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USE OF SIMULTANEOUS ANALYSES TO GUIDE FOSSIL-BASED CALIBRATIONS OF PINACEAE PHYLOGENY

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Uncertainties in the age and phylogenetic position of Pinaceae fossils present significant obstacles to our understanding of the timing of diversification in the family. We demonstrate that simultaneous phylogenetic analyses of chloroplast DNA (*matK* and *rbcL*) and nonmolecular characters that include both extant genera and a limited number of fossil taxa provide useful hypotheses for calibrating molecular trees. Root placements varied for Pinaceae, with Bayesian analyses recovering mutually monophyletic subfamilies Pinoideae and Abietoideae and parsimony analyses recovering Abietoideae as paraphyletic by placing the root between *Cedrus* and the remaining genera. The inferred phylogenetic positions of fossil taxa *Pityostrobus bernissartensis* as the sister group to *Pinus* and *Pseudolarix erensis* as the sister group to extant *Pseudolarix* were used to guide divergence-time calibrations; these calibrations yielded an Early Cretaceous and an Early Jurassic age for crown-group Pinaceae, respectively. The older age estimates based on *Pseudolarix erensis* are supported by weaker evidence from the fossil record but are consistent with recent reports of Early Cretaceous leaf fossils that appear to coincide with extant genera. There remains a great need to characterize the anatomy of extant and fossil species and to code additional nonmolecular characters.

Keywords: phylogenetics, Pinaceae, *Pinus*, *Pityostrobus*, *Pseudoaraucaria*, *Pseudolarix*.

Online enhancements: appendixes.

Introduction

Pinaceae, with 11 genera and ~225 species distributed almost exclusively in the Northern Hemisphere, is the largest extant conifer family (Farjon 2001). Genera such as *Pinus*, *Picea*, *Larix*, *Pseudotsuga*, and *Abies* are important components of Northern Hemisphere forests, and many species are of exceptional economic importance as sources of lumber, pulp, and resins. The family is characterized by the presence of linear leaves (needles), ovulate cones with independent cone scales (each of which has two ovaries), bisaccate pollen, a specialized proembryogeny, and an absence of biflavonoids (Hart 1987; Price 1989; Farjon 1990). Phylogenetic studies have recovered Pinaceae as sister to other extant conifers (Hart 1987; Chaw et al. 1997; Stefanovic et al. 1998; Quinn et al. 2002; Hilton and Bateman 2006; Rai et al. 2008), although molecular analyses have often found the conifers to be rendered paraphyletic by the inclusion of Gnetales, sometimes as sister to Pinaceae (Bowe et al. 2000; Gugerli et al. 2001; Magallón and Sander-son 2002).

Phylogenetic studies of extant Pinaceae genera using nonmolecular (Hart 1987) and molecular characters (Price et al. 1987;

Wang et al. 2000; Eckert and Hall 2006) have been fairly consistent with respect to generic relationships within the family. In contrast, simultaneous analyses of fossil taxa and extant genera using nonmolecular characters have yet to demonstrate robust relationships (Alvin 1988; Smith and Stockey 2001, 2002). Our limited understanding of the relationship between fossil and extant Pinaceae is a timely subject because fossils have been increasingly used to calibrate molecular phylogenies used, in turn, to estimate absolute divergence times and molecular rates (reviewed by Willyard et al. [2007]).

An important step in improving divergence-time calibration is to evaluate fossils that may be relevant in providing minimum ages for clades. The fossil record of Pinaceae extends at least to the Cretaceous (Miller 1976) and possibly to the Late Jurassic (LePage 2003). *Pinus belgica*, described from a single ovulate cone (Alvin 1960), has long been considered as the oldest unambiguous evidence of an extant genus. Although *P. belgica* was attributed to the Wealden of Belgium on the basis of the appearance of the rock matrix (Alvin 1960), its provenance is unconfirmed. Nevertheless, this fossil taxon has been used widely but inconsistently in the calibration of molecular phylogenies for *Pinus* and Pinaceae (Wang et al. 2000; Eckert and Hall 2006; reviewed by Willyard et al. [2007]). A competing claim for the oldest record of an extant genus has been proposed for fossil material, from localities in Mongolia,

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described as *Pseudolarix* (Krassilov 1982) and attributed to the Late Jurassic (Keller and Hendrix 1997; LePage 2003). Finally, extinct conifer taxa from the Cretaceous have been anatomically characterized, particularly ovulate cones attributed to the organ genera *Pseudoaraucaria*, *Obirastrobus*, and *Pityostrobus* (Smith and Stockey 2001, 2002). The provenance and age of many of these fossils are better known than those of *P. belgica*.

Phylogenetic analyses using combined morphological and molecular data matrices that code most fossil characters as missing may reveal better alternatives to *P. belgica* for calibrating Pinaceae phylogenies. The objectives of this study are (1) to evaluate Pinaceae fossil taxa for their utility in providing minimum-age calibrations within Pinaceae and within the genus *Pinus*, (2) to perform simultaneous analyses of extant and fossil Pinaceae using nonmolecular and molecular chloroplast (cp) *matK* and *rbcL* data sets, (3) to compare the resulting trees and evaluate each fossil for usefulness in calibration, and (4) to propose fossil-calibrated phylogenetic hypotheses for Pinaceae.

Material and Methods

Taxon Sampling

Nonmolecular and molecular data sets were constructed for all 11 extant genera of Pinaceae, including *Nothotsuga*. *Pinus* was represented by four extant species encompassing its sectional diversity because considerable uncertainty surrounds whether the fossil taxon *Pinus belgica* is sister to all living species or derived from an extant lineage of subgenus *Pinus*. The nonmolecular matrix also included fossil taxa *P. belgica* Alvin (1960), *Pseudolarix erensis* Krassilov (1982), *Pseudoaraucaria heeri* Alvin (1957), *Pityostrobus bernissartensis* Alvin (1953, 1957), *Pityostrobus californiensis* Smith and Stockey (2001), and *Pityostrobus corneti* Alvin (1953). *Pinus belgica* and *P. erensis* were chosen because they are hypothesized to represent the oldest records for modern Pinaceae genera (Miller 1976; LePage 2003). The choice of specific taxa of *Pityostrobus* and *Pseudoaraucaria* was guided by three criteria: (1) they were among the oldest fossils available (all from the Early Cretaceous), (2) all had relatively precise locality information, and (3) descriptions of these taxa were sufficiently detailed to score a limited number of additional morphological characters. Three species were selected for *Pityostrobus* because it is apparently polyphyletic (Miller 1976; Smith and Stockey 2001, 2002).

A cpDNA sequence matrix was assembled from a combination of GenBank accessions (Hipkins et al. 1990; Brunselfeld et al. 1994; Wakasugi et al. 1994; Wang et al. 1999, 2000; Conran et al. 2000; Gadek et al. 2000; Kusumi et al. 2000; Quinn et al. 2002; Rydin et al. 2002; Leebens-Mack et al. 2005) and new data (app. A in the online edition of the *International Journal of Plant Sciences*). For a subsequent cpDNA calibration, sampling of *Pinus* was increased from four to 33 species (one to five representatives for each subsection) to capture the major lineages of *matK* and *rbcL* found in a previous analysis of 101 species (Gernandt et al. 2005). Outgroups were selected from other gymnosperms. Results from Hart (1987), Smith and Stockey (2001, 2002), and Quinn et al. (2002) were

used to guide outgroup selection, with taxa chosen from Araucariaceae, Cupressaceae, Podocarpaceae, Sciadopityaceae, Taxaceae, and Ginkgoaceae. Alignment of Gnetales *matK* (*Gnetum gnemonoides*, AY449625; *Gnetum gnemon*, AY449621; and *Welwitschia mirabilis*, AY492030) was unsatisfactory because of a large number of indels and high sequence divergence, and these taxa were not considered further. The matrix used for analyzing the phylogenetic position of extant and fossil Pinaceae included a total of 26 taxa, and the matrix used for dating the age of Pinaceae diversification included 49.

Nonmolecular Character Matrix

A nonmolecular character matrix was compiled in MacClade 4.08 (Maddison and Maddison 2000) for Pinaceae and six outgroup taxa previously included in cladistic analyses of extant (Hart 1987; Farjon 1990) or extant and fossil Pinaceae (Smith and Stockey 2001, 2002). A total of 105 parsimony-informative characters were chosen: 46 first codified by Hart (1987), 11 added by Farjon (1990), four from Miller (1988), 33 from Alvin (1988) and Smith and Stockey (2002), five recognized as diagnostic for *Pseudolarix* by LePage and Basinger (1995), and six additional characters (app. B in the online edition of the *International Journal of Plant Sciences*). Hart (1987) and Smith and Stockey (2001, 2002) did not separate *Nothotsuga* from *Tsuga*. To code characters separately for these taxa, we relied on morphological descriptions by Frankis (1989) and Farjon (1990) and anatomical descriptions of wood and cones (Hu et al. 1989; Napp-Zinn and Hu 1989; Lin et al. 1995). Further modifications to the matrix were made on the basis of previous studies (Greguss 1955, 1972; Little and Critchfield 1969; Miller 1976, 1992; Alvin 1988; Tomlinson 1994; Takaso and Owens 1995; Biswas and Johri 1997; Wu and Hu 1997; Lin et al. 2002). Thirty-two characters were multistate. The resulting matrix of 26 taxa included 20 characters from wood, roots, and shoots, 15 from leaves, three from biochemistry, eight from pollen and pollen cones, and 59 from seed cones, seeds, and cotyledons.

DNA Character Matrix

New exemplars for this study were extracted from leaves (*Pinus pinea*, *Pinus krempfii*, *Pinus cembra*, *Abies hidalgensis*, *Cathaya argyrophylla*, *Keteleeria davidiana*, and *Picea chihuahuana*) with a modified CTAB protocol (Doyle and Doyle 1987) or from seed tissue (*Pinus nigra*) with a FastDNA Kit (Qbiogene). PCR amplification was performed in 50- μ L volumes with the following concentrations: 1 \times *Taq* buffer (Invitrogen), 1.5 mM MgCl₂, 0.2 mM for each dNTP (Invitrogen), 1 μ M for each primer, 1.5 U recombinant *Taq* polymerase, and \sim 0.2 ng of genomic DNA. PCR fragments were \sim 500 bp each, with three separate reactions per exemplar performed for *matK* and *rbcL*. Primers for *rbcL* were from Wang et al. (1999), and primers for *matK* were from Wang et al. (1999), Gadek et al. (2000), and Gernandt et al. (2005). Thermal-cycle conditions for PCR reactions were as follows: 94°C for 3 min; then 30 cycles of 94°C for 1 min, 55°C for 50 s, and 72°C for 80 s; and a final step of 72°C for 5 min. PCR products were purified with a GeneClean III kit (Qbiogene).

Purified PCR products were sequenced with a DYEnamic ET cycle sequencing kit (Amersham Biosciences) in 10- μ L

volumes as follows: 6 μL of purified PCR product, 1 μL of 2 μM primer, 1 μL of DYEnamic Terminator dilution buffer, and 2 μL of DYEnamic reaction mix. Thermal-cycle conditions for the sequencing reaction were 70 cycles of 96 °C for 30 s, 50 °C for 15 s, and 60 °C for 4 min. Sequencing reactions were precipitated in final concentrations of 66% ethanol and 0.08 M sodium acetate (pH = 4.6), and then eluted in 25 μL of MegaBACE loading solution (Amersham Biosciences) for sequencing on an ABI 310 automated sequencer with short (47 cm \times 50 μm) capillaries (PE Applied Biosystems).

Sequence reads were edited in BioEdit 6.0.7 (Hall 1999). Alignments were performed with CLUSTAL W (Thompson et al. 1994) and adjusted by eye.

Phylogenetic Analysis

Molecular and nonmolecular matrices are deposited in Treebase (study accession number S1973; matrix accession numbers Q1 M3674, M3675, and M3676). The nonmolecular and cpDNA matrices were analyzed separately and combined using parsimony in PAUP* 4.0b10 for Unix (Swofford 2003). Characters were equally weighted and unordered. The molecular characters were scored as missing for the fossil taxa. Heuristic searches included 10,000 random-addition sequence replicates and tree-bisection-reconnection (TBR) branch swapping. Bootstrap values were based on 10,000 replicates of a heuristic search, each with 10 random-addition sequence replicates and TBR branch swapping, saving all trees. Decay values were determined using converse constraints in PAUP*, with a 10,000-replicate heuristic search using random-addition sequence and TBR branch swapping.

Bayesian inference using separate molecular and morphological data partitions has been offered as a useful alternative to parsimony, for example, when the partitions provide heterogeneous signal (Nylander et al. 2004). For this reason, we performed separate analyses in MRBAYES 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). Separate models were applied for nonmolecular characters, *matK*, and *rbcl*. The nonmolecular characters were treated as standard discrete data, with all changes between states equally probable (Lewis 2001). The Akaike Information Criterion (AIC), as implemented in ModelTest 3.7 (Posada and Crandall 1998; Posada and Buckley 2004), was used to choose the best-fitting models for *matK* and *rbcl* separately. The nonmolecular and molecular partitions were analyzed separately and then combined using two independent Markov chain Monte Carlo (MCMC) runs performed on random starting trees with a temperature of 0.2. Each run included five million generations with four incrementally heated chains. A tree was sampled every 200 generations, with the number of trees to be excluded for the burn-in (20%) verified by identifying the stable phase of a plot of log-likelihood values across generations. The first 5000 trees, corresponding to one million generations, were excluded before calculation of the majority-rule consensus, which was based on 20,000 trees corresponding to the stable phase of the chain. Posterior probabilities (PPs) for each MCMC run were calculated from a majority rule of the sampled trees after burn-in.

To evaluate topological congruence between the nonmolecular and cpDNA data sets, parsimony trees were inspected for the presence of conflicting clades receiving >70% bootstrap

support (Mason-Gamer and Kellogg 1996). Two tests of topological congruence also were performed in PAUP* (excluding the fossil taxa). For the incongruence length difference (ILD) test (Farris et al. 1995), the sums of tree lengths resulting from searches on 1000 randomly assigned partitions were compared to the tree lengths of the actual partition and tested for significant differences. The Templeton (1983) test was also applied to determine whether trees resulting from either matrix resulted in a significantly poorer fit for the character state data from the other matrix.

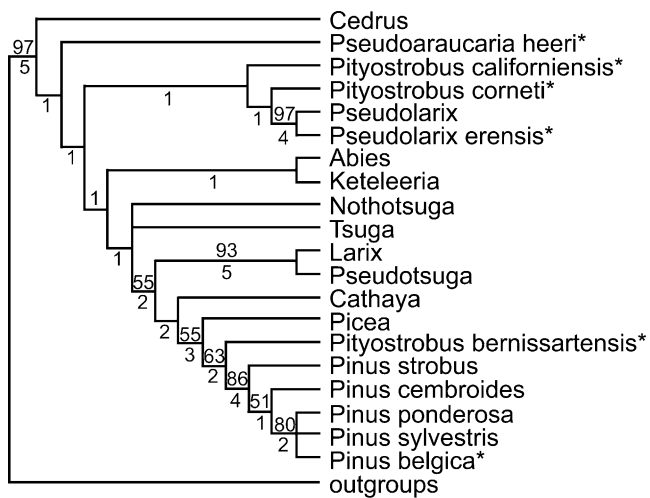
Age Estimation

Age estimation was performed on the 49-taxon molecular matrix. The tree with the best likelihood score was chosen from the sample of Bayesian replicates, and its branch lengths were estimated in PAUP* using maximum likelihood. Before model selection and branch length estimation, a 130-bp segment was trimmed from the 3' end of the *matK* partition because it was incomplete in the outgroups. A GTR + Γ + I model was applied to the unpartitioned cpDNA sequence matrix, with parameters estimated from the data.

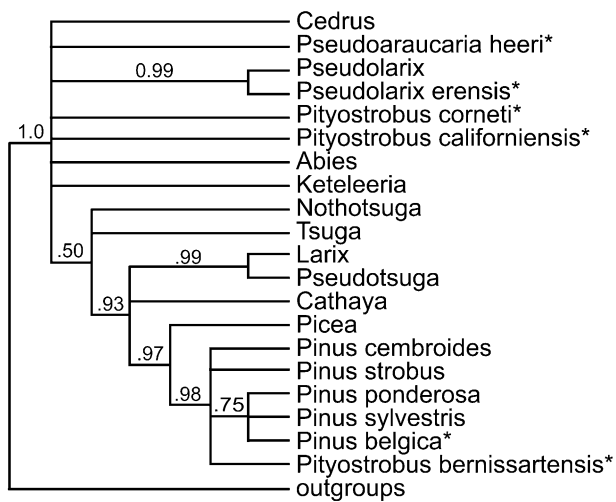
Absolute ages and molecular rates were estimated in r8s, version 1.7 for Unix (Sanderson 2002). All outgroups were pruned except for *Araucaria*; retaining that genus permitted inclusion of the crown-group node of extant crown conifers if Pinaceae is considered the sister group to the remaining families. Polytomies were treated as collapsed. The χ^2 test indicated that the Langley-Fitch molecular clock was rejected (results not shown). As a result, only age estimates using penalized likelihood are presented. The magnitude of the penalty for rate changes across branches, the smoothing parameter (λ), was optimized for each calibration with the branch-pruning cross-validation procedure, which used five guesses, three restarts, and a perturbation factor of 0.05. The error was estimated for \log_{10} cross-validation values ranging from 0 to 8 in increments of 0.2.

The r8s program allows for one or more nodes to be fixed in a calibration and for others to be constrained to a minimum or a maximum age. The fixed calibration points on the cpDNA tree were determined by the inferred phylogenetic position of fossil taxa with respect to extant genera. Additional fossils were taken into account to apply minimum-age constraints, which accommodate rate variability and place a lower limit on the age of specific nodes. When radiometric dates were unavailable, fossil ages were assigned on the basis of a recent geologic timescale (Gradstein and Ogg 2004). *Pseudolarix erensis* was described from the Gurvan-Eren, Manlaj, and Bon-Tsagan localities associated with the Tsagaan Tsav Formation, Mongolia, with a radiometric date of 156 ± 0.76 Ma (Late Oxfordian; Keller and Hendrix 1997; LePage 2003). The age of *P. bernissartensis* (Alvin 1957) was reported from the Puits Nègresse, Bernissart, Belgium (as *Pityostrobus bommeri*; Alvin 1953). The Sainte Barbe Formation at Bernissart was recently dated as mid-Barremian to earliest Aptian (Yans et al. 2005; range: 127.5–118.5 Ma, midpoint: 123 Ma). For the remaining Wealden taxa (*P. corneti*, *P. heeri*, and *P. belgica*), several recent publications were taken into account to establish an age range of 127.3–89.5 Ma (Robaszynski et al. 2001; Yans et al. 2005; Quinif et al. 2006; Dejax et al. 2007). However, after the phylogenetic results were considered (see “Phylogenetic

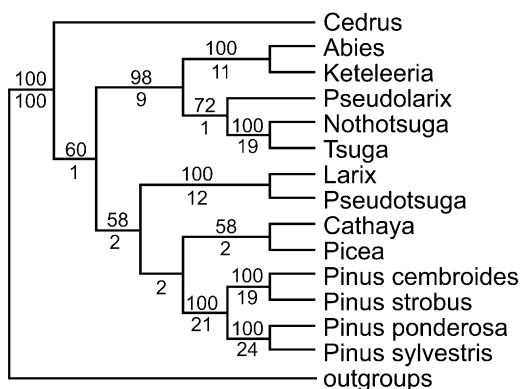
Q2



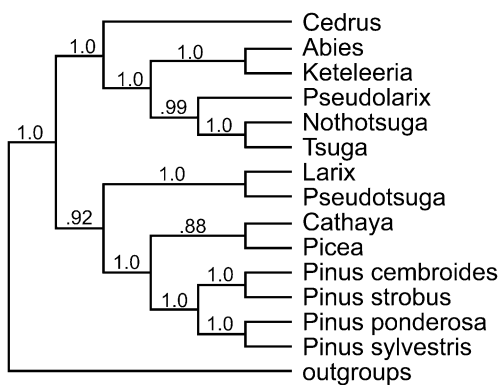
A. non-molecular parsimony



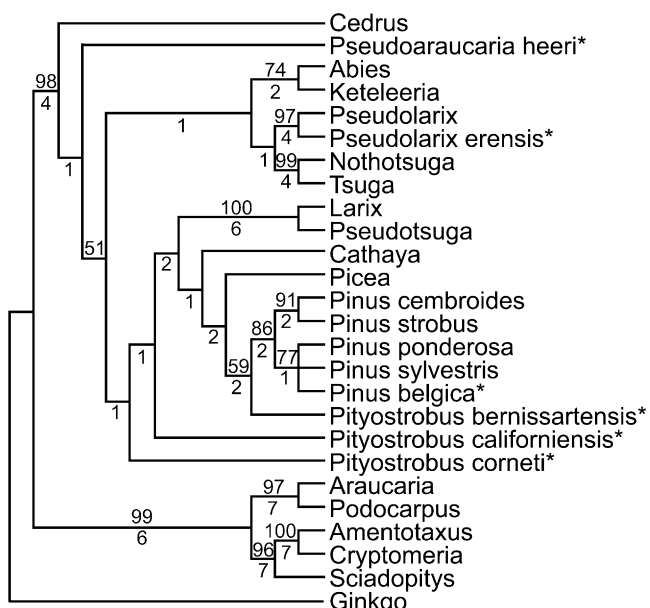
B. non-molecular Bayesian



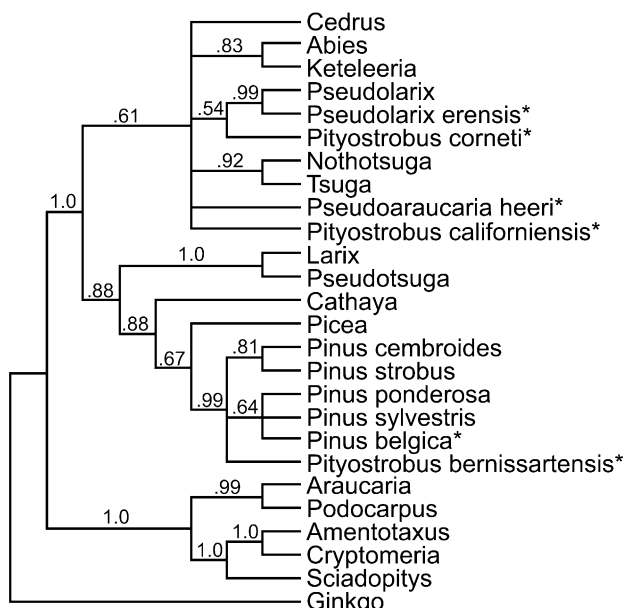
C. cpDNA parsimony



D. cpDNA Bayesian



E. combined parsimony



F. combined Bayesian

Analysis”), these taxa were ultimately not used for calibration or to apply constraints. A similar situation applied to *P. californiensis*, although a fairly precise age could be assigned to this taxon, corresponding to the midpoint of the Bedoulian stage from which it was described (Smith and Stockey 2001; middle Aptian, 118.5 Ma).

Additional minimum-age constraints were applied to extant genera and a *Pinus* section based on younger fossils not included in the phylogenetic analyses. Eocene records exist for *Abies* (46–45 Ma; Axelrod 1976; Erwin and Schorn 2005), *Larix* (44.85 Ma; LePage and Basinger 1991; LePage 2003; 45 Ma, based on radiometric dating; Schorn 1994), *Picea* (46–45 Ma; LePage 2003; Erwin and Schorn 2005), *Keleleeria* (44.4 Ma; LePage 2003), and *Tsuga* (44.85 Ma; LePage 2003). Oligocene fossils have been attributed to *Pseudotsuga* (32 Ma; Axelrod 1976; Schorn 1994). *Pinus* subsection *Balfouriana* and probably subsection *Cembroides* are present in the Oligocene (27.2 Ma; Wolfe and Schorn 1990), indicating that the crown group of section *Parrya* was present at the time. Age estimates were also evaluated with and without applying maximum-age constraints for crown-group conifers (the most recent common ancestor of Pinaceae-Araucariaceae) based on the first unequivocal appearance of conifers fossils in the Late Carboniferous (Pennsylvanian, 299–318 Ma, midpoint: 308.5 Ma; Rothwell et al. 1997).

Substitution rates were estimated for *rbcL* and *matK* separately with penalized likelihood and two calibration points. A silent rate was estimated for each gene separately on the basis of third-codon positions. An overall rate was also calculated using all sequence positions. The same tree topology was used for age estimation and substitution rates (see “Age Estimates”), but to avoid zero-length branches, only one species per section was used for *Pinus*. Nucleotide substitution models were chosen separately for each partition on the basis of on the AIC test, and branch lengths were calculated with maximum likelihood.

Results

Phylogenetic Analysis

The 26-taxon matrix included 105 parsimony-informative nonmolecular characters. The fossil taxa had a high number of cells scored as missing or inapplicable: 82 for *Pseudolarix erensis*, 58 for *Pinus belgica*, 58 for *Pseudoaraucaria heeri*, 61 for *Pityostrobus bernissartensis*, 66 for *Pityostrobus californiensis*, and 66 for *Pityostrobus corneti*. Among extant taxa, those with the most missing or inapplicable cells were *Ginkgo* (53), *Amentotaxus* (48), *Podocarpus* (36), *Araucaria* (31), and *Nothotsuga* (39). Fossil taxa were missing many characters related to wood, leaves, and male reproductive structures, while outgroups *Ginkgo*, *Amentotaxus*, and *Podocarpus* had many inapplicable characters for female reproductive structures.

The heuristic search of the nonmolecular matrix recovered three most parsimonious trees (MPTs) with a length (L) of 303 steps, a consistency index (CI) of 0.498, and a retention index (RI) of 0.675. For the Bayesian analysis of the same data, the average standard deviation of split frequencies was 0.00608 at the end of the MCMC run. More nodes were resolved in the parsimony strict consensus tree (fig. 1A) than in the Bayesian majority-rule tree (fig. 1B).

The cpDNA partition of the same matrix (with the six fossil taxa deleted) was 2838 bp in length (*rbcL*: 1262 bp; *matK* plus 289 bp of 3' spacer: 1576 bp). No gaps were needed for *rbcL*, but 14 insertion-deletion events were hypothesized to align *matK*. The matrix included 1042 variable characters, 569 of which were parsimony informative. Parsimony analysis recovered one MPT ($L = 1892$ steps, CI = 0.695, CI_{exc} = 0.571 [excluding variable but parsimony-uninformative characters], RI = 0.684; fig. 1C). Bayesian analysis (fig. 1D) used the Hasegawa-Kishino-Yano model (HKY + Γ + I) for *rbcL* and the general time-reversible model (GTR + Γ) for *matK*. The average standard deviation of split frequencies was 0.000444 at the end of the MCMC run. For both parsimony and Bayesian analyses, branch support on the cpDNA trees was equal to or higher than that on the nonmolecular trees.

The 26-taxon combined nonmolecular and cpDNA data matrix included 1147 variable characters, 674 of which were parsimony informative. The parsimony search yielded three MPTs ($L = 2211$ steps, CI = 0.663, CI_{exc} = 0.552, RI = 0.675). For the Bayesian analysis, the average standard deviation of split frequencies was 0.00866 at the end of the MCMC run. All branch-support measures were lower than in the analyses of cpDNA alone (fig. 1C, 1D), with the most marked reductions in decay indices, partly because of the unstable position of the fossil taxa in slightly suboptimal trees.

Parsimony trees based on nonmolecular, cpDNA-only, and combined matrices resolved subfamily Abietoideae as paraphyletic to subfamily Pinoideae, placing the root between *Cedrus* and the remaining members of Pinaceae (fig. 1A, 1C, 1D). In contrast, the Bayesian majority-rule trees for the nonmolecular matrix failed to resolve Abietoideae (fig. 1B), while both subfamilies were monophyletic for the cpDNA and combined matrices (fig. 1D, 1F). The fossil taxa *P. heeri*, *P. corneti*, *P. californiensis*, and *Pseudolarix* occurred within the Abietoideae clade or grade, but their positions within the group varied. In contrast, *P. erensis* consistently grouped with extant *Pseudolarix*. Relationships among the Pinoideae clade were more stable among analyses. *Cathaya*, *Picea*, (*P. bernissartensis*), and *Pinus* formed a paraphyletic grade with nonmolecular characters, while *Cathaya* and *Picea* formed a monophyletic sister group to *Pinus* with cpDNA. *Pityostrobus bernissartensis* was sister to *Pinus* in the parsimony analyses (fig. 1A, 1E) and formed a trichotomy with two lineages of *Pinus* in the Bayesian analyses (fig. 1B, 1F). *Pinus belgica* formed a clade with *Pinus ponderosa* and *Pinus sylvestris* in all analyses.

Fig. 1 Comparison of phylogenetic hypotheses in Pinaceae. A, Parsimony strict consensus of nonmolecular characters. B, Bayesian majority rule of nonmolecular characters. C, Parsimony strict consensus of chloroplast (cp) DNA. D, Bayesian majority rule of cpDNA. E, Parsimony strict consensus of combined characters. F, Bayesian majority rule of combined characters. Asterisks denote fossil taxa; values above branches are bootstrap values (A, C, and E) or posterior probability values (B, D, and F); values below branches (A, C, and E) are decay indices.

Topological differences were recovered between the nonmolecular and cpDNA-based matrices, although conflicting clades did not receive high branch support. After deletion of the six fossil taxa present only in the nonmolecular matrix, the ILD test found significant conflict between the nonmolecular and cpDNA matrices ($P = 0.002$). The Templeton (1983) test found that the nonmolecular matrix gave a significantly poorer fit on the single most parsimonious molecular tree than on the single fossil-pruned nonmolecular tree (292 vs. 275 steps; $P = 0.009$) and, conversely, that the molecular data gave a significantly worse fit on the nonmolecular tree (2007 vs. 1892 steps; $P < 0.0001$).

Diagnostic, nonmolecular synapomorphies for Pinaceae were identified by using the “trace all changes” option in MacClade to display the near-maximum number of changes on the combined parsimony tree (fig. 1E). The monophyly of Pinaceae was supported by 11 diagnostic synapomorphies: protein-accumulating sieve elements, absence of phloem fiber sclereids, tracheids of leaf transfusion tissue surrounding the vascular bundle, absence of biflavonoids, absence of sperm nuclear cell walls, ventral canal cell without a distinct wall, megaspore membrane thin at the micropylar end, primary type proembryo wall formation, four-tiered proembryo, two ovules per scale, and seed wing originating from scale epidermis. All but the last two characters were scored as missing in fossil taxa. In the parsimony tree, *Pseudoaraucaria* was the sister group to all members of Pinaceae except *Cedrus*, but only homoplasious character state changes supported its position. Extant *Pseudolarix* and *P. erensis* were united by brachioblasts with close annual rings, deltoid triangular cone scales, and semitrullate, pointed seed wings. *Nothotsuga* and *Tsuga* were united by the presence of a pendulous primary shoot, and support for *P. californiensis* as the sister group to subfamily Pinoideae was given by the absence of resin vesicles in the seed coat. Constraining subfamily Abietoideae to monophyly to reflect the Bayesian result revealed four diagnostic characters: abietoid immunology, seed scales with a pedicillate base, presence of a seed trace (scored as missing in *Nothotsuga*, fossil *Pityostrobus* spp., and *P. erensis*), and seed wings attached as a deep cup.

Monophyly of subfamily Pinoideae was supported by the presence of horizontal resin ducts in the rays; piceoid, pinoid, or fenestriform cross-field pits; two root resin canals; pinoid chemical immunology; absence of a seed trace; and seed wings attached as a shallow cup or a claw. Diagnostic characters supporting the sister relationship of *Larix* and *Pseudotsuga* were the presence of phloem fiber sclereids and an asymmetric micropyle. These two genera were in a sister position to a clade consisting of *Cathaya*, *Picea*, *Pinus*, and *P. bernissartensis* and supported by the presence of plicate mesophyll parenchyma (scored as missing or polymorphic in *Picea* and the fossil taxa). Monophyly of *Pinus* and *P. bernissartensis* was supported by tongue-shaped ovulate cone scales and clawlike seed wing attachment. *Pityostrobus bernissartensis* formed a trichotomy with two *Pinus* lineages in the Bayesian analysis, whereas *Pinus* was monophyletic in the parsimony tree, as supported by the fusion of the vascular traces leading to the bract and scale in the ovulate cone. Extant *Pinus* species were united by needlelike leaves spirally arranged in fascicles on short shoots and by a thin epithelium of vertical resin canals (scored as missing in *P. belgica*). *Pinus cembroides* and *Pinus strobus* were sister groups, but no diagnostic character state

was identified. *Pinus ponderosa*, *P. sylvestris*, and *P. belgica* were united by platelike thickenings of transverse tracheid walls.

The 49-taxon (with expanded *Pinus* sampling) cpDNA matrix had 1074 variable characters, 614 of which were parsimony informative. The heuristic search recovered 1480 MPTs ($L = 2045$ steps, $CI = 0.668$, $CI_{exc} = 0.551$, $RI = 0.785$). The family Pinaceae was monophyletic in the strict consensus tree (not shown). The root of Pinaceae in the strict consensus tree was unresolved, with 90% of the MPTs indicating that the root was placed between *Cedrus* and the remaining genera. Neither subfamily Pinoideae nor subfamily Abietoideae was resolved as monophyletic in the strict consensus tree. The only generic relationships resolved in the strict consensus tree for subfamily Pinoideae was the sister position of *Pseudotsuga* and *Larix*. Except for *Cedrus*, subfamily Abietoideae had the same topology as that recovered in the cpDNA analyses of 20 taxa (fig. 1C, 1D).

For the 49-taxon data set, the best *rbcl* model was $K81uf + \Gamma + I$, and the best *matK* model was $TVM + \Gamma$. The mean standard deviation of split frequencies between the two MCMC runs was 0.00362 after five million generations. The tree with the best log-likelihood value ($\ln = 14,582.626$) was found in generation 4,199,800 of the first run (fig. 2; fig. C1, available in the online edition of the *International Journal of Plant Sciences*, shows a majority-rule tree with PP values). Pinaceae divided into two clades corresponding to subfamilies Pinoideae and Abietoideae. Generic relationships were identical to those found in the Bayesian analysis of 20 taxa (fig. 1D). PP support for intergeneric relationships was >0.95 in all cases, except for the sister relationship between *Picea* and *Cathaya* (PP = 0.79) and monophyly of subfamily Pinoideae (PP = 0.90). Within *Pinus*, the subgeneric, sectional, and subsectional relationships were identical to those in previous cpDNA studies (Gernandt et al. 2005; Eckert and Hall 2006).

Age Estimates

With the results from the combined analyses (fig. 1E, 1F), two calibration scenarios were evaluated. A conservative scenario (fig. 3) took into account the position of *P. bernissartensis* as the sister group to *Pinus* by calibrating the node corresponding to the most recent common ancestor of *Pinus*, *Picea*, and *Cathaya* at 123 Ma (hereafter the *Pinus-Picea* calibration). A second scenario (fig. 4) took into account the position of *P. erensis* as the sister group to extant *Pseudolarix* by calibrating the node for the most recent common ancestor of *Pseudolarix*, *Tsuga*, and *Nothotsuga* at 156 Ma (hereafter the *Pseudolarix-Tsuga* calibration). *Pinus belgica*, *P. californiensis*, *P. corneti*, and *P. heeri* were not used to set minimum-age constraints in the conservative *Pinus-Picea* calibration because their positions were not considered robust enough. The last three fossils were too young to be relevant in the *Pseudolarix-Tsuga* calibration.

The *Pinus-Picea* calibration (fig. 3) yielded a younger age at all nodes than did the *Pseudolarix-Tsuga* calibration (fig. 4). The Pinaceae crown group was estimated to have first diversified in the Early Cretaceous (*Pinus-Picea* calibration) or the Early Jurassic (*Pseudolarix-Tsuga* calibration). Calibration decisions had a great influence on age estimates, particularly at the deepest nodes. For example, for the *Pinus-Picea* calibration

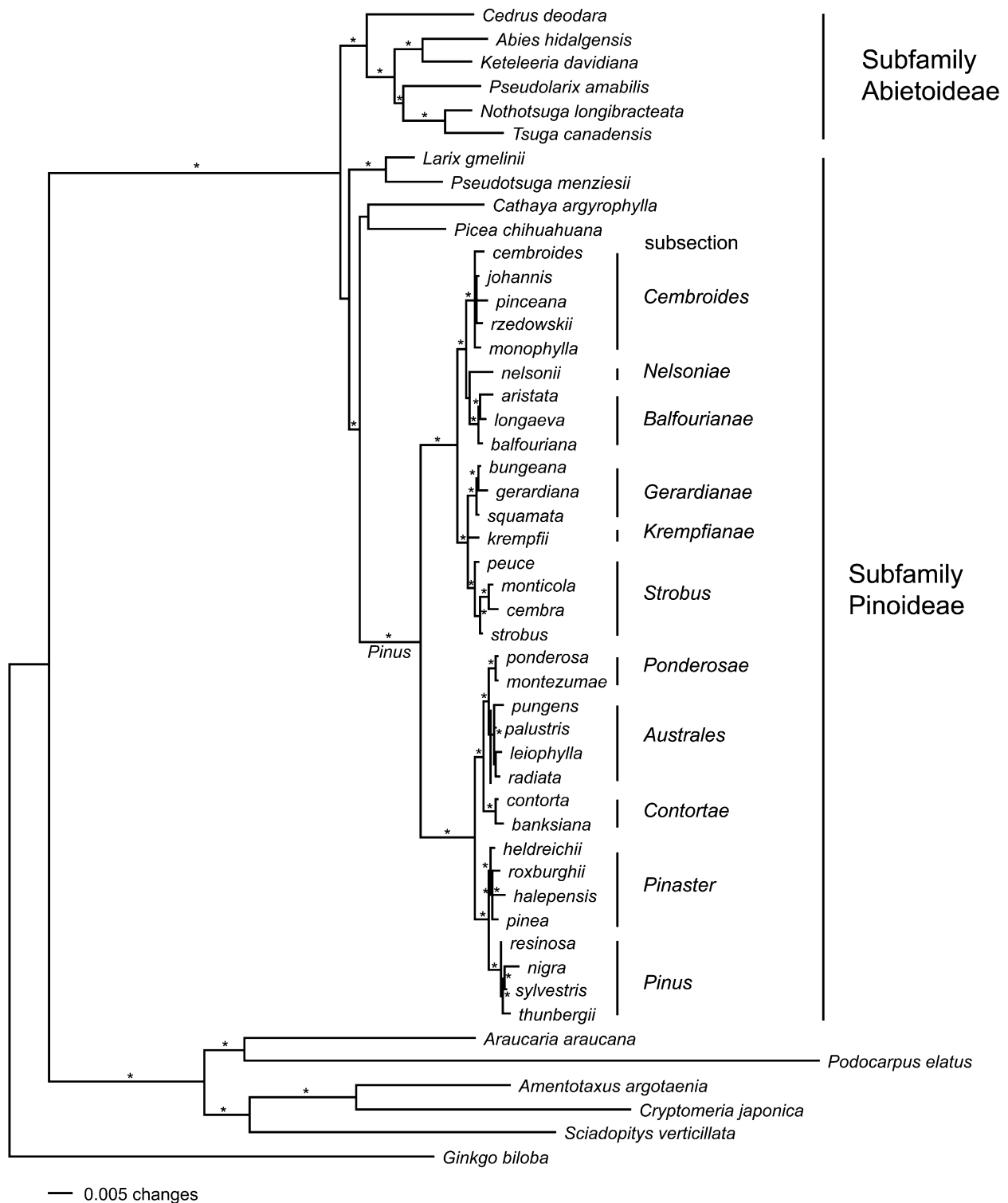


Fig. 2 Best Bayesian tree for Pinaceae. *Pinus* subsections and Pinaceae subfamilies are indicated on the right. Posterior probability values >0.95 are indicated by asterisks. The branch lengths are proportional to the number of nucleotide changes, inferred with maximum likelihood.

(fig. 3), the *Pseudolarix-Tsuga* node was estimated at 74 Ma, the *Pinus-Picea* node was calibrated at 123 Ma, and the age of the subgenus *Pinus-Strobis* node was estimated at 72 Ma. In contrast, for the *Pseudolarix-Tsuga* calibration (fig. 4), the

Pseudolarix-Tsuga node was calibrated at 156 Ma (82 Myr older), the *Pinus-Picea* node was estimated at 155 Ma (32 Myr older), and the subgenus *Pinus-Strobis* node was estimated at 87 Ma (15 Myr older).

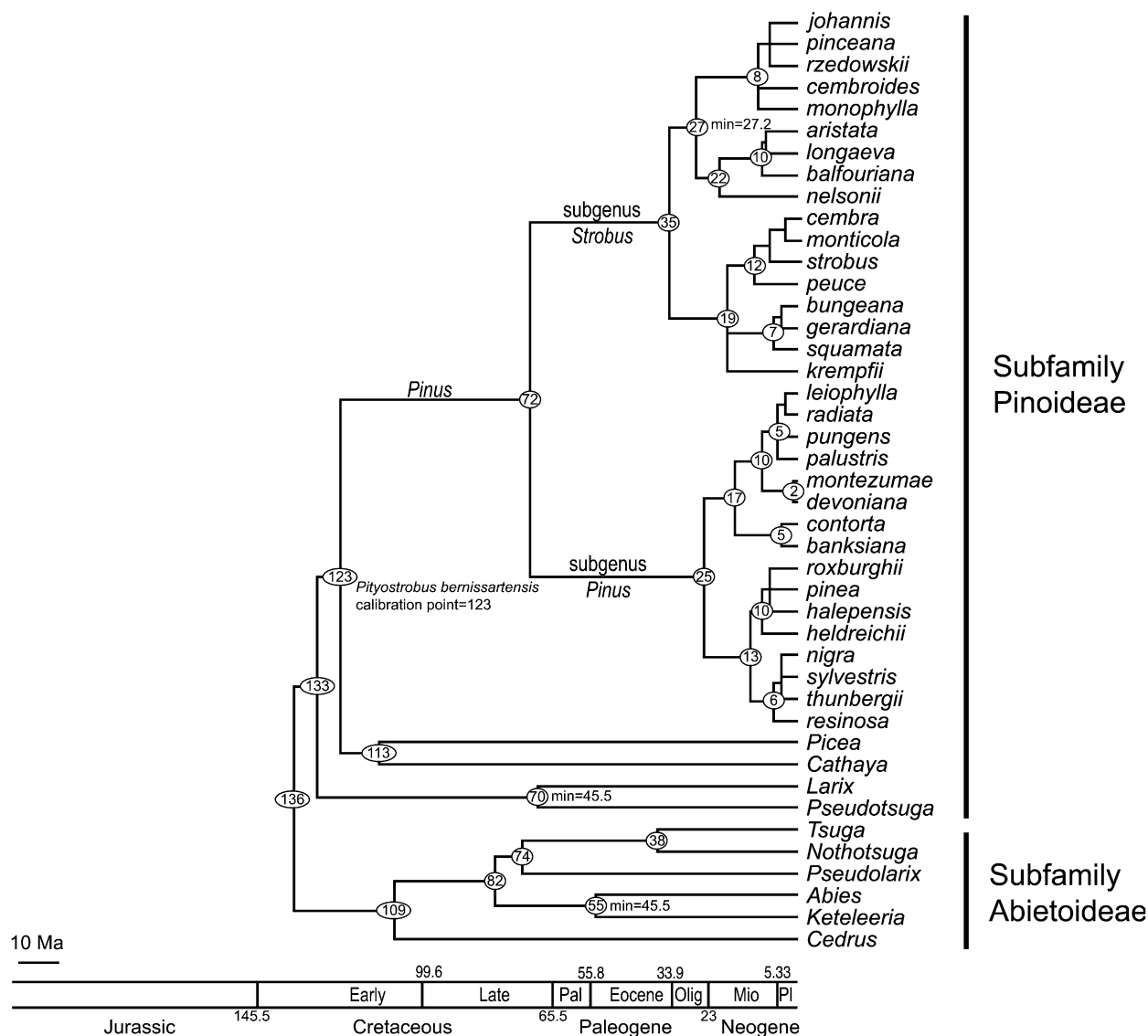


Fig. 3 Chronogram for Pinaceae based on a conservative fossil calibration with penalized likelihood. The fixed calibration point is the *Pinus-Picea* split at 123 Ma.

To evaluate the effect of constraining the Pinaceae-Araucariaceae divergence to a maximum age of 308.5 Ma, ages for five representative nodes were compared with and without the age constraint and by increasing or decreasing the constrained value by 20% (table 1). The ages estimated for the deepest nodes in the tree were unrealistically old when maximum-age constraints were not enforced. The removal of the maximum-age constraint also changed the results of the cross-validation test, resulting in lower error rates at higher smoothing values (allowing for more clocklike substitution rates). Constraining the Pinaceae-Araucariaceae node to 370.2, 308.5, or 246.8 Ma had a minimal effect on the optimal smoothing parameter (λ), but a greater effect was observed on the age estimates, particularly at the deepest nodes (table 1).

With the *Pinus-Picea* calibration, the *rbcL* silent-substitution rate estimated with penalized likelihood was 3.28×10^{-10} substitutions/site/year (421 third-codon positions; range: 3.26×10^{-10}

to 3.30×10^{-10}). The rate for all 1262 sites was 1.46×10^{-10} substitutions/site/year (range: 0.878×10^{-10} to 2.17×10^{-10}). For the *Pseudolarix-Tsuga* calibration, the *rbcL* silent-substitution rate was 2.61×10^{-10} substitutions/site/year (range: 1.91×10^{-10} to 3.75×10^{-10}). The rate for all 1262 sites was 0.962×10^{-10} substitutions/site/year (range: 0.537×10^{-10} to 1.84×10^{-10}). The *Pinus-Picea* calibration yielded a *matK* silent-substitution rate estimate of 4.02×10^{-10} substitutions/site/year (471 third-codon positions; range: 3.52×10^{-10} to 4.50×10^{-10}). The rate for 1414 sites, excluding 152 bp of a 3' flanking region, was 2.80×10^{-10} substitutions/site/year (range: 1.51×10^{-10} to 3.84×10^{-10}). With the *Pseudolarix-Tsuga* calibration, the *matK* silent-substitution rate was 2.91×10^{-10} substitutions/site/year (range: 1.59×10^{-10} to 4.43×10^{-10}), and the rate for 1414 sites was 2.25×10^{-10} substitutions/site/year (range 1.69×10^{-10} to 3.22×10^{-10}).

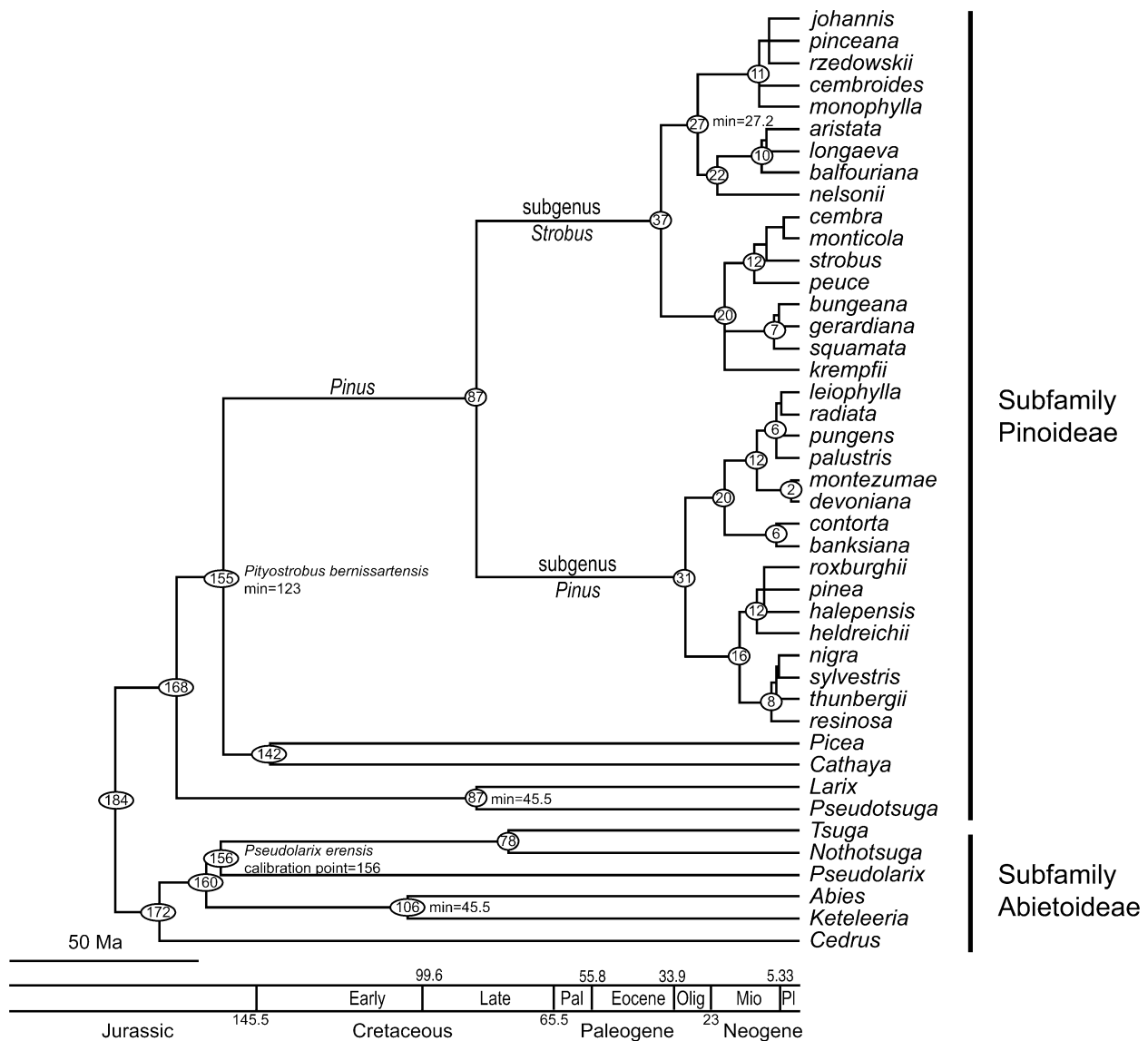


Fig. 4 Chronogram for Pinaceae based on a liberal fossil calibration with penalized likelihood. The fixed calibration point is the *Pseudolarix-Tsuga* split at 156 Ma.

Discussion

Assigning Ages to the Oldest Pinaceae Fossil Cones

Pinaceae is well represented in the fossil record throughout the Cretaceous, but with the exception of *Pinus* and *Pseudolarix*, ovulate cone fossils from that time do not belong to extant genera but are assigned to organ genera *Pseudoaraucaria*, *Pityostrobus*, and *Obiraostrobus* (Smith and Stockey 2002). In Europe, the oldest Cretaceous fossils reported for *Pinus*, *Pseudoaraucaria*, and *Pityostrobus* are from the Wealden facies, mainly the Mons Basin of Belgium (Alvin 1953, 1957, 1960) but also sites in northern France and southern England (Alvin 1988). The ages for Cretaceous deposits in Belgium have been revised, and works on specific localities have offered an age range for the Belgian Wealden of 127.3–89.5 Ma (Barremian-Turonian), although the Wealden is highly complex and some

formations may be found to extend to the Late Jurassic (Robaszynski et al. 2001). Pinaceae fossils from these sites usually lack collection information precise enough to link them to a specific excavation or a specific depth. The most problematic example is that of *Pinus belgica*, which was attributed to the Wealden on the basis of adhering particles but is of unknown provenance (Alvin 1960). Although *P. belgica* ultimately was not used for calibration, we note that it may not be accurate to assign the midpoint age of the Wealden (108.4 Ma) to this taxon. The upper age limit of the Wealden, 89.5 Ma, corresponds better to the next appearance of an anatomically characterized *Pinus* ovulate cone in the fossil record, *Pinus mutoi* (Saiki 1996) from the Coniacian (85.8–89.3 Ma) of Japan. Historical misassignment of the age of *P. belgica* may explain the perceived gap in the Cretaceous fossil record between the first and subsequent appearances of *Pinus* (Miller 1976).

Table 1
The Effect of Maximum-Age Constraints on the Ages of Five Pinaceae Nodes

Node	<i>Pinus-Picea</i> calibration				<i>Pseudolarix-Tsuga</i> calibration			
	None	370.2 Ma	308.5 Ma	246.8 Ma	None	370.2 Ma	308.5 Ma	246.8 Ma
Optimal smoothing (λ)	6.0	2.2	2	2	6.0	2.4	2.4	2.2
Pinaceae crown (Myr)	148.1	139.8	136.0	131.5	271.2	195.6	183.9	173.2
<i>Pinus-Picea</i> ^a (Myr)	123	123	123	123	227.7	165.3	155.1	148.1
<i>Larix-Pseudotsuga</i> (Myr)	62.3	69.4	70.5	68.7	113.8	94.8	87.0	85.8
<i>Nothotsuga-Tsuga</i> (Myr)	44.9	38.8	37.7	35.9	81.5	78.7	78.4	79.6
Subsect. <i>Cembroides</i> (Myr)	9.3	10.1	10.5	10.4	16.0	11.0	10.6	10.7

Note. Columns represent maximum-age constraints of 308.5 Ma, 370.2 Ma (308.5 + 20%), and 246.8 Ma (308.5 – 20%) and the absence of a constraint.

^a Constrained node.

A second relevant example is the age assigned to *Pityostrobus bernissartensis*. This taxon was reported from a Bernissart locality, Puits Négresse, that has not been precisely dated, but the age and palynological composition of a nearby Bernissart locality have been subject to several studies, particularly at the depth of 322 m, where many *Iguanadon* fossils were found (Yans et al. 2005; Quinif et al. 2006; Dejax et al. 2007). Seward (1900) reported that Pinaceae fossils from the *Iguanadon* pit at Bernissart were taken from a depth of greater than 250 m, and conifer pollen is reported from 322 m (Dejax et al. 2007). The age assigned here for *P. bernissartensis*, based on the age of the *Iguanadon* site (range: 127.5–118.5 Ma, midpoint: 123 Ma), is slightly older than the midpoint age of the Wealden as a whole (range: 127.3–89.5 Ma, midpoint: 108.4 Ma).

In contrast to the relatively wide age intervals associated with the Wealden, the provenance of *Pityostrobus californiensis* was reported in detail (Smith and Stockey 2001), allowing for the assignment of a more precise age (middle Aptian, 118.5 Ma). The age and inferred phylogenetic position of this and the Wealden taxa considered here support the hypothesis that modern Pinaceae genera began to diverge no later than the Aptian.

Considering the diversity of Pinaceae in the Cretaceous, Alvin (1960) expressed surprise that extant Pinaceae genera were not known from the Jurassic. More recently, LePage and Basinger (1995) reviewed the fossil record of *Pseudolarix* and concluded that fossil seeds, cone scales, brachioblasts, and leaves extending throughout the Cretaceous are morphologically indistinguishable from those of the extant species *Pseudolarix amabilis*. Cretaceous fossils accepted by LePage and Basinger (1995) were recorded from the Portlandian/Berriasian, Berriasian, Neocomian, Neocomian/Aptian, Barremian, Albian, Cenomanian, and Turonian. Subsequent ⁴⁰Ar/³⁹Ar dating of fossil deposits correlated to localities bearing *Pseudolarix* in Mongolia (Keller and Hendrix 1997) would extend the fossil record of the genus to the Oxfordian (Late Jurassic; Krassilov 1982; LePage 2003). Dates for the specific formations where *Pseudolarix* was described are needed, but these findings suggest that *Pseudolarix* has the oldest and most continuous fossil record of any extant Pinaceae genus.

Miller (1976) interpreted *Compsostrobus neotericus* from the Late Triassic of North Carolina as suggestive that extant Pinaceae may have been present at that time. Age estimates for crown-group Pinaceae were heavily influenced by use of a

maximum-age constraint one node removed (table 1), but only use of a higher maximum-age constraint (370.2 Ma) with the *Pseudolarix-Tsuga* calibration gives a crown-group Pinaceae age estimate that approaches support for the possibility suggested by Miller (195.6 Ma, Early Jurassic; table 1).

Phylogenetic Relationships among Extant Genera and Six Fossils

Integration of fossil taxa into phylogenetic hypotheses is a critical step in understanding the evolutionary history of Pinaceae. Analyses of nonmolecular data, alone (fig. 1A, 1B) and in combination with molecular characters (fig. 1E, 1F), yield phylogenies that resolve six fossil taxa within the crown of Pinaceae. Inferred tree topologies incorporating nonmolecular and molecular data that are unavailable for the fossil taxa bore a much stronger resemblance to molecular results than did trees inferred solely from ovulate cone characters (Smith and Stockey 2001, 2002). This and prior molecular studies are generally consistent with the classification by Van Tieghem (1891), who divided Pinaceae into Cédreés (subfamily Abietoideae: *Tsuga*, *Cedrus*, *Abies*, *Keteleeria*, and *Pseudolarix*), united by the presence of a single central resin canal in the taproot primary vascular region, and Pinées (subfamily Pinoideae: *Pinus*, *Picea*, *Larix*, and *Pseudotsuga*), united by resin canals positioned “adjacent to each protoxylem pole.” *Cathaya* and *Nothotsuga* were described after Van Tieghem’s classification (Chun and Kuang 1958; Page 1989); *Cathaya* has pinoid root anatomy (Hu and Wang 1984), but we have not located a description for the root anatomy of *Nothotsuga*. Wood resin canal characters support these groupings, as does the presence of resin vesicles in the seed coats of all members of subfamily Abietoideae (Price et al. 1987; Wang et al. 2000; Liston et al. 2003) and the distinctive form of their ovulate cone scales. However, placement of the root between *Cedrus* and the rest of Pinaceae often has rendered subfamily Abietoideae paraphyletic to subfamily Pinoideae; this is the case in all parsimony trees presented here (fig. 1A, 1C, 1E) and in cp *matK*, mitochondrial *nad5*, and nuclear *4CL* data sets analyzed separately (Wang et al. 2000). In contrast, the subfamilies were mutually monophyletic in Bayesian analyses of cpDNA alone (figs. 1D, 2) and of combined cpDNA and nonmolecular data (fig. 1F), a result recovered in two prior studies with reduced generic sampling (Prager et al. 1976; Price et al. 1987). Other

positions for the root of Pinaceae were found with nonmolecular characters (Hart 1987), with cpDNA restriction sites (Tsumura et al. 1995), and with a combined analysis of *rbcL* and nuclear ITS regions (Vining 1999). Paraphyly of subfamily Abietoideae is supported by the observation that the simpler wood anatomical characters in subfamily Abietoideae are considered plesiomorphic (Greguss 1955); the upright ovulate cones and their disarticulation to release seeds, features also observed in Araucariaceae, might also be plesiomorphic.

Q3 Several other generic relationships in Pinaceae are uncertain. *Cedrus*, besides having been recovered as sister to the remaining Pinaceae or sister to subfamily Abietoideae, has also been recovered as sister to *Abies* and *Keteleeria* (Price et al. 1987) or sister to *Pseudolarix* (Smith and Stockey 2001, 2002). In the three-genome data set of Wang et al. (2000), *Pseudolarix* was the sister group either to *Tsuga* and *Nothotsuga* (*matK* and *nad5*) or to *Tsuga*, *Nothotsuga*, *Keteleeria*, and *Abies* (*4CL*). The sister position of *Pseudolarix* spp. (with *P. californiensis* and *Pityostrobus corneti*) to *Tsuga*, *Nothotsuga*, *Keteleeria*, *Abies*, and subfamily Pinoideae in the nonmolecular parsimony tree (fig. 1A) is consistent with the nuclear result of Wang et al. (2000) and better accommodates the older fossil record of *Pseudolarix* with respect to other genera in Pinaceae (LePage and Basinger 1995). In subfamily Pinoideae, a sister relationship between *Picea* and *Cathaya* has been found repeatedly in DNA-based studies, but branch support is not high (Wang et al. 2000; Eckert and Hall 2006; fig. 1C, 1D). Alternatively, *Cathaya* or *Picea* may be sister to *Pinus* (Hart 1987; Wang et al. 2000; fig. 1A, 1B, 1E, 1F).

The six fossil taxa analyzed in this study occupied three separate lineages within the Pinaceae crown group. *Pseudoaraucaria*, *P. californiensis*, *P. corneti*, and *Pseudolarix erensis* occurred within Abietoideae in the combined analyses (though at varying positions), and *P. bernissartensis* and *Pinus* were monophyletic (fig. 1E, 1F). The position of *Pseudoaraucaria*, as either unresolved within subfamily Abietoideae in the Bayesian tree (fig. 1F) or sister to all members of Pinaceae except *Cedrus* in the parsimony tree (fig. 1E), is similar to that found by Alvin (1988) with a phenetic analysis of 22 characters, in which *Pseudoaraucaria* was the sister group to all extant members of Abietoideae. Whether *Pseudoaraucaria* had deciduous cone scales remains an open question, but we included two characters identified by Alvin (1988) referring to less developed, gelatinous “mechanical tissue” that could play a role in the shedding of ovulate cone scales and could point to a closer relationship between *Cedrus* and *Pseudoaraucaria* than was found by Smith and Stockey (2001, 2002).

Pityostrobus corneti was sister to *Pseudolarix* in the nonmolecular parsimony tree and the combined Bayesian tree (fig. 1A, 1F) but was sister to *P. californiensis* and subfamily Pinoideae in the combined parsimony tree (fig. 1E). The taxon had been described previously as similar to two other members of subfamily Abietoideae, *Keteleeria* and *Cedrus* (Alvin 1953). *Pityostrobus californiensis* does not bear a strong resemblance to any particular extant genus (Smith and Stockey 2001), and although it seems to have an affinity for subfamily Abietoideae, it lacks resin vesicles in its seed coat and appears as sister to subfamily Pinoideae in the combined parsimony tree (fig. 1E).

Pseudolarix is well represented in the fossil record (LePage and Basinger 1995; LePage 2003). Inclusion of *P. erensis* (*Pseudolarix* sp. sensu LePage and Basinger 1995) in a phylogenetic analysis supports both its interpretation as *Pseudolarix* (fig. 1) and its use as a Late Jurassic calibration point within crown Pinaceae (table 1; fig. 4). The interpretation of these fossils as *Pseudolarix* was described as “unequivocal” by LePage (2003). The high branch support for this relationship (fig. 1E, 1F) is intriguing but deceptive, because the anatomy of the fossil taxon is unknown; as a result, 78% of its cells were scored as missing in the nonmolecular matrix. Calibration based on the inferred age and phylogenetic position of *P. erensis* gives ages at most nodes that are much older than the appearance of ovulate cones attributed to extant genera in the fossil record but coincide well with recent findings of fossil leaves (Stockey and Wiebe 2006). Until detailed anatomical studies are published, these fossils must be treated with a greater degree of caution than anatomically characterized Pinaceae fossils from the Cretaceous.

The sister position between *P. bernissartensis* and *Pinus* supports the assertion by Miller (1976) that a “strong *Pinus* influence” is apparent in *Pityostrobus*. However, the artificial genus also includes taxa more closely related to genera in subfamily Abietoideae, such as *Keteleeria*, *Abies*, and *Cedrus* (fig. 1; Alvin 1953; Smith and Stockey 2001, 2002). Exploratory analyses of the combined nonmolecular and molecular matrix expanded to include all fossil taxa scored by Smith and Stockey (2002) suggest that at least 12 of the 25 *Pityostrobus* species belong to subfamily Pinoideae. Simultaneous analysis of an expanded version of the combined matrix presented here should greatly improve our understanding of the phylogenetic position of anatomically characterized fossil cones with respect to extant taxa.

The total-evidence analyses resolved *P. belgica* as a crown-group member of *Pinus* (fig. 1E, 1F). The taxon was described from a single ovulate cone with anatomical and morphological synapomorphies for *Pinus*, including scales with a raised area (apophyses) in a dorsal position and a vascular trace system leading to an ovuliferous scale that conforms to the pattern defined for *Pinus* rather than to the fossil organ genus *Pityostrobus* (Miller 1976). It is similar to extant species, such as *Pinus sylvestris* of section *Pinus* (Alvin 1960). Some studies have treated *P. belgica* as the earliest reliable evidence that *Pinus* had diverged from other extant genera of Pinaceae (Wang et al. 2000), while others have used the fossil to calibrate an internal node of *Pinus*, either the divergence of the subgenera *Pinus* and *Strobos* or the divergence of sections *Pinus* and *Pinaster* (Eckert and Hall 2006; reviewed by Willyard et al. [2007]).

Although our understanding of nonmolecular character evolution within *Pinus* is limited, fossils attributed to infrageneric groups of *Pinus* have been important in shaping hypotheses regarding the age of Pinaceae. The oldest fossil member plausibly assignable to *Pinus* subgenus *Strobos* appears in the Late Cretaceous Aachen Formation of Belgium (Santonian, 83.5–85.8 Ma; Meijer 2000): fossil wood with pinoid cross-field pits and conspicuously dentate ray tracheids representing the organ genus *Pinuxylon*. This fossil was considered an upper bound for the age of the *Pinus* subgeneric split in the rate estimations of Willyard et al. (2007). Paleobotanists have varied in their willingness to attribute this and other fossil taxa to extant infrageneric ranks in *Pinus*. Axelrod (1986) argued that six extant

Pinus subsections (*Strobos*, *Cembroides*, *Balfourianae*, *Pinus*, *Ponderosae*, and *Contortae*) first appeared in North America during the Eocene (55.8–33.9 Ma). This controversial hypothesis has never been supported by an explicit phylogenetic analysis, but these ages for extant subsections were generally accepted by Millar (1993, 1998), interpreting fossil taxa as having affinities to extant infrageneric categories. The subsectional age estimates here also conflict with the assignment of Middle Eocene taxa *Pinus arnoldii*, *Pinus princetonensis*, and *Pinus baileyi* to subsection *Pinus* (Erwin and Schorn 2006). These taxa, like *P. belgica*, possess symplesiomorphic characters of *Pinus* and at present are best considered unassignable to extant subsections. Similarly, assignment of Oligocene *Pinus escaletis* to subsection *Contortae* (Erwin and Schorn 2006) is inconsistent even with the *Pseudolarix-Tsuga* calibration (fig. 4), but the taxon may suggest the ancestral morphology of North American hard pines. These conflicts highlight the importance not only of revising other North American Tertiary floras but also of including both fossil and extant taxa in phylogenetic analyses.

The calibrations proposed here recover an Early Jurassic or Early Cretaceous crown-group diversification of Pinaceae, with the final generic divergence between *Nothotsuga* and *Tsuga* occurring during the Eocene (fig. 3) or the Late Cretaceous (fig. 4). For both calibrations, the cpDNA silent-substitution rate estimates (range: 2.61×10^{-10} to 4.02×10^{-10} substitutions/site/year) are within the range reported by Willyard et al. (2007) from an independent calibration of the *Pinus* subgenus *Pinus-Strobos* divergence (2.2×10^{-10} to 4.2×10^{-10} substitutions/site/year). Calibrating the *Pinus-Picea* split at 123 Ma from the position of *P. bernissartensis* and avoiding calibration with *Pinus belgica* yield a reasonable minimum bound for ages in the family (fig. 3). The *Pinus-Picea* calibration at 123 Ma is conservative because it avoids assigning ages on the basis of fossils with a more tentatively phylogenetic place or age, such as *P. californiensis*, *P. erensis*, and *P. belgica*. This calibration yields a crown-group age for *Pinus* of 72 Ma, which requires that *P. belgica* and *P. mutoi* (both dated at >89 Ma) be interpreted as stem- rather than crown-group members of the genus. In the *Pseudolarix-Tsuga* calibration, the estimated age of the subgenus *Pinus-Strobos* node was 87 Ma, slightly younger than the minimum age of the Wealden considered here (89.5 Ma), which indicates that using *P. belgica* for calibrating any crown node of *Pinus* would yield ages older than those suggested by other Pinaceae fossils.

Significant character incongruence was found between non-molecular and molecular matrices in this study. Nevertheless, no incongruent clades received high levels of branch support (fig. 1). Other studies have suggested that the Templeton (1983) and ILD tests can detect significant congruence between different data partitions as a result of factors related more to signal heterogeneity than to divergent genealogies (Cunningham 1997; Barker and Lutzoni 2002; Hipp et al. 2004). In this study, combining nonmolecular and molecular data for Pinaceae caused a reduction in phylogenetic resolution compared to separate analyses. This observation, together with the lower levels of branch support in the nonmolecular than in the molecular trees, indicates that, beyond the need to acquire more DNA characters from all three genomes, there is a need for more-refined scoring and analysis of nonmolecular characters, as has been done in Cupressaceae (see Little 2006). Functional correlation is widely thought to exist among certain morphological characters scored separately, such as the presence of winged pollen and pollination drops, and between cone scale and seed morphology (Hart 1987; Tomlinson 1994). Furthermore, the ontogeny of highly homoplasious nonmolecular characters could be examined in more detail.

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Queries

- Q1 The accession numbers have been added for the sake of completeness and because it will be easier to remove them than to add them after the proofs stage. If you wish to retain them, the matrix accession numbers can be shortened to “M3674–M3676.”
- Q2 Should “Bon-Tsagan” be “Bon-Tsagaan,” as in “Tsagaan Tsav”?
- Q3 In the last sentence of the paragraph, the passage after the Greguss citation has been revised in light of query 16 in the e-mail correspondence. Does the revised sentence express your intended meaning accurately