rbcL*, particularly the average number of nucleotide substitutions at nonsynonymous sites (K_a) in *matK* was 2-times higher than that for *rbcL*. The average number of nucleotide substitutions in both noncoding regions was 2.5-times lower than the average number of nucleotide substitutions at synonymous sites in *rbcL* and *matK*. Interestingly, comparing the average numbers of nucleotide substitutions among the four regions between the two lineages revealed similarly low divergence within each lineage, except

for *matK*. The Eurasian clade exhibited a much higher rate of nucleotide substitutions in *matK* than did the North American clade, particularly at nonsynonymous sites, which was 3.2-times higher in the Eurasian clade. Heterogeneity in the substitution rate in *matK* and the higher nonsynonymous to synonymous ratio (>1) suggest a positive selection in the lineage of Eurasian pines.

The P values ($P > 0.05$ for all comparison) resulting from the relative rate test performed on *rbcL* were not significantly different, supporting the molecular clock hypothesis within the subgenus (data not shown). Thus, based on the age of the *Diploxylon-like* fossil and the sequence divergence for *rbcL* between the Eurasian and North American sister lineages (0.0095 ± 0.0021), the substitution rate was estimated to be $3.65 \pm 0.81 \times 10^{-11}$ per site per year. Application of this rate to all pairs of the clades indicated an approximate lineage-divergence

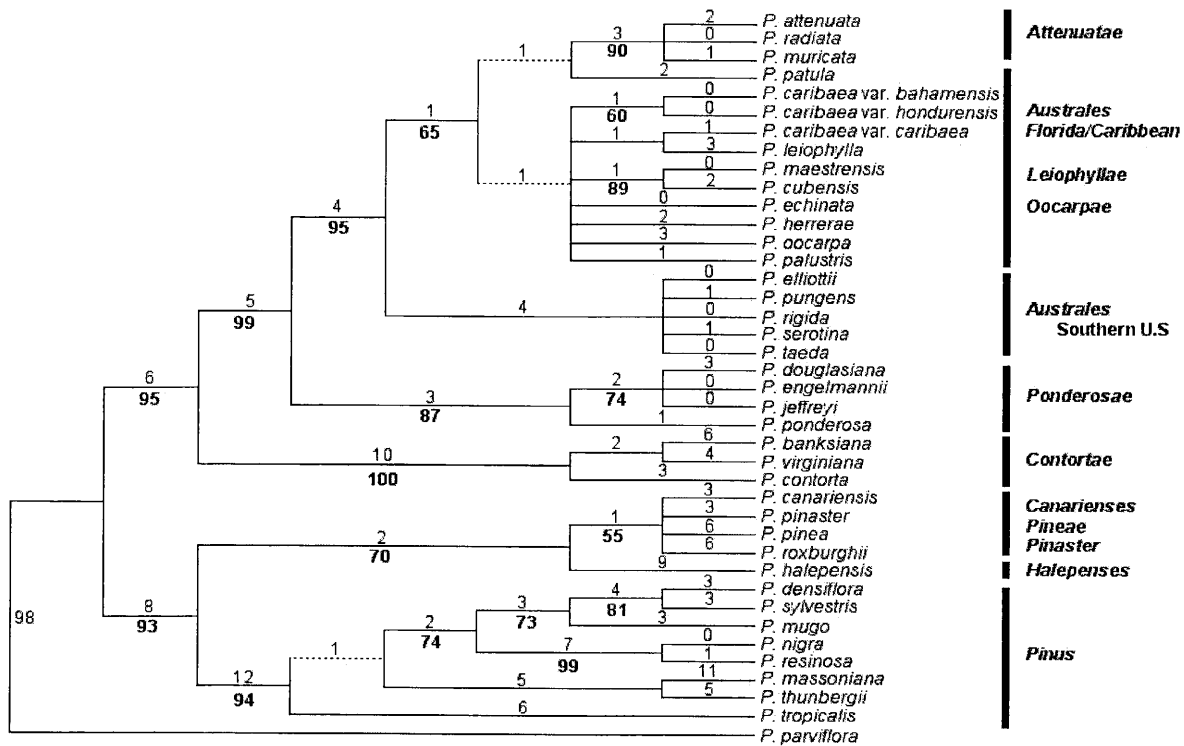


Figure 1. One of the eight most-parsimonious trees based on the 3932 bp. Numbers below each branch show the bootstrap values and above indicate the number of steps. Subsections are indicated on the right. The dashed branch collapses in the strict consensus tree.

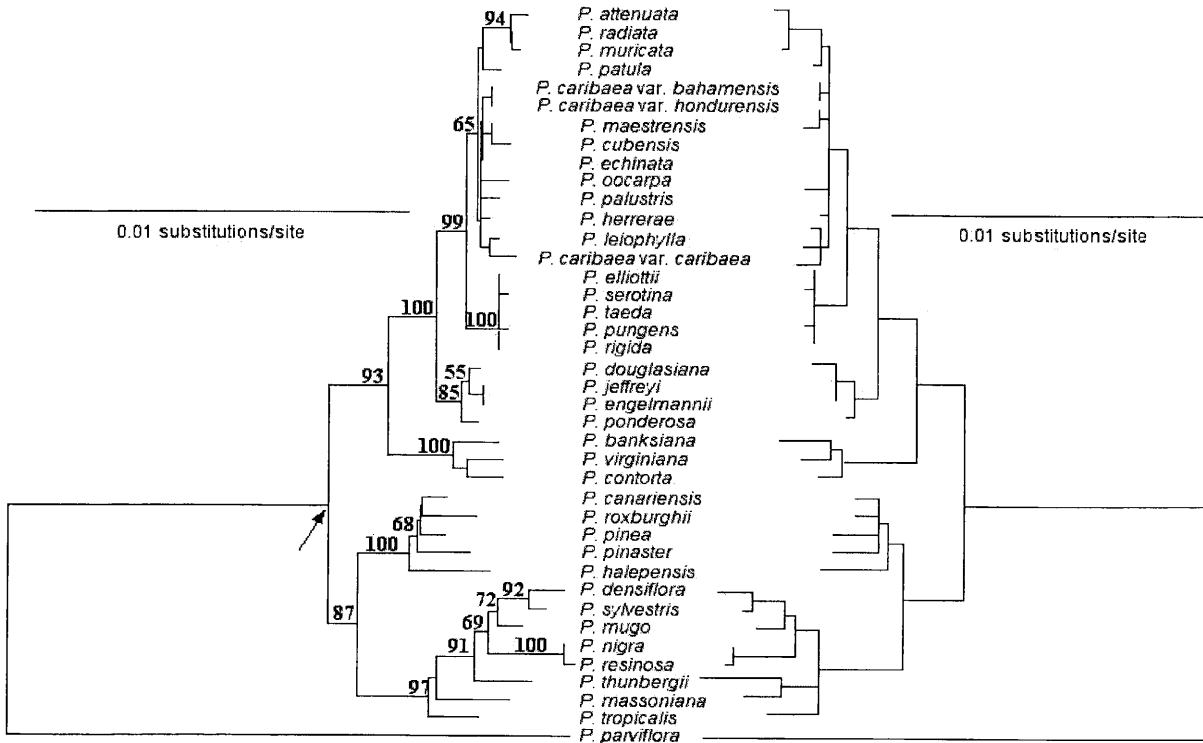


Figure 2. Neighbor-joining tree (left) and Maximum likelihood tree (right). Bold numbers show the bootstrap values. Arrow shows the landmark event used for the calibration.

date of 104 MY for subsection *Contortae* (with 95% confidence limits of 61–169 MY), 65 MY for *Ponderosae* (34–114 MY), 37 MY for *Australes* (south of U.S.) (34–114 MY), 20 MY for *Attenuatae* (7 and 43 MY), and 21 MY for the *Oocarpae* – *Leiophyllae* – *Australes* (Florida/Caribbean species) clade (11 and 38 MY). Within the Eurasian clade, the split between subsection *Pinus* and subsections *Pinaster* – *Canarienses* – *Pineae* – *Halepenses* was estimated to be 64 MY (27 and 119 MY).

DISCUSSION

Phylogenetic Relationships

Our phylogenetic analysis confirmed the view of PRICE *et al.* (1998), who identified two sections in subgenus *Pinus*: section *Pinus* comprising the Eurasian species (with the exception of *P. tropicalis* and *P. resinosa*) and the section informally named “New World hard pines” for the North American species.

The Cuban pine, *Pinus tropicalis*, has been considered as a member of subsection *Pinus* (MIROV 1967, RUSHFORTH 1987) and a close relative of the northeast North American pine *P. resinosa* due to its geographical distribution (LITTLE & CRITCHFIELD 1969, VAN DER BURGH 1973, PRICE *et al.* 1998). *Pinus tropicalis* has also been associated with subsections *Oocarpae* (KLAUS 1989) and *Australes* (FARJON 1984) because of its growth characteristics. Our result clearly indicates that *P. tropicalis* is a typical member of subsection *Pinus*. Thus, the “grass-stage” seedlings seen in *P. tropicalis* and various “New World pines” have apparently evolved in parallel. Parsimony analyses using ITS recovered *P. tropicalis* and *P. resinosa* in subsection *Pinus* (LISTON *et al.* 1999), but could not resolve the relationship between them. Our data do not support the idea of a close relationship between *P. tropicalis* and *P. resinosa*.

In the second Eurasian clade, the close relationship between *P. pinaster* and the Mediterranean pines described here is consistent with previous classifications in which all the Mediterranean pines are included in subsection *Pinaster* (RUSHFORTH 1987). This classification is further supported by data from artificial and natural hybridization experiments (VIDAKOVIĆ 1991), electrophoretic profiles of seed proteins (SCHIRONE *et al.* 1991), and analysis of the ITS region (LISTON *et al.* 1999). Thus, the inclusion of *P. pinaster* as a member of subsection *Pinus* (PRICE *et al.* 1998) needs to be reconsidered.

Our analyses placed *P. roxburghii* in the Mediterranean clade, but its close relationship with *P. canariensis* was not well resolved. Strong morphological similitude of *P. roxburghii* to *P. canariensis* permitted its classification in subsection *Canarienses*

(LITTLE & CRITCHFIELD 1969, FARJON 1984, KLAUS 1989, PRICE *et al.* 1998), while RUSHFORTH (1987) classified *P. roxburghii* into subsection *Pinaster*. Our data support the Mediterranean origin of *P. roxburghii* proposed by KLAUS (1989). In the analysis of Eurasian pines based on these chloroplast regions (WANG *et al.* 1999), the taxon labelled as *P. roxburghii* seems to be a misidentified taxon.

Our data indicates that the “New World hard pines” fall into five clades. Subsection *Contortae* appeared to be the first to speciated. However, the affinity of *Contortae* with *Australes* has generally been considered based on morphological characters, the sympatric association in the south-eastern United States and artificial hybridisation between some species and *P. virginiana* (FARJON 1984, PRICE 1989, FARJON & STYLES 1997). Furthermore, based on the restriction analysis Strauss and DOERKSEN (1990) suggested a close relationship between subsections *Contortae* and *Oocarpae* while, LISTON *et al.* (1999) using ITS data could not resolved the relationships between the subsections *Leiophyllae*, *Oocarpae*, *Australes*, *Attenuatae* and *Contortae*. The monophyletic subsection *Contortae* is strongly supported as being the basal sister group of the remaining North American subsections.

Subsection *Ponderosae* comprised the western North American pines. The monophyly of this subsection was clearly illustrated by our chloroplast analysis as well as by ITS analysis (LISTON *et al.* 1999). The greatest discrepancy between cpDNA and ITS topologies relied on the basal position of the “New World hard pines”. The cpDNA positioned *Contortae* in the basal position of the North American pines, whereas the ITS topology placed *Ponderosae*. The total congruence among the MP, NJ and ML trees, support the basal position of subsection *Contortae* among the “New World hard pines”.

Subsection *Attenuatae* is supported as being a unique taxonomic group, which is consistent with allozyme data (WU *et al.* 1999), cpDNA restriction site analysis (HONG *et al.* 1993), and RAPD (DVORAK *et al.* 2000). However, sister relationship with *Australes* proposed by KRUPKIN *et al.* (1996) is contrary to our cpDNA analyses. Unexpectedly, *Australes* was paraphyletic in our phylogeny. The *Australes* species from southern U.S. appeared as a well-supported monophyletic clade separated from the remaining species of *Australes*, which were in the latter clade (Fig. 1). Similarly, DVORAK *et al.* (2000) found an unambiguous division of *Australes* using RAPD markers when he assessed the relationships among *Oocarpae*–*Australes*, consisting in two groups: southern U.S. and Florida/Caribbean

species of *Australes*. However, this subsection has resulted a monophyletic clade in the previous molecular studies (KRUPKIN *et al.* 1996 and LISTON *et al.* 1999). Indeed, the limited number of taxa sampled in the previous studies could be the reason of the inaccurate relationship of this subsection; *i.e.*, the Central American and Caribbean Sea species of *Australes* have been seldom included. The subsections *Oocarpae* and Florida/Caribbean species of *Australes* have been treated as a 'species complex' (MIROV 1967 and FARJON & STYLE 1997). The lower substitution rates among the species in these subsections indicate that these subsections could have radiated and speciated recently.

LÓPEZ (1982) suggested that *P. cubensis* is the older species of *Australes*, while *P. maestrensis* might have evolved through hybridisation between *P. cubensis* and *P. occidentalis*. However, morphological similarities between *P. cubensis* and *P. maestrensis*, and their sympatric association in Sierra Maestra have complicated the taxonomy of these taxa. For example, *P. maestrensis* has been misidentified and classified as being *P. occidentalis* (MIROV 1967), while FARJON and STYLES (1997) concurred that *P. occidentalis* is absent in Cuba. Alternatively, BISSE (1975) and LÓPEZ (1982) concluded that *P. maestrensis* is endemic to the Sierra Maestra. Our data supports the idea that *P. cubensis* and *P. maestrensis* are genetically different. Further studies with multiple accessions of those species are needed to sets the inter-specific relationship.

Subsection *Leiophyllae* appeared in the latter clade, which echoes the notion of its recent origin. However, based on cone and seed morphologies it has been associated to section *Pinus* or classified as an independent section (See review PRICE *et al.* 1998). Thus, those characters in *P. leiophylla* seem to have evolved in parallel with section *Pinus*'s pines particularly with some Mediterranean pines.

Paleobotanical Interpretations

The utility of molecular divergence data has provided an important tool for evolutionary studies. Here we presented a re-evaluation of the time of divergence for the main subsections based on *rbcL* sequence data. The evolutionary scenarios suggested here are very much speculative; but represents the authors' best attempt to describe the phylogenetic patterns generated by cpDNA sequences data.

By the late Cretaceous, pines had reached the eastern and western edges of Laurasia (MILLAR 1998). Eurasian subsections appear to have evolved during the early Cretaceous and migrants to North America arrived during the mid-Cretaceous (MILL-

AR 1998). The North American pines, *Pinus resinosa* and *P. tropicalis* do not appear to be part of the lineage that gave rise to the American subsections ("New World hard pines") (Fig.1). They were instead on the clade of subsection *Pinus*, which appears to have diverged from the progenitor of the "New World hard pines" in the early Cretaceous (Results). Thus, the North American hard pines comprise two independent lineages.

The very long branch that separates the common ancestor of the "New World hard pines" from the section *Pinus*: subsection *Pinus* suggest a long period of isolation between eastern and western North America likely because of the expansion of the Western Interior Seaway, the dramatic decrease of temperature from the Middle Miocene, and the cordillera in western North America became effective barriers to biotic interchange between eastern and western North America (TIFFNEY 2000). The *Diploxylon* ancestor used different corridors to reach America. *Pinus resinosa* and *P. tropicalis* ancestors (resembling *P. sylvestris*) might have used the North-Arctic land bridge, which was not impeded until late Miocene (TIFFNEY & MANCHESTER 2001). Although they are the only members of subsection *Pinus* in North America, we speculate they migrated into North America independently. We calculated the time of divergence between taxa, *P. tropicalis* and *P. resinosa*, giving a value of 61~75 MY. Indeed, the only reported *P. resinosa*-like ancestor dated back to Middle Eocene (STOCKEY 1984), whereas the fossil of pollen "sylvestris-like" as *P. tropicalis*-ancestor dated back to Oligocene (ARECES 1987).

Meanwhile, the Beringian corridor might be used for the progenitors of the remaining North American subsections. The second subsection that reached America was *Contortae*; this subsection has limited fossils records, which does not become abundant until the Pleistocene. These contradictory findings could be a result of a narrow pre-Pleistocene distribution in North America (AXELORD 1986, MILLAR 1998). Periods of active mountain building and major changes in climate characterized the Palaeogene (65–54 MY), when the newer North American subsections evolved (MILLAR 1998). The earliest cladogenesis seemed to have occurred in western North America, into subsection *Ponderosae*. Our cladogram supposes that the immediate ancestor of the remaining North American species could be originated from *Ponderosae* during the Eocene. From the Eocene refugia multiple radiations could have happened: to the west to originate *Attenuatae*, to the south into Mexico to derive the *Oocarpae* and to the eastern-

south to establish the members of *Australes*'s southern U.S.

The ancestral *Attenuatae* moved westward from the Eocene refugia. Based on the fossils records (AXELROD & COTA 1993) and morphological similarities, FARJON and STYLES (1997) suggested a common "*oocarpae-like*" ancestor for the Mesoamerican and Californian pines. It is likely that the Californian and Mesoamerican pines were spatially and temporally contemporaneous during the Miocene and evolved under similar climatic conditions (DVORAK *et al.* 2000). While elements of *Oocarpae* were moving south from the northern Mexico into Central America, the *Australes* shifted west to east during Miocene-Pleistocene and entered to the eastern Cuba, which could serve as Pleistocene refugia of the *Australes* (Florida/Caribbean species). These double refugia in *Australes* have been proposed in several occasions (MIROV 1967, WATTS 1983), one of them for Texas/Mexico (*Australes*'s southern U.S.) and the other more to the south perhaps in the Caribbean Sea. However, our results suggest that both occurred independently in *tempo*, i.e. during the Eocene (Texas/Mexico) and during the Pleistocene for the Florida/Caribbean species (in Cuba). The southern Florida was submerged until the Pleistocene, so early pines establishment of this area was not possible. Our study supports the idea that the colonization of Florida is a relatively recent event (DVORAK *et al.* 2000) and that the establishment of pines in the south U.S. was a result of multiple *Australes* migrations (MIROV 1967).

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