Silent mutation rate estimates for *Pinus* vary 50-fold, ranging from angiosperm-like to among the slowest reported for plants. These differences either reflect extraordinary genomic processes or inconsistent fossil calibration, and they have important consequences for population and biogeographical inferences. Here we estimate mutation rates from 4 *Pinus* species that represent the major lineages using 11 nuclear and 4 chloroplast loci. Calibration was tested at the divergence of *Pinus* subgenera with the oldest leaf fossil from subg. *Strobus* (Eocene; 45 MYA) or a recently published subg. *Strobus* wood fossil (Cretaceous; 85 MYA). These calibrations place the origin of *Pinus* 190–102 MYA and give absolute silent rate estimates of 0.70–1.31 × 10⁻⁹ and 0.22–0.42 × 10⁻⁹ site⁻¹ year⁻¹ for the nuclear and chloroplast genomes, respectively. These rates are approximately 4- to 20-fold slower than angiosperms, but unlike many previous estimates, they are more consistent with the high per-generation deleterious mutation rates observed in pines. Chronograms from nuclear and chloroplast genomes show that the divergence of subgenera accounts for about half of the time since *Pinus* diverged from *Picea*, with subsequent radiations occurring more recently. By extending the sampling to encompass the phylogenetic diversity of *Pinus*, we predict that most extant subsections diverged during the Miocene. Moreover, subsect. *Australae*, *Ponderosae*, and *Contortae*, containing over 50 extant species, radiated within a 5 Myr time span starting as recently as 18 MYA. An Eocene divergence of pine subgenera (using leaf fossils) does not conflict with fossil-based estimates of the *Pinus–Picea* split, but a Cretaceous divergence using wood fossils accommodates Oligocene fossils that may represent modern subsections. Because homoplasy and polarity of character states have not been tested for fossil pine assignments, the choice of fossil and calibration node represents a significant source of uncertainty. Based on several lines of evidence (including agreement with ages inferred using calibrations outside of *Pinus*), we conclude that the 85 MYA calibration at the divergence of pine subgenera provides a reasonable lower bound and that further refinements in age and mutation rate estimates will require a synthetic examination of pine fossil history.

Introduction

Morphological and molecular analyses of the pine genus (*Pinus*; *Pinaceae*) reveal conflicting estimates of age and evolutionary rates that are difficult to reconcile. *Pinus* contains 2 monophyletic subgenera, *Pinus* (diploxylon or “hard pines”) and *Strobus* (haploxylon or “soft pines”), diagnosable by 2 versus 1 fibrovascular bundle per leaf (Gernandt et al. 2005). Origin of the genus is thought to date to the Early Cretaceous (Millard 1998), whereas estimates for the divergence of the subgenera range from the Late Cretaceous (Millard 1998) to the Mid-Eocene (Miller 1976). Limited morphological differentiation in the approximately 110 species has been attributed to an exceptionally slow rate of change, but morphological homoplasy (Gernandt et al. 2005) and retention of ancestral molecular polymorphism (Syring et al. 2007) are common. Several studies have reported slow molecular divergence rates for pines (Krupkin et al. 1996; Dvornyk et al. 2002; Geada López et al. 2002; Brown et al. 2004; Sokol and Williams 2005; Ma et al. 2006), but these rates appear inconsistent with estimates of per-generation deleterious mutation rates which are known to be at least 10-fold higher in pines than in self-compatible annual flowering plants (Kärkkäinen et al. 1996; Klekowski 1998). In contrast, mutation rate estimates comparable to angiosperms have been reported for pines based on antigenic distances (Prager et al. 1976) and for a retrotransposon (Kossack and Kinlaw 1999). Unlike many plant groups, *Pinus* is prominent in paleofloras (Miller 1976; Millar 1998; Price et al. 1998), has a long history of taxonomic inquiry (Price et al. 1998; Gernandt et al. 2005), and extensive genomic resources are available (Brown et al. 2001; Temelesgen et al. 2001; Chagné et al. 2003; Komulainen et al. 2003; Krutovsky et al. 2004). These enhance its usefulness for studying the interrelationship between morphological and molecular evolution. However, the paleontological data cannot be utilized to full advantage until critical tests of fossil–phylogenetic associations are conducted (e.g., Magallón and Sanderson 2005). The earliest fossil attributable to *Pinus, Pinus belgica* (Alvin 1960), is a Cretaceous ovulate cone apparently originating from the Wealden Formation in Belgium 145–125 MYA. Attempts to incorporate this and other *Pinus* fossils into molecular phylogenetic analyses have taken strikingly different approaches. For calibration, *P. belgica* has been placed at the divergence between *Pinus* and other modern *Pinaceae* genera (Wang et al. 2000; García-Gil et al. 2003) or at the divergence of subgenera (Sokol and Williams 2005; Ma et al. 2006). Because Alvin (1960) further ascribed *P. belgica* to subsect. *Pinus*, its age has been commonly applied to the divergence between representatives from the sections of subg. *Pinus* (Krupkin et al. 1996; Dvornyk et al. 2002; Geada López et al. 2002; Brown et al. 2004; Eckert and Hall 2006). Alternative calibrations used 195 MYA based on a presumed Jurassic origin of the genus that lacks explicit fossil evidence (Kutil and Williams 2001) or 45 MYA as the divergence of subgenera based on the earliest fossils representing both subgenera (Kossack and Kinlaw 1999). As a consequence of inconsistent calibration, synonymous mutation rate estimates vary 50-fold, ranging from angiosperm-like (2.8 × 10⁻⁹ substitutions site⁻¹ year⁻¹) for “gypsy”-like retrotransposons; Kossack and Kinlaw...
1999) to far slower than angiosperms (0.05 × 10⁻⁹ for Adh; Dvornyk et al. 2002). Similarly, divergence rates calculated for pine chloroplast DNA (cpDNA; 0.06 × 10⁻⁹; Krupkin et al. 1996) are among the slowest reported for any plant.

Here we evaluate hypotheses concerning the age of *Pinus* and explore the impact of different calibrations on estimated absolute mutation rates across 11 nuclear and 4 chloroplast loci. A phylogenetic framework was created with an exemplar from each of the 4 monophyletic sections (a “quartet” of species). Using a critical evaluation of the fossil record, we test 2 calibrations at the crown, inferred as the divergence between subg. *Strobus* and subg. *Pinus*, and represented by competing hypotheses for the oldest fossils from subg. *Strobus* (putative lower age of 85 MYA and an upper bound of high certainty at 45 MYA). We also use our multilocus data set to recalculate ages and rates with the calibration scenarios used in recent studies (Krupkin et al. 1996; Dvornyk et al. 2002; Geada López et al. 2002; Brown et al. 2004; Eckert and Hall 2006; Ma et al. 2006) to test whether highly heterogeneous rates are an intrinsic feature of the loci and taxa sampled in those studies or if the variation can be attributed to calibration.

**Materials and Methods**

**Plant Materials**

Each subgenus comprises 2 monophyletic groups—sections *Trifoliate* and *Pinus* from subg. *Pinus* and sections *Paryya* and *Quinquefoliae* from subg. *Strobus*. For this study, 1 species was chosen from each section: *Pinus taeda* L. (Mississippi, United States) for section *Trifoliate*; *Pinus thunbergii* Parl. (Cheonnam, South Korea) or *Pinus merkusii* Jungh. & deVriese s.l. (Thailand) for section *Quinquefoliae*; and *Pinus nelsonii* Shaw (Nuevo León, Mexico) for section *Paryya*. Haploid genomic DNA from seed megagametophyte tissue was isolated for amplifying nuclear DNA (nDNA) (FastDNA, Qbiogene, Irvine, CA), whereas leaf DNA was the source for cpDNA (Gernandt et al. 2005).

**Loci Evaluated**

Nuclear loci included in this study map to 7 of 12 linkage groups in *Pinus* (see table S1, Supplementary Material online). Represented are LG2, *aquaporin*; LG3, chlorophyll-binding protein type II precursor (*LHC-CAB*) and late embryogenesis abundant-like protein (*LEA*-like); LG5, arabinogalactan-like protein (*AGP6*) and *ferritin*; LG6, phenylalanine ammonia lyase (*PAL1*); LG7, 4-coumarate:CoA ligase (*4CL*); LG9, cinnamyl alcohol dehydrogenase (*CAD*) and open stomata (*OST1*); LG10, chloroplast-localized Cu–Zn superoxide dismutase (*SOD*); and the unmapped early response to dehydration (*ERD3*). Loci from LG3, LG5, and LG9 are sufficiently distant such that they are effectively unlinked (Krutovsky et al. 2004). Chloroplast loci included in this study are *matK*, *rbcL*, the *rpl20*/*rpsS18* intergenic spacer, and the *trnV* intron. See table S1, Supplementary Material online for primer and amplicon descriptions.

**Amplification, Sequencing, and Analysis**

Polymerase chain reaction (PCR) products were directly sequenced using BigDye v. 3.1 (Applied Biosystems, Foster city, CA) and visualized on an Applied Biosystems 3730 Genetic Analyzer. DNA alignments were made using ClustalW (Thompson et al. 1994) as implemented in BioEdit v. 7.0.1 (Hall 1999) or an iterative process of BlastN analysis (http://www.ncbi.nlm.nih.gov), followed by hand alignment, to align cDNA sequences from GenBank (http://www.ncbi.nlm.nih.gov/GenBank) to genomic sequences. Alignments were made at 3 levels: 1) species within a subgenus, that is, *P. taeda* to *P. thunbergii* and *P. monticola* to *P. nelsonii*; 2) the quartet of *Pinus* species, excluding unalignable regions (Syring et al. 2005); and 3) the quartet of *Pinus* species with an outgroup sequence. For 10 loci (*4CL*, *AGP6*, *aquaporin*, *CAD*, *LHC-CAB*, *PAL1*, *matK*, *rbcL*, *rpl20*/*S18*, and *trnV*; see table S1, Supplementary Material online for details), outgroup sequences from *Picea* were obtained using PCR (as described for ingroup sequences) or by searching GenBank for putative genomic or expressed sequence orthologs. Outgroup sequences were not isolated for *ERD3* and *SODchl* because of orthological concerns regarding some ingroup amplicons (see Results). Amplification of the LEA-like locus failed in *Picea*, so a GenBank sequence from *Pseudotsuga* was used as the outgroup. The closest GenBank matches available for *ferritin* and *OST1* (*Arabidopsis thaliana*) were used to provide a root for these 2 loci. New nucleotide sequences were submitted to GenBank (see table S2, Supplementary Material online for details); alignments are available as Supplementary Material online.

Pairwise substitution rates for silent (ds; synonymous plus noncoding) and nonsynonymous (dn) sites were calculated using DnaSP (Rozas et al. 2003) using the approximate method of Net and Gojobori (1986) with a Jukes–Cantor correction for multiple substitutions. DnaSP (Rozas et al. 2003) was also used to calculate GC content. Models of sequence evolution (number of substitution categories, base frequencies, shape parameter, and proportion of invariant sites) were tested independently for all sites and for the silent sites (approximated by including noncoding and third-codon positions) of each locus using Modeltest 3.7 (Posada and Crandall 1998). Models selected by the hierarchical likelihood ratio tests in Modeltest 3.7 were used to obtain maximum likelihood (ML) estimates of phylogeny with PAUP* ver. 4.0b10 (Swofford 2002). To test for rate equality (i.e., clock-like evolutionary history) among lineages, we used the likelihood ratio test (Muse and Weir 1992) to compare clock-enforced and clock-relaxed likelihood scores that were obtained using the selected evolutionary models described above (1-tailed probability, α = 0.05).

A partition homogeneity test (Cunningham 1997; 10 replicates using a heuristic search) was used to test for significant conflict between loci (F = 0.01) for all sites as well as silent sites from 3 different data sets (see table S3, Supplementary Material online for details). The 3 data sets are 1) 9 nuclear loci with different outgroup species (*Picea, Pseudotsuga, or Arabidopsis*; *4CL*, *AGP6*, *aquaporin*, *CAD*, *ferritin*, *LEA*-like, *LHC-CAB*, *OST1*, *PAL1*; 8,346
bp; 6,098 silent bp); 2) 5 nuclear loci with *Picea* as the outgroup (4CL, AGP6, aquaporin, CAD, LHC-CAB; 3,973 bp; 2,348 silent bp); and 3) 4 chloroplast loci with *Picea* as the outgroup (matK, rbcL, rpL20/S18, trnV; 3,903 bp; 1,914 silent bp). Because data sets did not exhibit significant conflict, they were concatenated, and models of sequence evolution were selected for concatenated data sets as described above. To investigate the impact of increased taxon density on rate and divergence estimates, we examined data from Syring et al. (2005) that include 4 loci evaluated in this study (4CL, AGP6, LEA-like, LHC-CAB; 5,338 bp; 3,766 silent bp) for 12 pine species serving as exemplars for subsections with *Picea* as the outgroup. Evolutionary model selection, likelihood ratio tests, and partition homogeneity tests were performed on each locus and on the concatenated silent sites as described above.

Fossil Calibration

Because all pine fossil reports that were considered as calibration sources lack radiometric dating, ages were adjusted to the midpoint of the currently assigned range for their geological Epoch or Stage (Gradstein and Ogg 2004). *Pinus* pollen, pollen cones, and ovulate cone casts lack diagnostic characters for subgeneric identification (Miller 1976; Phipps et al. 1995), so only anatomical reports from ovulate cones, leaves, and wood were considered for calibration sources. The earliest *Pinus* fossil, *P. belgica* (145–125 MYA; Alvin 1960), shows affinities to subg. *P. nigra* (Miller 1976) and has been considered part of subsect. *Pinus* (Millar 1998). However, the collection locality for *P. belgica* (Wealden Formation, Belgium) was inferred from the lignitic state and adhering particles (Alvin 1960), leaving its geographic and stratigraphic origins uncertain. There is an approximate 35-Myr gap until the next *Pinus* fossils (100–75 MYA ovulate cones from North America, Japan, and Europe), and these fossils also have affinity to subg. *Pinus* (Fliche 1896; Alvin 1960; Robison 1977; Blackwell 1984; Miller and Malinky 1986; Stockey and Nishida 1986; Saiki 1996). Because these early fossils show symplesiomorphic features and lack synapomorphies to support infrageneric nodes, they cannot be used as reliable calibration sources (Magallon and Sanderson 2001).

The node corresponding to the divergence of the subgenera is well supported with both morphological and molecular synapomorphies (Gernandt et al. 2005). Because all of the oldest *Pinus* fossils have affinity to subg. *Pinus*, the first fossil representing subg. *Strobus* supports the minimum age of divergence of the subgenera. The appearance of subg. *Strobus* dates to the Late Cretaceous based on permineralized wood anatomy (Santonian, 83.5–85.8 MYA, midpoint approximately 85 MYA, *Pinus xylon* sp.; Meijer 2000), or to the Mid-Eocene based on either leaf anatomy (37.2–48.6 MYA, midpoint approximately 45 MYA, *Pinus similikameeneensis*; Miller 1973), or ovulate cones (ca. 43 MYA; Axelrod 1986). It is important to note that Cretaceous fossils have been attributed to subg. *Strobus* (Jeffrey 1908; Stopes and Kershaw 1910; Penny 1947), but these fossils are not useful for calibration because they have been reassigned to subg. *Pinus* or to other genera (see Discussion). Therefore, we calibrated the divergence of the subgenera with a putative lower age of 85 MYA based on wood anatomy and an upper age of 45 MYA based on leaves and ovulate cones.

Rates and Ages

Absolute rates of silent-site changes in the concatenated data sets were estimated using 2 approaches. First, we computed silent-site divergence (*dS*) for comparisons between subgeneric representatives using 9 nuclear and 4 chloroplast loci. Lower and upper rate estimates (i.e., 2 calibration ages) were calculated using the formula \( \mu = dS/2T \), where \( \mu \) is the silent divergence-site\(^{-1}\).year\(^{-1} \), *dS* is the mean of silent substitutions-site\(^{-1} \) (weighted by number of sites in each locus), and *T* is time in years. Second, we calculated ML branch lengths (using the selected model of sequence substitution described above; see Table S3, Supplementary Material online for details) from clock-enforced phylogenograms using silent sites from pine quartet alignments of nDNA and cpDNA. Silent-site data sets were used for age inference to maintain consistency with silent rate estimation and to minimize the potential for selection to distort the evolutionary history or rate at a locus. Ages were also inferred using “all” nucleotide sites to provide corroborative estimates. To account for departures from clock-like behavior in the 12-species data set, we used a non–clock-enforced tree based on silent sites to assign dates and to estimate a range of local rates using penalized likelihood (PL), with a smoothing factor estimated using cross-validation (r8s ver. 1.70; Sanderson 2002).

Results

Sequence Variation across Loci, Genomes, and Lineages

Alignments of 11 nuclear and 4 chloroplast loci from 4 *Pinus* species included 11,481 bp and 10,643 bp from subg. *Pinus* and *Strobus*, respectively (Table 1). Mean lengths of nuclear sequences were shorter than chloroplast sequences (690 bp vs. 972 bp in subg. *Pinus*), but the larger sample of nuclear loci yielded a substantially larger nDNA data set (e.g., 7,594 bp nDNA vs. 3,887 bp cpDNA in subg. *Pinus*). In total, 65% of nDNA and 77% of cpDNA was exonic, whereas the noncoding portion included introns (4CL, aquaporin, CAD, ERD3, ferritin, LEA-like, OST1, SOD\(_{\text{chl}}\), and trnV), 5′ (OST1) and 3′ (ferritin) untranslated regions, and an intergenic spacer (rpL20/S18). Noncoding regions provided 51% of nDNA silent sites (1,959 bp vs. 1,882 bp at third-codon positions) and 40% of cpDNA silent sites (876 bp vs. 1,289 bp at third-codon positions). Nine of the mapped nuclear loci lacked heterozygosity when amplified from haploid megagametophyte tissue, providing evidence for target specificity and orthology. Two loci—*SOD\(_{\text{chl}}* and *ERD3*—showed evidence of paralogy and were excluded from rate analyses. These loci were unexpectedly heterogeneous in subg. *Pinus*, indicative of gene duplication or nonspecific priming. The *SOD\(_{\text{chl}}* sequence from *P. monticola* also lacked 4 expected introns, raising the possibility that it is a reverse transcribed pseudogene. GC% showed considerable variation across loci. For nDNA, the average GC content was 47.3% (range = 32.9–66.7%), with coding slightly higher (51.3%; range = 40.5–67.0%) and noncoding
considerably lower (35.8%; range = 23.6–44.5%). For cpDNA, the average GC content was 37.6% (range = 31.4–45.4%), and the GC content in coding (37.8%; range = 32.5–45.4%) and noncoding (36.2%; range = 31.4–45.4%) regions were very similar.

Table 1 Length (L), Noncoding Length (NC), Silent (dS; synonymous plus noncoding), and Nonsynonymous (dN) Substitutions per Site across 11 Nuclear and 4 Chloroplast Genes within Subg. Pinus, Subg. Strobus, and across Subgenera

<table>
<thead>
<tr>
<th>Locus</th>
<th>Section Trifoliæ–Pinus</th>
<th>Section Quinquefoliæ–Parrya</th>
<th>Subg. Pinus–Strobus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L</td>
<td>NC</td>
<td>dS</td>
</tr>
<tr>
<td>nDNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4CL</td>
<td>996</td>
<td>321</td>
<td>0.040</td>
</tr>
<tr>
<td>AGP6</td>
<td>444</td>
<td>0</td>
<td>0.047</td>
</tr>
<tr>
<td>CAD</td>
<td>653</td>
<td>338</td>
<td>0.039</td>
</tr>
<tr>
<td>ERD3*</td>
<td>656</td>
<td>202</td>
<td>0.162</td>
</tr>
<tr>
<td>Ferritin</td>
<td>767</td>
<td>367</td>
<td>0.042</td>
</tr>
<tr>
<td>LEA-like</td>
<td>1,115</td>
<td>1,037</td>
<td>0.040</td>
</tr>
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<td>LHC-CAB</td>
<td>701</td>
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<td>0.072</td>
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<td>641</td>
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<tr>
<td>PALI</td>
<td>409</td>
<td>0</td>
<td>0.021</td>
</tr>
<tr>
<td>SOD*</td>
<td>852</td>
<td>658</td>
<td>0.066</td>
</tr>
<tr>
<td>Meanb (SD)</td>
<td>690</td>
<td>331</td>
<td>0.042 (0.013)</td>
</tr>
<tr>
<td>cpDNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>matK</td>
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<td>rtl20/S18</td>
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<tr>
<td>trnV</td>
<td>546</td>
<td>543</td>
<td>0.007</td>
</tr>
<tr>
<td>Meanb (SD)</td>
<td>972</td>
<td>226</td>
<td>0.010 (0.008)</td>
</tr>
<tr>
<td>Total</td>
<td>11,481</td>
<td>4,549</td>
<td></td>
</tr>
</tbody>
</table>

a Values are excluded from mean due to possible paralogy (SOD, ERD3), short partition length (trnV), or unusually high dN (> 9 SD from mean; LEA-like).

b dS and dN means are weighted by number of sites.

Fossil-Calibrated Mutation Rates and Divergence Dates

Single-locus ML phylogenies (results not shown) using “all” characters in the quartet alignments showed the expected species relationships in all cases except OST1 (section Quinquefoliæ is sister to section Parrya and subg. Pinus) and ferritin (ingroup nodes were unresolved). Rooting of these 2 loci with Arabidopsis is likely responsible for the topological differences, and we excluded these loci from our chronograms. Rate equivalence (as inferred by the likelihood ratio test) was statistically supported for all individual loci except LHC-CAB, ferritin, and PALI using all characters and for all individual loci except PALI using “silent” sites (see table S3, Supplementary Material online for details). A concatenated alignment of the 5 clock-like loci that share Picea as outgroup (4CL, AGP6, aquaporin, CAD, and LHC-CAB) exhibited rate equality using

Table 2 Estimated Absolute Mutation Rates (μ; substitutions site⁻¹ year⁻¹) Based on Silent (dS) and Nonsynonymous (dN) Substitutions in Comparisons between subg. Pinus and subg. Strobus. Rates Are Averages of 9 Nuclear Loci or 4 Chloroplast Loci, Using Divergence Dates of 85 or 45 MYA. Values in Parentheses Are 1 SD

<table>
<thead>
<tr>
<th>Genome</th>
<th>Rate</th>
<th>dS</th>
<th>dN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear</td>
<td></td>
<td>0.118 (0.045)</td>
<td>0.70 (0.27)</td>
</tr>
<tr>
<td>Chloroplast</td>
<td></td>
<td>0.020 (0.013)</td>
<td>0.12 (0.04)</td>
</tr>
</tbody>
</table>

μ (×10⁻⁵)

For each genome; ages were calculated using all-site and silent-site data sets.

Based on a calibration with 85 or 45 MYA, absolute silent mutation rates (μ) for 9 nuclear loci average 0.70 or 1.31 × 10⁻⁵ silent substitutions site⁻¹ year⁻¹ for nDNA, and 0.22 or 0.42 × 10⁻⁵ for cpDNA (table 2). To address the impact of unequal base frequencies and among-site rate heterogeneity on rate estimates, the absolute silent mutation rate (μ) was also calculated from ML branch lengths for each calibration test (table 3). ML-based rates are marginally

The partial ML tree (fig. 5) and 3 trees inferred from strict and relaxed clock assumptions (fig. 6) all support the clade Pinus clade, as sister to section

Pinus Molecular Evolutionary Rates 93
higher than \( dS \)-based rates for nDNA (\( \mu = 0.75 \) or \( 1.41 \times 10^{-3} \)), whereas cpDNA rates are almost identical (\( \mu = 0.21 \) or \( 0.40 \times 10^{-3} \)).

Chronograms based on silent sites from nDNA and cpDNA reveal identical topologies, but disparities in the branch lengths (especially at deeper nodes) yield different estimated divergence dates for Pinus lineages (fig. 1 and table 3). Most notably, estimates from the 5-locus nDNA data set predict older divergence events between Pinus and Picea (190–102 MYA) than does cpDNA (164–136 MYA). Both genomes show sections of subg. Strobus diverging before those of subg. Pinus, although nDNA estimates are more ancient (48–25 for subg. Strobus vs. 30–16 MYA for subg. Pinus) than those from cpDNA (37–19 MYA for subg. Strobus vs. 25–13 MYA for subg. Pinus). Divergence dates inferred from the same data sets using all nucleotides were nearly identical at sectional divergences but somewhat younger for the Pinus–Picea node (table 3). Divergence dates were also estimated using silent sites in the clock-enforced data set that included 4 additional nuclear loci (ferritin, LEA-like, OST1, and PAL1; 6,998 bp). The use of different outgroups for 3 of these loci precluded us from estimating the divergence of Pinus from Picea (node D), but estimated sectional divergences within subg. Pinus (31–16 MYA; node A) and subg. Strobus (48–25 MYA; node B) were not different from estimates based on 5 clock-like loci (table 3).

Cross-validation yielded an optimum smoothing factor of 63 for PL in the 12-taxon data set. In general, results from PL indicate more recent divergence events than predicted from the analysis of only 4 exemplar species (fig. 2 and table 3). For example, using an 85 MYA calibration, subg. Strobus sections are predicted to have diverged 37 MYA or 10 Myr more recently than predicted from 4-taxon comparisons (fig. 1). Similarly, subg. Pinus sections are predicted to have diverged 28 MYA, which is 2 Myr more recent than indicated by 4-taxon estimates (fig. 1). An important finding highlighted by this additional taxon sampling is that modern pine subsections radiated in a narrow time span in the relatively recent past. For example, using the 85 MYA calibration, the 3 subsections of *Trifoliae* (fig. 2b, nodes E–G), with approximately 51 extant species, radiated within a 5 Myr time span starting 18 MYA.

**Discussion**

Mutation rate estimates are used widely for hypothesis testing in molecular, population, and evolutionary genetic studies (Muse 2000), and they are increasingly used to estimate genetic parameters of conifers (Dvornyk et al. 2002; García-Gil et al. 2003; Brown et al. 2004; Ma et al. 2006). Numerous estimates of mutation rates have been made for angiosperms (e.g., Wolfe et al. 1987, 1989; Gaut et al. 1996; Koch et al. 2000; Clark et al. 2005), but comparable conifer estimates are limited to studies of single-locus variation across multiple species (Geada López et al. 2002), multilocus variation in a single species (García-Gil et al. 2003; Brown et al. 2004), or multilocus variation in a few closely related species (Ma et al. 2006). The perspective added by our synthesis of multiple nuclear and chloroplast loci based on exemplar taxa and 2 fossil calibration points indicates that divergence times separating pine lineages have been frequently overestimated, with a concomitant underestimation of absolute mutation rates (Krupkin et al. 1996; Dvornyk et al. 2002; Geada López et al. 2002; Brown et al. 2004; Sokol and Williams 2005; Ma et al. 2006). This new perspective supports a relatively recent radiation of extant pine sections in the Early Miocene, a hypothesis that has been proposed (e.g., Miller 1973; Strauss and Doerksen 1990) (but often ignored) to explain the modest morphological and genetic divergence between pine species.

**Sources of Error**

Confidence intervals (CI) for molecular clock estimations must consider the simultaneous uncertainties of rate and time that are confounded within divergences, as well as the age and placement of calibration points. Because fossils document minimum divergence times, the elapsed time from taxon origin to the earliest fossil cannot be known. Additional uncertainty is introduced by variation in deposition, discovery, and the quality of our understanding of the age of...
Table 3
Estimated Divergence Dates for *Pinus* Nodes Shown in Figure 1 Based on Silent Sites (or all sites) from nDNA or Chloroplast DNA. Calibration Is at Node C at 85 or 45 MYA. Ages and Silent Mutation Rates (μ) Are Estimated Using ML Branch Lengths for Data Showing Rate Equality or Using PL for the Nonclocklike 12-Taxon Data Set

<table>
<thead>
<tr>
<th>Data set&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Outgroup</th>
<th>Method</th>
<th>Sites</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>μ (×10^-9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>5 Loci—4 taxa</td>
<td><em>Picea</em></td>
<td>ML</td>
<td>Silent</td>
<td>30</td>
<td>48</td>
<td>85</td>
<td>190</td>
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<td>29</td>
<td>47</td>
<td>85</td>
<td>172</td>
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<td></td>
<td></td>
<td></td>
<td>Silent</td>
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<td>25</td>
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<td>102</td>
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<tr>
<td>9 Loci—4 taxa</td>
<td>Multiple&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ML</td>
<td>Silent</td>
<td>31</td>
<td>48</td>
<td>85</td>
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<td>Silent</td>
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<td>45</td>
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<td>4 loci—4 taxa</td>
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<td>25</td>
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*Note:*—NA, not available.
<sup>a</sup> Data set composition described in text.
<sup>b</sup> OST1, ferritin = Arabidopsis thaliana; LEA-like = Pseudotsuga menziesii.

the source formation as well as the taxonomic affinity of the fossil. There are promising approaches for estimating CI for divergence times (e.g., Kumar et al. 2005; Yang and Rannala 2006), but these will require multiple robust fossil calibrations for *Pinus*. In addition, molecular divergence rates may vary across the genome and across lineages. We addressed this complex problem using several approaches to provide a perspective on the relative impact of various factors on the error ranges of our rate estimates.

First, both of the fossils selected for calibration lack radiometric dating. Because midpoint ages of the ranges for geological Epochs or Stages were used to create the chronograms (figs. 1 and 2 and table 3), we recalculated divergence dates using the upper and lower bounds of these periods. The stratigraphic age ranges for wood from the Santonian *Pinus* sp. (Meijer 2000) and leaves from the Middle Eocene *P. similkameenensis* (Phipps et al. 1995) are currently considered to be 83.5–85.8 MYA, and 37.2–48.6 MYA, respectively (Gradstein and Ogg 2004). The inferred age ranges for node D (cf. table 3), using these stratigraphic ranges to calculate at node C, are 187–192 MYA and 83–109 MYA for calibrations based on wood and leaves, respectively. Inferred age ranges for node A are 29.6–30.4 and 13–17 MYA for calibrations based on wood and leaves, respectively, whereas node B ages are 47–48 and 21–27 MYA, respectively. Clearly, the use of midpoint ages introduces uncertainty in our estimates, particularly for Eocene fossils because that Epoch is much longer than the Santonian (11.4 vs. 2.3 Myr). Nonetheless, this uncertainty is small relative to the differences in alternative calibration points suggested by fossil wood (85 MYA) versus fossil leaves (45 MYA) for the origin of *Pinus* subgenera.

Second, the use of the mean substitution rates from multiple loci reduces stochastic deviation resulting from rate variation among genomic regions, and limiting the sample to silent sites reduces the potential for selection to influence rates. To assess the amplitude of this variation among our 9 nuclear loci, we also calculated absolute substitution rates using the mean dS plus and minus 1 standard deviation (SD). This results in μ = 0.43–0.96 × 10^-9 for a calibration based on wood and 0.81–1.81 × 10^-9 for leaves (cf. table 2). We also projected the “combined” effects of locus-specific rate variation (mean dS plus 1 SD and the upper bound for the stratigraphy; dS minus 1 SD and the lower bound) for each calibration. This results in “inclusive” ranges of μ = 0.42–0.98 × 10^-9 for a calibration based on wood and 0.75–2.2 × 10^-9 for leaves (cf. table 2). Among the 9 loci that were used for rate calculation, distortion may have been introduced by our use of different outgroups for ferritin, LEA-like, and OST1 (see table S2, Supplementary Material online for details). Further, PALI silent sites showed a significant departure from rate equivalence even with *Picea* as the outgroup. However, ages and rates inferred from the 5- and 9-locus data sets (table 3) are largely consistent. Further, data accumulated for 50 *Pinus* loci (Cronn R, unpublished data) show a median silent rate that is very similar to the silent rate described in these results. This implies that despite locus-specific rate variation, our 5-locus data set is useful as a first approximation of μ in *Pinus*.

Finally, we derived CIs for age estimates using the method of Haubold and Wiehe (2001), which uses nonoverlapping pairs of phylogenetic distances to infer the unknown mutation rate for the other pair. Using this method at node A (fig. 1b), with subg. *Strobus* as the reference and ages calculated from the 85 MYA crown calibration, yields 95% CIs of 14–67 MYA for the divergence of subg. *Pinus*. Applying this technique to node B (with subg. *Pinus* as the reference) yields intervals of 22–106 MYA for subg. *Strobus*. The magnitude of these intervals far exceeds error ranges introduced by stratigraphic ranges, intralocus rate variation, or even fossil choice between per-mineralized wood (85 MYA) or leaves (45 MYA). These large uncertainties clearly highlight the challenge inherent...
in the simultaneous uncertainties of rate and time over evolutionary time scales.

Although the magnitude of these sources of error highlight the importance of considering molecular rate variation and stratigraphic uncertainty, past calibration scenarios applied to Pinus show that incorrect fossil assignment can be a far more dramatic source of error. For example, the common practice of using *P. belgica* to calibrate the divergence of sections within subg. *Pinus* (node A, fig. 3e) pushes the divergence of pine subgenera (node C) to 339 MYA and the divergence of *Pinus–Picea* (node D) to 758 MYA in the Precambrian. The attendant silent mutation rate in the nuclear genome is exceptionally slow (\(\mu = 0.19 \times 10^{-9}\)). This unrealistic (but commonly cited) calibration is clearly responsible for many of the exceptionally low \(\mu\) values reported for pine nDNA and cpDNA (Krupkin et al. 1996; Dvornyk et al. 2002; Geada López et al. 2002; Brown et al. 2004; Ma et al. 2006). This calibration can also cause substantial distortion in biogeographical interpretations. For example, Eckert and Hall (2006) recently hypothesized dispersal and vicariance events using *P. belgica* to calibrate the divergence of sections within subg. *Pinus* (node A, fig. 3f) in a cpDNA data set. In doing so, they fail to consider the unrealistic estimate that this calibration indicates for the divergence of *Pinus* and *Picea* (i.e., 720 MYA) based on our clock-like cpDNA branch lengths (fig. 3f). In the same manner, associating *P. belgica* with the divergence of pine subgenera also conflicts with the fossil record. Such a calibration (e.g., 136 MYA, Sokol and Williams 2005; 130 MYA, Ma et al. 2006) indicates a *Pinus–Picea* split of approximately 300 MYA (fig. 3d) and produces absolute silent mutation rates of \(\mu = 0.47–0.49 \times 10^{-9}\). These projected ages for *Pinus* (Carboniferous or Early Permian, respectively) predate *P. belgica* by more than 150 Myr. In summary, the errors resulting from incorrect fossil/node association far exceed the cumulative error estimated with CIs that account for the confounding effect of simultaneous rate and time variation. In contrast, using *P. belgica* to represent the divergence of *Pinus* and *Picea* in our clock-like data set (node D, fig. 3c; Wang et al. 2000; García-Gil et al. 2003) places the divergence of *Pinus* subgenera at 63 MYA. This estimate lies between the earliest putative subg. *Strobus* wood and leaf fossils.

Implications of a Moderate Tempo for Pine Mutation Rates

Several authors have argued that a high mutation rate is necessary to account for the extremely high level of inbreeding depression found in conifer species capable of partial self-fertilization (Lande et al. 1994; summarized in Scofield and Schultz 2006). Because plants do not segregate a germ line, somatic mutations may accumulate in meristems and be incorporated into gametes. Hence, longevity and large stature contribute to a large number of mitoses, both of which may serve to elevate the per-generation mutation rate (Scofield and Schultz 2006). Evidence for a high per-generation mutation rate in *Pinus* compared with other plant groups is provided by observations of the frequency of chlorophyll-deficient mutants. Assuming an equal number of loci capable of mutating to chlorophyll deficiency and equivalent lengths across loci, the per-generation rate of deleterious mutations for *Pinus sylvestris* (\(U = 1–3 \times 10^{-12}\); Kärkkäinen et al. 1996) is comparable to the mutation rate for *Rhizophora mangle*, another long-lived woody perennial (\(U = 1.5 \times 10^{-7}\); Klekowski and Godfrey 1989). These mutation rates are approximately 100-fold higher than the average for 10 annual flowering plant species (\(U = 1–3 \times 10^{-13}\); Klekowski 1992).

Mutation rates in *Pinus* can be evaluated by comparing absolute rates derived from fossil calibrations in other plant groups. Our 85 MYA calibration provides an estimate for nDNA (\(\mu = 0.70 \times 10^{-9}\) synonymous substitutions-site \(^{-1}\)-year \(^{-1}\); table 2) that is 14-fold higher than previous *Pinus* estimates (Dvornyk et al. 2002), but approximately 4-fold slower than the rate for palms (\(\mu = 2.61 \times 10^{-9}\); Gaut et al. 1996) and 7- to 40-fold slower than the range reported for herbaceous angiosperms (\(\mu = 5–33 \times 10^{-9}\); Gaut et al. 1996; Koch et al. 2000; Clark et al. 2005). Similarly, our estimated rates for chloroplast silent sites (\(\mu = 0.22 \times 10^{-9}\) synonymous substitutions-site \(^{-1}\)-year \(^{-1}\); table 2) are approximately 6-fold slower than those reported for angiosperm cpDNA (\(\mu = 1.1–1.6 \times 10^{-9}\); Wolfe et al. 1987). Expressed on a per-year basis, these comparisons
suggest that substitution rates in *Pinus* are still far slower than most angiosperms. In this context, it is important to note that molecular substitution rates can also show a "generation-time effect" (e.g., Gaut et al. 1992, 1996; Kay et al. 2006). Based on our estimate of the average rate \( (\mu = 0.70 \times 10^{-9} \text{ synonymous substitutions-site}^{-1} \text{year}^{-1}) \) and correcting for longevity (assuming a 25-year generation time; Brown et al. 2004), the per-generation nuclear substitution rate for *Pinus* averages \( 1.75 \times 10^{-8} \) substitutions-site\(^{-1}\) generation\(^{-1}\). This rate is nearly equivalent to the \( 1.5 \times 10^{-8} \) substitutions-site\(^{-1}\) year\(^{-1}\) rate inferred for short-lived Brassicaceae (Koch et al. 2000). The near-equivalence between these values closes the gap between gymnosperm and angiosperm rates relative to prior rate estimates (e.g., Dvornyk et al. 2002; Brown et al. 2004), but they still fall short of the 100-fold difference that might be expected based on chlorophyll-deficiency mutations.

Clearly, these calculations hinge on an appropriate association between a calibration time and a phylogenetic node; the choice of a node with which to associate a fossil is the largest determinant of the rate. The availability of *Pinus* fossils with synapomorphies supporting the divergence of *Pinus* subgenera (a well-supported node in a molecular phylogeny) is more robust than options available for many plant groups, and we suggest that this will be an important factor for future, more refined, rate comparisons. This study illustrates the variability of divergence rates among loci because \( dS \) for 9 nuclear loci ranges from 0.063 to 0.205 (table 1). Even though fossil dates were used as fixed calibration points in our calculations, each fossil represents a "minimum" age for a lineage. It follows then that the rates calculated from mean \( dS \) \( (\mu = 0.70-1.31 \times 10^{-9} \text{ substitutions-site}^{-1} \text{year}^{-1}) \); table 2) represent "maximum" absolute rates, although the same caveat applies to angiosperm rates used for comparison.

Our revised estimates of mutation rates in pines have important implications for studies of population and molecular genetic parameters. For example, an effective population size \( (N_e) \) of \( 5.6 \times 10^5 \text{ was calculated for } P. \text{taeda using } 19 \text{ loci and a substitution rate } (\mu = 1.17 \times 10^{-10}; \text{ estimated by calibrating divergence between members of section } Trifoliae \text{ with the age of } P. \text{belgica; Brown et al. 2004). If we apply our estimate of } \mu = 0.70 \times 10^{-9} \text{ (calibrated at } 85 \text{ MYA between the subgenera; table 2), we find that the predicted } N_e \text{ for } P. \text{taeda is far lower at } 9.4 \times 10^4 \text{. This value of } N_e \text{ contrasts sharply with census population estimates for } P. \text{taeda (which may exceed } 10^{10}; \text{ Brown et al. 2004), and it suggests that this species has experienced dramatic population growth following a relatively recent genetic bottleneck in the history of this species.}

**Fig. 3.—Application of some recently published fossil calibration scenarios (see Discussion), using clock-enforced branch lengths (shown in fig. 1) from our nDNA or cpDNA data sets. ML-projected ages are shown below each node; ML-calculated silent rates are given for each scenario. Node letters correspond to figure 1.**
**Pinus** Fossil Status

Despite the diversity and abundance of *Pinus* fossils (see Millar 1998), several obstacles need to be overcome before integrating additional fossils into a multipoint calibration. To date, pine fossil reports have been based on unattached organs (ovulate or pollen cones, leaves, or wood fragments). Two of these organs have yet to be found on a contiguous fossil, although species have been named based on the hypothesized common origin of separate organs. Retention of ancestral character states and homoplasy among extant *Pinus* species are sufficiently frequent that characters from a single organ are inadequate for discriminating among extant pine subsections or sections (Gernandt et al. 2005). Because *Pinus* subgenera are diagnosable by leaf vasculature (Gernandt et al. 2005) and by a combination of wood features (Van der Burgh 1973), this key divergence event provides the most reliable calibration point for *Pinus*.

Historically, fossil descriptions used a typological approach for assigning affiliations with extant taxa. This process is complicated by historical revisions of *Pinus* sectional affiliations, and it lacks the necessary phylogenetic framework for making assignments. As previously noted, the number of fibrovascular bundles per leaf is the only nonhomoplasic character known to date that distinguishes *Pinus* subgenera (Gernandt et al. 2005). However, the presence of a single fibrovascular bundle at the base of a leaf fossil may not be diagnostic for the subgenus because bundles branch distally into the 2 bundles characteristic of subg. *Pinus* (Stockey and Nishida 1986). Based on this information, *Pinus* sp. leaf fossils from Staten Island, New York (Jeffrey 1908) are now thought to represent subg. *Pinus* rather than subg. *Strobos*. Similarly, Stockey and Nishida (1986) suggest that Cretaceous *P. yeoensis* leaf fossils from Japan (Stopes and Kershaw 1910), also once believed to represent subg. *Strobos*, are more representative of *Cedrus*.

Although needles are informative within *Pinus*, ovulate cones are considered the most dependable evidence for assigning fossils to the genus. Miller (1976) outlined 4 characteristics that together define *Pinus*: inflated scale apex, bract and scale traces united at origin, all resin canals abaxial to vascular tissue in scale base, and scale strands curved on adaxial side. Critically, members of the extinct cone genus *Pityostrobus* can share at least 2 of these features, complicating the identification of Cretaceous pine ovulate cones. Indeed, using Miller’s criteria, some early “pine” fossils have been reassigned to *Pityostrobus*, *Picea*, or *Cedrus*. For example, Miller and Malinky (1986) referred cone scales from the Magorthy Formation of Delaware, originally described as representing subg. *Strobos* (Penny 1947) to *Pityostrobus*. Because many fossil cones are found in marine deposits, abrasion during transport can remove important scale features (Smith and Stockey 2002), misleading attempts at classification (Wolfe and Schorn 1989).

**Evidence for an Eocene Divergence of Subgenera**

Some authors have suggested a relative recent origin for subg. *Strobos* in the Eocene (Miller 1973; Phipps et al. 1995; Kossack and Kinlaw 1999), as reflected by our 45 MYA crown calibration. This hypothesis is based on numerous ovulate cone fossils assigned to subg. *Strobos* that appear in the fossil record starting approximately 40 MYA (Millar 1998), closely following the oldest known leaves attributable to subg. *Strobos* (45 MYA). Unattached leaves (with subg. *Strobos* features), deposited near ovulate cones (with subg. *Pinus* features), in the Princeton Chert in British Columbia were originally described as 2 species: *P. similicamerenensis* and *P. arnoldii* (Miller 1973). More recently, Phipps et al. (1995) reinterpreted these fossils as a single species (*P. similicamerenensis*) and proposed that the mosaic of characters in this hypothetical species may represent a lineage that predates the divergence of subg. *Strobos*. These authors did note that alternative explanations (e.g., organs derived from a lineage with no extant descendent or from sympatric pine species) cannot be ruled out.

A 45-MYA calibration also shows surprising agreement with a recent cpDNA-based estimate of seed plant divergence times using entirely different calibration sources (Magallón and Sanderson 2005). In that analysis, the divergence of Gnetophytes and Pinaceae was constrained with a fossil date of 216 MYA; estimated divergence dates for *Pinus* subgenera (ca. 50 MYA) and synonymous cpDNA substitution rates (\( \mu = 0.26 \times 10^{-9} \)) nearly match our results. Because fossils represent minimum ages, calibration at the divergence of *Pinus* subgenera with 45-MYA leaf fossils appears to provide a reasonable upper bound for this event.

**Evidence for a Cretaceous Divergence of Subgenera**

The description of a *Pinuxylon* sp. from the Aachen (Meijer 2000) may push the age of subg. *Strobos* more than 40 Myr before the oldest leaf and cone fossils, but this is not a simple determination. The cone genus *Pityostrobus* was abundant and diverse during the Cretaceous, but associated wood remains undescibed and its exact relationship to *Pinus* is unresolved (Smith and Stockey 2002). Further, the 2 characters used to affiliate the Aachen *Pinuxylon* wood with subg. *Strobos* (cross-field pitting and ray tracheid dentations) are not unequivocal synapomorphies (data not shown), even in extant pine species, indicating the possibility of retention of ancestral polymorphism or parallel evolution. Both of these tracheid features are quantitative traits for which intraspecies variation has been noted (Shaw 1914; Hudson 1960; Van der Burgh 1973) and for which the ancestral state is unclear (Hart 1987). Perhaps most challenging is that diagnosis of subg. *Strobos* fossil wood is made by determining that a specimen has “weak-to-absent” dentations. In this context, the description of the Aachen *Pinuxylon* sp. as possessing “ray tracheids faintly dentate but owing to rather poor state of preservation only locally visible” (Meijer 2000) may best be considered tantalizing evidence for the existence of subg. *Strobos* in the Cretaceous, but evidence requiring corroboration. The evidence for subg. *Strobos*—like wood in the Cretaceous is bolstered by fossil wood of about the same age that shows similarities to modern-day members of subg. *Pinus* (Blackwell 1984), hence providing wood fossils flanking the crown node. Although the possibility that the subg. *Pinus*—like fossils may simply be the ancestral state in
Pinaceae cannot be excluded, they do lend credence to the hypothesis that the Aachen *Pinuxylon* represents *Pinus* rather than another member of the Pinaceae.

The 45-MYA calibration (even when viewed as a minimum age) suggests a more recent *Pinus–Picea* split (102–87 MYA; fig. 1) than is indicated by the existence of *P. belgica* at approximately 135 MYA (Miller 1976). However, there are unresolved issues concerning the age of the genus. First, the approximately 35-Myr gap between *P. belgica* and the next *Pinus* fossil, combined with uncertainties about the source of *P. belgica*, deserves note. Second, the only other evidence for an “Early” Cretaceous origin for *Pinus* is pollen from Alaska (Langenheim et al. 1960). The age of the Kuk River fossil formation that is the source for these reported 145–100 MYA *Pinus* microfossils is now estimated to be the Mid- to Late Albian (104–97 MYA; Koteja and Poinar 2001). In addition, Erwin and Schorn (2006) recommend caution when relying on pollen without associated megafossils due to the unknown status of pollen associated with seed cone genera such as *Pityostrobus*. If one were to discount the inference of an “Early” Cretaceous age for *P. belgica* and the previous estimate for the age of the Alaskan pollen, the oldest evidence for *Pinus* would be ovulate cones from the Albian–Cenomanian (ca. 103 MYA; Fliche 1896). This does not appear to fit with the 190–164 MYA *Pinus–Picea* split implied by a Cretaceous divergence of the subgenera (fig. 1). On the other hand, the earlier age estimate for our node D (fig. 1) that results from the 85 MYA calibration appears consistent with an older divergence of *Pinus* and *Picea* because there is support for an early independent divergence of these 2 genera from the *Pityostrobus* grade (Smith and Stockey 2002). The 85 MYA calibration also agrees with a study of Pinaceae genera, which calibrated the *Pinus–Picea* split at 140 MYA, yielding an estimated age of approximately 84 MYA for the divergence of *Pinus* subgenera (Wang et al. 2000).

Perhaps the strongest evidence that subg. *Strobus* diverged in the Cretaceous are the numerous Oligocene (ca. 26 MYA) fossils that potentially represent recent lineages. Oligocene ovulate cone fossils have been affiliated with 6 different *Pinus* subsect.: *Pinaster* and *Pinus* in section *Pinus* (Mai 1986; Erwin and Schorn 2006); *Ponderosae* in section *Trifoliae* (Wolfe and Schorn 1989); *Balfourianae* and *Cembroides* in section *Parrya* (Wolfe and Schorn 1989); and *Strobus* in section *Quinquefoliae* (Mai 1986). Existence of these subsections approximately 26 MYA is in better agreement with our predicted age of these groups. These subsections approximately 26 MYA are in better agreement with our predicted age of these groups. Existence of these subsections approximately 26 MYA is in better agreement with our predicted age of these groups. Existence of these subsections approximately 26 MYA is in better agreement with our predicted age of these groups.
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Spencer Muse, Associate Editor

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