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## An overview of extant conifer evolution from the perspective of the fossil record

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# An overview of extant conifer evolution from the perspective of the fossil record

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**PREMISE OF THE STUDY:** Conifers are an important living seed plant lineage with an extensive fossil record spanning more than 300 million years. The group therefore provides an excellent opportunity to explore congruence and conflict between dated molecular phylogenies and the fossil record.

**METHODS:** We surveyed the current state of knowledge in conifer phylogenetics to present a new time-calibrated molecular tree that samples ~90% of extant species diversity. We compared phylogenetic relationships and estimated divergence ages in this new phylogeny with the paleobotanical record, focusing on clades that are species-rich and well known from fossils.

**KEY RESULTS:** Molecular topologies and estimated divergence ages largely agree with the fossil record in Cupressaceae, conflict with it in Araucariaceae, and are ambiguous in Pinaceae and Podocarpaceae. Molecular phylogenies provide insights into some fundamental questions in conifer evolution, such as the origin of their seed cones, but using them to reconstruct the evolutionary history of specific traits can be challenging.

**CONCLUSIONS:** Molecular phylogenies are useful for answering deep questions in conifer evolution if they depend on understanding relationships among extant lineages. Because of extinction, however, molecular datasets poorly sample diversity from periods much earlier than the Late Cretaceous. This fundamentally limits their utility for understanding deep patterns of character evolution and resolving the overall pattern of conifer phylogeny.

**KEY WORDS** fossil calibration; molecular dating; paleobotany; seed cone evolution.

Conifers are the most diverse and widespread group of extant gymnosperms, with ~615 species and a global distribution (Farjon, 2010; Farjon and Filer, 2013). Although vastly outnumbered by angiosperm species, conifers are a major component of woody biomass in many temperate and boreal forests in the Northern Hemisphere and in many tropical montane forests as well (Enright and Hill, 1995; Eckenwalder, 2009). From the Late Carboniferous onward (Hernandez-Castillo et al., 2001; Plotnick et al., 2009), conifers have been an important component of terrestrial ecosystems, and they have one of the longest and best-known fossil records of any seed plant group (see Brodribb and Hill, 1999; Taylor et al., 2009). Given their diversity and ecological importance, understanding conifer evolution has been a long-standing goal of neobotanical and paleobotanical research (e.g., Florin, 1938; Miller, 1977; Rothwell et al., 2005).

As with other organisms, molecular phylogenetics has reshaped our understanding of conifer evolution (e.g., Bowe et al., 2000;

Chaw et al., 2000; Rai et al., 2008). Many parts of the conifer tree remain difficult to resolve, especially among closely related species (e.g., Gernandt et al., 2001, 2009; Campbell et al., 2005; Parks et al., 2012; Gaudeul et al., 2012; Ruhsam et al., 2015), but results from molecular datasets are consistent with respect to backbone relationships among major extant conifer clades (Stefanović et al., 1998; Gugerli et al., 2001; Rai et al., 2008; Leslie et al., 2012; Wickett et al., 2014). Some analyses have also used fossil-calibrated molecular clocks to estimate divergence ages in various conifer clades (Wang et al., 2000; Gernandt et al., 2008; Biffin et al., 2012; Leslie et al., 2012; Mao et al., 2012), and more recent dating techniques promise better (or at least new; see Bapst et al., 2016) ways in which to integrate fossils into molecular phylogenies by analyzing morphological and molecular data together in a single model (Ronquist et al., 2012; Heath et al., 2014; O'Reilly et al., 2015; Zhang et al., 2015). These techniques are already being incorporated into analyses that

include fossil plants (Grimm et al., 2014; Renner et al., 2016; Saladin et al., 2017) and are likely to become widespread in future analyses of conifers.

In order to provide context for future studies seeking to integrate fossils with molecular data, we survey the current landscape of conifer molecular phylogenetics in light of the fossil record. Our aim is not to provide an in-depth discussion of all published conifer phylogenies or an exhaustive compilation of conifer fossils, but rather to give a broad overview of apparent instances of congruence and conflict between evidence from fossils and molecules, as well as to identify areas where their integration could improve our understanding of conifer evolution. We use a new time-calibrated tree that updates previous work from our group (Leslie et al., 2012) to focus our discussion, which centers on family-level conifer clades that have a good fossil record. Within these clades, we specifically ask whether molecular tree topology and estimated divergence dates for major genera and subclades are congruent with the known fossil record. Finally, we discuss some of the inherent limitations of a molecular approach to study conifer history, using the evolution of seed cones as a case study.

## MATERIALS AND METHODS

### Estimating conifer divergence times

The phylogeny used in this study expands on the phylogeny of Leslie et al. (2012) by including more species and additional fossil calibration points. In particular, this analysis improves taxon sampling in the previously undersampled genera *Abies* Mill. (increased from 26 to 55 spp.), *Callitris* Vent. (from 4 to 10 spp.), and *Podocarpus* L'Her. ex Pers. (from 58 to 74 spp.). We also expanded sampling for *Agathis* Salisb. (increased from 13 to 16 spp.), *Cupressus* L. and *Hesperocyparis* Bartel et R.A. Price (from 7 to 11 spp.), *Picea* A. Dietr. (from 32 to 35 spp.), *Pinus* L. (from 102 to 116 spp.), and *Prumnopitys* Phil. (from 5 to 8 spp.). The new phylogeny includes 578 species, or ~90% of recognized extant diversity (88% if based on the more conservative taxonomy of Farjon [2010], which does not recognize some species in the current tree). The current phylogeny is based on sequences from two chloroplast genes (*rbcL*, *matK*) and one nuclear ribosomal gene (18S); our previous phylogeny used four genes (*rbcL*, *matK*, *PHYP*, 18S), but *PHYP* was dropped from this study in order to assure more even gene sampling across clades.

We primarily assembled sequence data from GenBank using the PHyLogeny Assembly With Databases pipeline (PHLAWD, Smith et al., 2009). PHLAWD uses a "baited" sequence comparison approach, where a small subset of sequences for a clade of interest are provided by the user, which are then used to filter GenBank sequences and to determine whether these sequences are homologous to the gene regions of interest. We cleaned the initial PHLAWD data using preliminary phylogenetic analyses and then combined them with unpublished sequences to fill significant gaps, particularly from *Abies*. New *matK* and *rbcL* sequences were also obtained according to Cronn et al. (2008). In total, the dataset contains sequences from 578 conifer taxa and three cycad species (*Cycas micronesica* K.D. Hill, *Encephalartos lehmanii* Lehm., *Zamia furfuracea* L.f.) as outgroups, with 18S sequences from 126 taxa, *rbcL* sequences from 557 taxa, and *matK* sequences from 565 taxa (Appendix S1; see Supplemental Data with this article). We estimated initial sequence alignments using MAFFT version 6 (Kato and Toh, 2008)

and profile alignments using MUSCLE version 3.6 (Edgar, 2004). The concatenated sequence matrix contained 4523 sites, and we performed a maximum likelihood (ML) phylogenetic analysis of this alignment using RAxML version 7.2.6 (Stamatakis, 2006). We conducted tree searches under the GTR+CAT approximation of rate heterogeneity, partitioned by gene region.

We estimated divergence times in the phylogeny using Markov chain Monte Carlo (MCMC) methods implemented in BEAST version 1.7.5 (Drummond and Rambaut, 2007). For the BEAST analysis, we applied a separate GTR+ $\Gamma$  substitution model to the ribosomal and chloroplast genes and constrained the topology to reflect the optimum ML tree from RAxML. We used this tree as a fixed constraint because the goal of this analysis was to establish a consistent temporal framework for conifer divergence ages, not to refine or test conifer phylogenetic relationships, and the optimum RAxML tree resolved the same phylogenetic relationships among family-level and genus-level clades as previous studies. To assess support for nodes in this tree, we conducted a bootstrap analysis with the number of bootstrap replicates determined by the MRE-based stopping criterion (Pattengale et al., 2010).

We used 26 fossil calibrations as minimum divergence ages in the BEAST analysis, which were associated with log-normal prior age distributions in most cases (see below and Appendix S2). To overcome the problem of inferring a zero probability during the initial parameter search in BEAST, the branch lengths of the constraint tree were smoothed to time in treePL (Smith and O'Meara, 2012) using the minimum age of each fossil calibration point. We then ran three independent MCMC runs of 100 million generations, sampling every 1000th generation. To ensure that the posterior distribution of branch lengths came from the target distribution, we used Tracer version 1.5 to assess convergence and proper sampling of the likelihood surface (effective sample size >200), with the first 25 million generations discarded as burn-in for each run. We then sampled every 10,000th tree from the post-burn-in chains and combined all chains using LogCombiner version 1.7.5. The final tree, summarized with TreeAnnotator, represents the maximum clade credibility tree with the consensus ages being the median estimate.

### Fossil calibrations

In our previous study (Leslie et al., 2012), we used calibration fossils conservatively; we only included fossils of reproductive organs that shared unambiguous apomorphies with extant clades (see "best practices" of Parham et al., 2011), and in all cases we treated fossils as stem members of extant genera. We often followed the same approach here (13 of the 26 calibration fossils are used in the same way as Leslie et al., 2012), but we have added additional fossils in an effort to increase coverage and to create a more even phylogenetic and temporal distribution of calibrated nodes. For example, we have added four additional stem calibrations (for *Agathis*, *Dacrydium* Sol. ex Lamb., *Retrophyllum* C.N. Page, and *Abies*) and two calibrations that date infra-generic splits in *Pinus*, all following the same standards as in Leslie et al. (2012) where the fossils share either key vegetative or reproductive synapomorphies with extant clades (see Appendix S2).

Such a strict approach not only limits the potential number of calibration fossils, but may also bias analyses toward estimating younger divergence ages because most of the calibration fossils will be relatively young taxa that share obvious features with extant genera. Our previous results, in particular, have been suggested to

be underestimates (see Wilf and Escapa, 2015), and we have therefore specifically included a wider range of fossils in this study. For example, we now include seven additional fossils that date infrageneric splits but that lack strong synapomorphies (including within *Araucaria* Juss., *Dacrycarpus* (Endl.) de Laub., *Podocarpus*, *Juniperus* L., and three in *Pinus*). While these fossils clearly belong to these genera, they possess suites of characters that are suggestive of affinities with specific crown subclades rather than exhibiting unambiguous synapomorphies for them (which may not even exist for these subclades). We place these fossils within crown genera following the suggestions of the original authors, but we recognize that they do not have as strong support as the other calibration fossils (see Appendix S2).

Many of the calibration fossils occur in formations that have been radiometrically dated, and these ages are used in the study as minimum ages for the calibrated node. Other fossils are known only to the geologic epoch or age, and in these cases, we generally used the youngest boundary of the interval in which they occur in order to set the minimum age of the calibration. In cases where the age of the fossil was poorly constrained, we also extended the confidence intervals of the log-normal prior age distribution to encompass the possibility that the fossil was considerably older than the minimum age (see Appendix S2).

## RESULTS

### Topology

Our concatenated alignment consisted of 1686 sites (672 were informative) from the nuclear 18S gene and 2087 sites from our combined chloroplast alignment of *matK* and *rbcL* (2087 were informative). The inferred topology of the tree (Fig. 1; see also full tree in Appendix S3) is similar to those of other conifer-wide molecular analyses (e.g., Stefanović et al., 1998; Gugerli et al., 2001; Rai et al., 2008). The tree has good (>90%) bootstrap support for nearly all ordinal, family, and genus-level clades, which are the main focal areas of this study, as well as for major splits within genera (Appendix S4). Although our tree topology is similar by design to those of previous studies, we briefly summarize them in the following paragraphs.

Pinaceae are monophyletic and sister to a large “cupressophyte” (Cantino et al., 2007) or “conifer II” clade (Ran et al., 2010). Within cupressophytes, the primarily Southern Hemisphere families Araucariaceae and Podocarpaceae are sister groups and form the Araucariales clade. This clade is, in turn, sister to a clade referred to here as the Cupressales, consisting of the monotypic genus *Sciadopitys* Siebold et Zucc. (Sciadopityaceae) and the Taxaceae and Cupressaceae. Within Cupressales, *Sciadopitys* is sister to a clade composed of the Cupressaceae and the Taxaceae (including *Cephalotaxus* Siebold et Zucc. ex Endl., a genus sometimes treated as its own separate family).

Within the major conifer clades, the basic relationships that we recover are also largely consistent with those of previous studies. In Pinaceae, molecular data consistently support a deep split between pinoid genera and abietoid genera (Wang et al., 2000; Gernandt et al., 2008; Gernandt et al., 2016), although the position of the *Cedrus* Mill. lineage can vary; in our analysis, *Cedrus* is sister to the other abietoid genera (see Appendix S3). Within the Araucariales clades, our data generally support a broad split in Podocarpaceae between a primarily southern temperate “prumnopityoid” clade

(here used in the sense of Knopf et al. [2012], although this clade is not resolved in all studies; see Biffin et al., 2011) and a larger, “tropical clade” (here used in a modified sense from Kelch, 1998). Within Araucariaceae, our data resolve a basic split between *Araucaria* and an “agathoid” clade consisting of *Agathis* and *Wollemia* Jones, Hill, et Allen; this result is consistent with other studies using multiple genes (Rai et al., 2008; Mei, 2010) or using both molecules and morphology (Escapa and Catalano, 2013).

Within the Cupressales clades, our results and those of other studies support a Taxaceae clade composed of *Cephalotaxus* sister to the traditional “core” genera of Taxaceae such as *Amentotaxus* Pilg., *Taxus* L., and *Torreya* Arn. (see also Cheng et al., 2000; Elpe et al., 2017). Within Cupressaceae, molecular data resolve a paraphyletic grade of lineages (the “taxodiaceous Cupressaceae”) with a generally consistent topology (Gadek et al., 2000; Kusumi et al., 2000; Mao et al., 2012; Yang et al., 2012) that we discuss in more detail in subsequent sections. Finally, previous studies have shown that derived Cupressaceae consist of a pair of clades, including a Northern Hemisphere cupressoid clade and a Southern Hemisphere callitroid clade (Mao et al., 2012), which we also recover.

### Estimated divergence ages

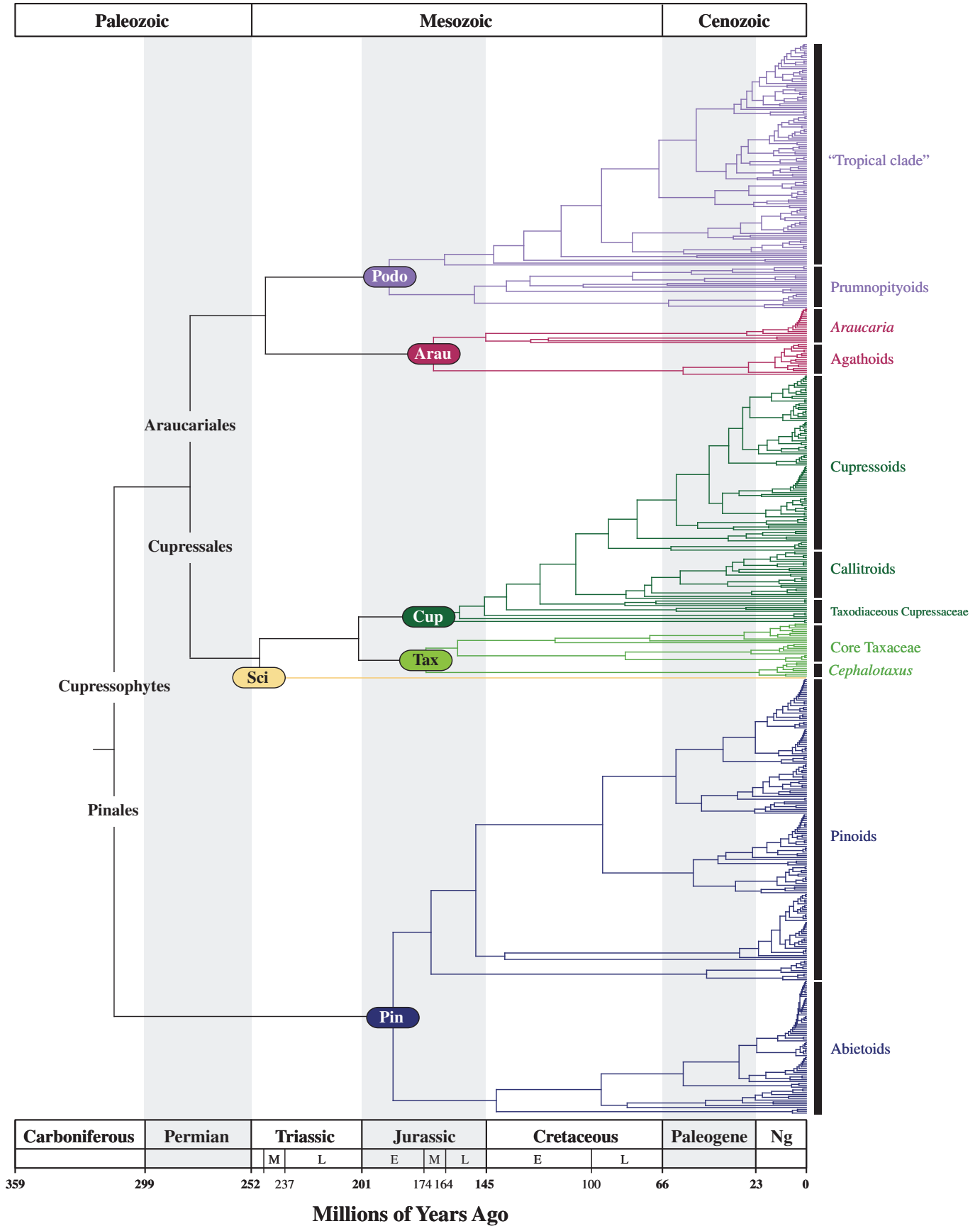
Our analysis estimates late Paleozoic to early Mesozoic divergence ages for the extant ordinal and family-level conifer clades, with initial crown splits in families occurring between 190 and 160 mya in the Early to Middle Jurassic (Fig. 1; see the same figure for absolute ages associated with the geologic periods mentioned throughout the text), although confidence intervals on all these nodes are wide and generally span the Jurassic (Appendices S3 and S5). Among extant genera, mean estimated divergence ages are concentrated in the Late Cretaceous through the early Cenozoic, with the mean age of most initial infrageneric crown splits occurring in the later half of the Cenozoic (Fig. 2A). Most extant conifer species richness is therefore estimated to be of relatively recent origin, appearing over the Oligocene and Neogene, which is consistent with our previous study (Leslie et al., 2012). The widths of the 95% highest posterior density (HPD) intervals on these age estimates generally increase with estimated node age (Fig. 2B). HPD intervals for Neogene nodes average 9 million years, and those for Paleogene, Cretaceous, Jurassic, and earlier nodes average 26, 50, 57, and 49 million years, respectively.

The use of additional calibration fossils in the current analysis, compared to Leslie et al. (2012), did not substantially alter the overall distribution of estimated mean species divergence ages (Fig. 2C), although it did result in consistently older mean estimated ages of genera and initial infrageneric crown splits, particularly in the Araucariaceae, Podocarpaceae, and Taxaceae clades (Fig. 2D; see below for a discussion of the unusually wide variation in the Araucariaceae).

## DISCUSSION

### Estimated divergence ages for conifers

Previous estimates of divergence ages in conifers have varied widely (see Gernandt et al., 2008; Biffin et al., 2012; Leslie et al., 2012; Mao et al., 2012), and, as in other groups, these ages depend heavily on the specific calibration fossils and dating methodologies employed



(see Sauquet et al., 2011; Wilf and Escapa, 2015; Saladin et al., 2017). It is often difficult to make direct comparisons among different dating studies because they can differ in their focal clades and evolutionary scope, but our mean estimated divergence ages for genera generally fall between those of other comparable conifer analyses (Fig. 3). In particular, our estimated divergence ages are between those of studies that used calibration fossils less conservatively than our study (i.e., the “liberal” analysis of Gernandt et al., 2008) and those that use a smaller sample of well-placed fossils exhibiting clear synapomorphies (i.e., the “conservative” analysis of Gernandt et al., 2008). In the following sections, we ask how well our estimated divergence ages accord with current knowledge of the fossil record.

### Congruence and conflict in molecular and fossil data

Broadly speaking, estimated divergence ages for the deepest splits within and among the ordinal and family-level conifer clades in our tree tend to be older than might be expected from the fossil record, while estimates of species divergence ages within genera are often very young, typically concentrated in the last several million years. Within the context of these general patterns, however, specific conifer clades show greater or lesser degrees of concordance between the fossil record and estimated molecular divergence dates, which we discuss in more detail in the following sections. We focus our discussion on backbone splits and divergences among genera within family-level clades, because these higher-level clades are much more likely than individual species divergences to be recorded in the fossil record.

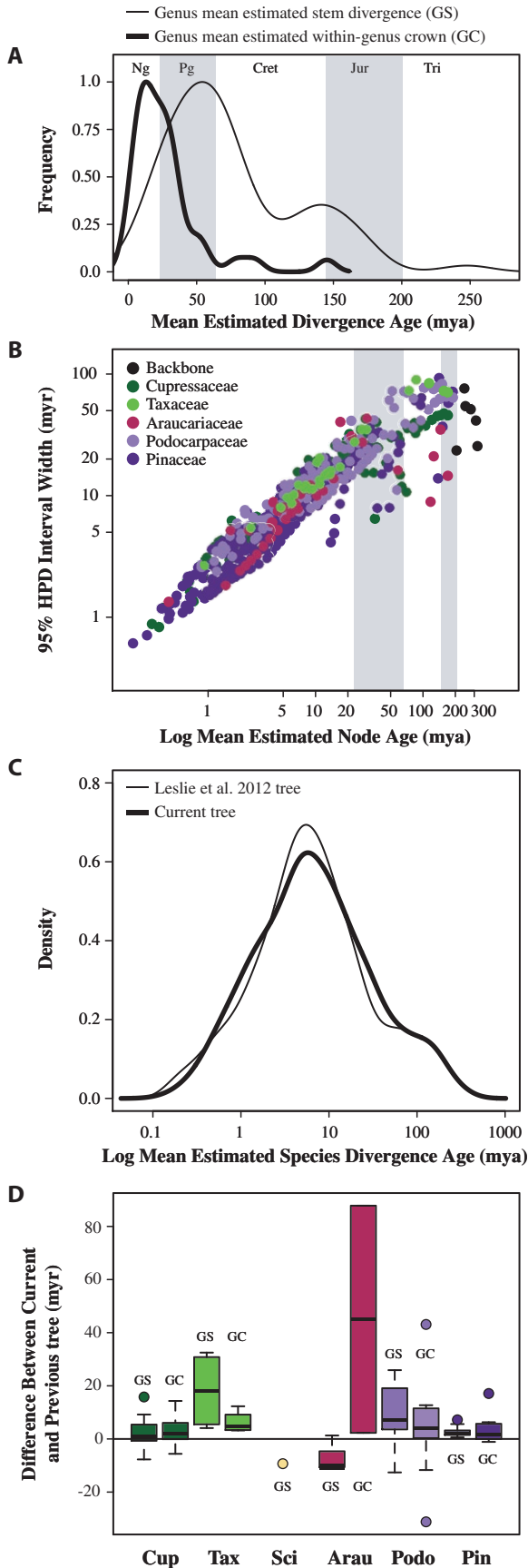
**Cupressaceae: Extensive fossil record, high congruence**—Cupressaceae show a high degree of congruence among mean estimates of molecular ages, the consensus molecular topology, and the stratigraphic appearance of fossil groups (Fig. 4). The first-diverging extant clades, *Cunninghamia* R. Br. in Rich and *Taiwania* Hayata, are estimated in our analysis to have diverged in the Middle to Late Jurassic, between 171 mya (95% HPD: 146–192 mya) and 157 mya (95% HPD: 133–179 mya). These mean ages and age ranges are consistent with the first appearance of unambiguous Cupressaceae in the Early to Middle Jurassic of Patagonia (*Austrohamia minuta* Escapa, Cúneo, et Axsmith; Escapa et al., 2008; Bodnar and Escapa, 2016) and the Middle Jurassic of England (*Elatides williamsonii* Lindley et Hutton; Harris, 1979). *Austrohamia* and at least some species of *Elatides* also share reproductive characteristics with extant *Cunninghamia* and *Taiwania*, including reduced or fused ovuliferous scales and pollen cones in clusters (Shi et al., 2014). Recent work has continued to highlight the diversity of *Cunninghamia*-like conifers from the Middle Jurassic through the early Late Cretaceous (Atkinson et al., 2014; Shi et al., 2014; Spencer et al., 2015), further suggesting that this lineage formed a major component of an initial radiation of Cupressaceae.

The fossil record of *Athrotaxis* D. Don, typically resolved as sister to the remaining Cupressaceae (Fig. 4; but see Yang et al., 2012), is more difficult to interpret because its vegetative features and gross cone morphology are not highly diagnostic (see Dong et al., 2014; Escapa et al., 2016). Nevertheless, our estimated molecular divergence date for the *Athrotaxis* lineage (145 mya, 95% HPD: 122–167 mya) is consistent with the presence of putative stem members (e.g., *Athrotaxis ungeri* Halle [Florin], *Athrotaxites berryi* Bell, and *Athrotaxites yumenensis* Dong, Sun, Wu, Du, Xu, et Jin) in the Late Jurassic and Early Cretaceous (see Escapa et al., 2016). The Late Cretaceous appearance of stem sequoioids and taxodioids is delayed in relation to their estimated molecular dates (divergences at 135 [95% HPD: 114–159 mya], and 120 mya [HPD: 101–143 mya], respectively), but the stratigraphic order of their appearance does follow the molecular topology (Fig. 4). *Sequoia*-like reproductive cones (e.g., *Quasisequoia* Shrinivasan et Friis emend. Kunzmann; *Yezosequoia* Nishida, Nishida, et Ohsawa; *Krassilovidendron* Sokolova, Gordenko, et Zavalova; Srinivasan and Friis, 1989; Nishida et al., 1991; Sokolova et al., 2017) appear at the end of the Early Cretaceous, whereas unambiguous members of the taxodioid clade (*Cryptomeria* D. Don, *Glyptostrobus* Endl., and *Taxodium* Rich) become abundant later in the Late Cretaceous (by ~70 mya) and especially over the Paleogene (Aulenback and LePage, 1998; Stockey et al., 2005).

The fossil record suggests that the cupressoid and callitroid clades (see Fig. 4) also diversified from the Late Cretaceous through the early Cenozoic. The earliest members appear ~95 mya, although we do not consider these fossils to be *Widdringtonia* Endl. as originally suggested by McIver (2001; see Leslie et al., 2012). The earliest stem representatives of modern genera, as based on unambiguous reproductive material, first appear in the Late Cretaceous (*Thuja smileya* LePage; 94–90 mya; LePage, 2003) and early Paleogene (*Papuacedrus prechilensis* [Berry] Wilf, Little, Iglesias, Carmen Zamalao, Gandolfo, Cúneo, et Johnson in the Early Eocene, ~51 mya; Wilf et al., 2009). These genera are also resolved as the sister groups to remaining cupressoids and callitroids, respectively, whereas more derived genera such as *Tetraclinis* Mast. and *Juniperus* appear later, from the Middle to latest Eocene (48–33 mya; see Kvaček et al., 2000; Kvaček, 2002). As in other parts of the Cupressaceae clade, fossil morphology, stratigraphy, molecular topology, and estimated molecular divergence dates (both means and HPD intervals) are thus in considerable agreement.

Although we used fossil Cupressaceae as calibrations in our analysis, the concordance between molecular results and fossil data was not imposed by constraining all nodes to fossils; divergence times for the earliest lineages in particular were not directly constrained (see Fig. 4). The general agreement instead appears to reflect a good representation of past diversity by extant lineages, where surviving clades record the diversification history of the Cupressaceae with reasonable accuracy. Specifically, we believe that the earliest-diverging extant lineages capture the remains of an initial radiation

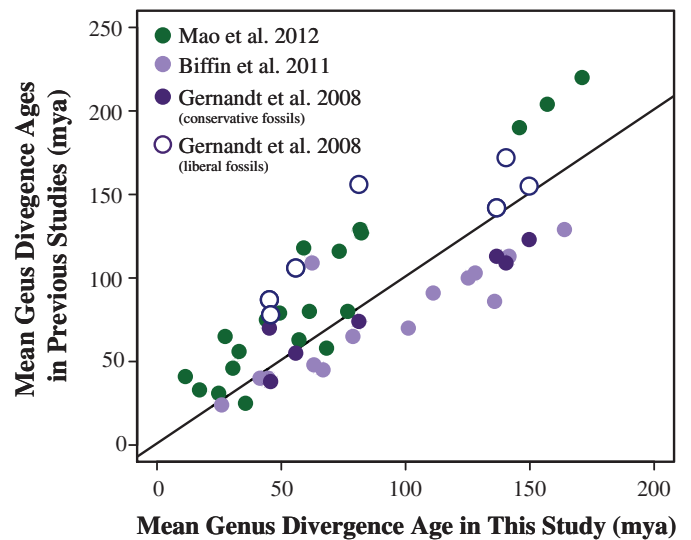
**FIGURE 1.** Time-calibrated molecular phylogeny of conifers presented in this study. There are six extant family-level conifer clades (Araucariaceae, Cupressaceae, Podocarpaceae, Pinaceae, Sciadopityaceae, and Taxaceae) whose oldest estimated crown divergence dates are indicated by a bubble containing an abbreviation of the clade name. Important subclades within the families are indicated to the right of the tree. Multi-family or ordinal-level clades are labeled on the tree over their respective branches. The 95% highest posterior density (HPD) intervals on these age estimates have been omitted for clarity but are shown in Appendix S3. Divisions between Early (E), Middle (M), and Late (L) epochs are shown for the Mesozoic geologic periods. Arau = Araucariaceae, Cup = Cupressaceae, Ng = Neogene, Pin = Pinaceae, Podo = Podocarpaceae, Sci = Sciadopityaceae, Tax = Taxaceae.



**FIGURE 2.** (A) Scaled density distributions of mean estimated divergence ages for extant conifer genera (thin line) and the first crown split within each extant genus (thick line) based on the time-calibrated conifer phylogeny presented in this study. (B) Relationship between the mean estimated divergence age of a node and its 95% highest posterior distribution (HPD) for major conifer clades. (C) Scaled density distributions of mean estimated species divergence ages in our previously published conifer phylogeny (Leslie et al., 2012) and the current one. (D) Boxplot comparing mean estimated stem divergence ages for genera (GS) and the mean estimated age of the first crown split within genera (GC) in this study and Leslie et al. (2012). Points indicate outlier values, and positive values indicate that this study estimates older ages for these nodes.

of stem cunninghamioid and taiwanioid Cupressaceae over the Jurassic, followed by the sequential radiations of sequoioids and taxodioids over the Cretaceous and Paleogene, and finally the diversification of the cupressoids and callitroids from the Late Cretaceous through the Neogene. The Cupressaceae appear to be unique among major conifer clades in showing this sequential set of diversification events, and this history may explain, in part, the concordance between molecular dates and the fossil record. By contrast, the early diversification of most other conifer clades is either not as well preserved in the fossil record or not as well sampled by extant lineages.

**Pinaceae: Extensive fossil record, limited congruence**—Pinaceae are the most species-rich clade of living conifers (Farjon and Filer, 2013), but they enter the fossil record later than other conifer groups and their origins remain uncertain (Taylor et al., 2009). Putative stem members have been traced to the Late Triassic (Delevoryas and Hope, 1973; Leslie et al., 2013b), but unambiguous Pinaceae do not appear until the Late Jurassic (Kimmeridgian, 151–156 mya; Rothwell et al., 2012). Following their appearance,



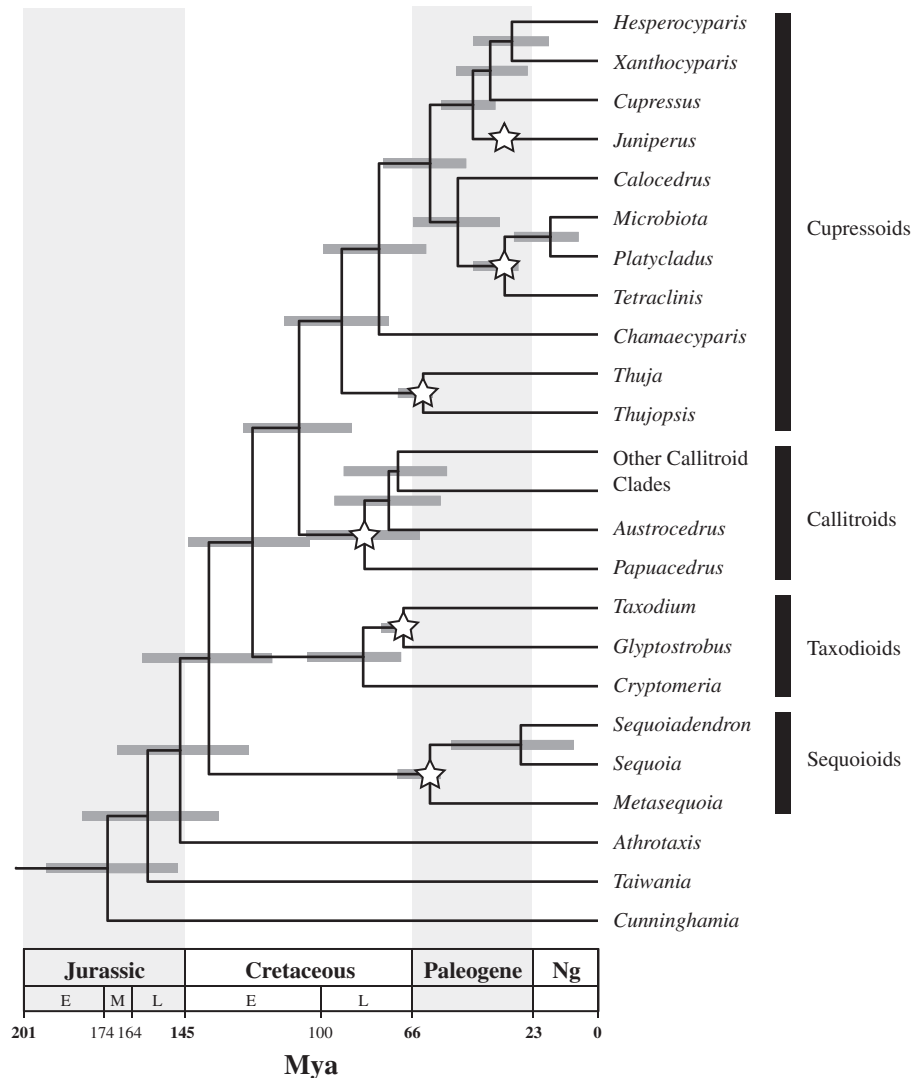
**FIGURE 3.** Comparison of mean estimated stem divergence ages for genera in this study with those estimated in comparably broad-scale dated conifer phylogenies. Colors correspond to the focal clades of the particular study (Cupressaceae in Mao et al., 2012; Podocarpaceae in Biffin et al., 2011; Pinaceae in Gernandt et al., 2008).



however, Pinaceae rapidly radiate over the Early Cretaceous (Miller, 1976; Smith and Stockey, 2001). At least in terms of seed cone anatomy, which has been the primary means of classifying fossil Pinaceae (Miller, 1976), Cretaceous taxa show a wider range of character combinations than extant members (see Smith et al., 2017). The fossil record thus suggests that Pinaceae underwent a rapid burst of initial diversification followed by subsequent extinction that “pruned” the group into the relatively few lineages represented by extant genera. Such a process would be consistent with the structure of the dated molecular tree, which shows a striking gap between old backbone ages and much younger crown diversity (Fig. 1).

The probable rapid burst of diversification in the Pinaceae complicates the comparison of molecular results with the fossil record. In terms of interpreting molecular ages, a rapid radiation may bias age estimates in deep nodes, because high evolutionary rates can artificially inflate them (Beaulieu et al., 2015). If the initial burst of radiation by the Pinaceae was indeed associated with high rates of evolution, this effect may contribute to the old ages that we infer for the deepest splits in this family. For example, the mean estimated age for the initial crown split in Pinaceae between abietoids and pinoids is in the Early Jurassic, ~188 mya (Fig. 1 and Appendix S5). This date is >30 million years before the first unambiguous appearance of Pinaceae (Rothwell et al., 2012), although these early fossils do fall within the broad HPD intervals estimated for this split (95% HPD: 155–227 mya). In terms of interpreting fossil data, on the other hand, the rapid radiation of Pinaceae is associated with a high degree of variation in anatomical and morphological characters, particularly as regards the seed cone features most often used in their systematics (Miller, 1976). This variation has historically made many Cretaceous taxa difficult to place in relation to modern genera (Smith and Stockey, 2001, 2002) and therefore difficult to use as either fossil calibration points or as a means to evaluate putative molecular topologies and estimated divergence ages.

Despite these challenges, recent paleobotanical advances are helping to clarify relationships among early Pinaceae, allowing for a more fruitful comparison between the fossil record and molecular results. For example, some recent phylogenetic analyses suggest that the earliest-known member of the Pinaceae, *Eathiestrobus mackenziei* Rothwell, Mapes, et Stockey (Rothwell et al., 2012), is actually derived within the pinoids and may be more closely related to *Pinus* than to other extant lineages (Smith et al., 2017). If this result is accurate, then our inferred Late Jurassic to Early Cretaceous divergence for stem *Pinus* (mean = 150 mya, 95% HPD: 136–173 mya) is reasonable and backbone pinoid splits must have occurred prior to the Late Jurassic despite the lack of any current fossil evidence



**FIGURE 4.** Detail of Cupressaceae phylogeny showing 95% highest posterior distribution intervals. Nodes associated with calibration fossils used in this study are indicated by stars; in *Juniperus*, the calibration fossil was used to date a split within the genus and is therefore shown along the branch at the appropriate age.

for them. Recent analyses have also made progress toward resolving relationships among other early taxa as well (Ryberg et al., 2012; Gernandt et al., 2016; Smith et al., 2017), suggesting that various species of the widespread Cretaceous form genus *Pityostrobus* are stem members of both extant abietoids and pinoids. Stem abietoids appearing over the Cretaceous would be consistent with our estimated backbone divergence dates and HPD intervals for the extant genera in this clade (see Appendices S3 and S5).

Although fossil studies are improving our knowledge of the systematics (Ryberg et al., 2012; Gernandt et al., 2016; Smith et al., 2017) and biogeography of early Pinaceae (Smith et al., 2017), the rapidity of their radiation makes understanding the evolution of Pinaceae fundamentally challenging. Because extant lineages of Pinaceae appear to represent a subsample of the morphological and anatomical diversity that existed in the group during this initial radiation, living taxa provide relatively little information to help polarize or untangle deeper patterns of character evolution. Properly understanding the

radiation of Pinaceae, and properly assessing the conflicts between morphological and molecular data, therefore requires more input from the fossil record.

**Araucariaceae: Extensive fossil record, poor congruence**—The fossil record of Araucariaceae is extensive and well documented (see Stockey, 1982, 1994; Kunzmann, 2007), but of all the major conifer clades, theirs is the most difficult to reconcile with molecular topologies and estimated divergence ages. Putative Araucariaceae first appear in the Late Triassic (Axsmith and Ash, 2006), with the earliest unambiguous members present by the Lower Jurassic (Arrondo and Petriella, 1980; Axsmith et al., 2008). By the Middle Jurassic, seed cones consistent with placement in the extant genus *Araucaria* are present in both the Northern and Southern Hemispheres (Stockey, 1975, 1980). Some of the best-known Middle Jurassic taxa (*Araucaria mirabilis* [Spegazzini] Windhausen and *A. sphaerocarpa* Caruthers) can even be placed within the modern *Araucaria* Section *Bunya* M. Wilde et A.J. Eames clade (which includes one living species, *Araucaria bidwillii* Hook.), due to the anatomy of their seed cones (Stockey, 1975; Stockey, 1994).

The placement of these early fossils is not consistent with the topology or estimated divergence dates of molecular trees, however; extant Section *Bunya* is not resolved as the sister group to remaining *Araucaria*, as might be expected, but is instead nested within a broader clade of Australasian and South American species (see Setoguchi et al., 1998). Molecular divergence ages also do not generally support a Middle Jurassic Section *Bunya*, because if crown nodes in *Araucaria* are left completely unconstrained, the clade is estimated to have diverged quite recently, ~10 mya (Leslie et al., 2012). The age of this clade in our current analysis (Early Cretaceous ~117 mya, 95% HPD: 114–123 mya) is a consequence of an additional calibration fossil that dates the appearance of its sister clade (see Appendix S2) and therefore changes crown ages across the genus. The extreme sensitivity and instability of estimated divergence ages in Araucariaceae (see Fig. 2D) reflects these conflicts between molecular and fossil data.

Such conflicts are not restricted to Section *Bunya* but occur throughout Araucariaceae. For example, the molecular phylogeny implies a mid-Jurassic split between *Araucaria* and an “agathoid” clade consisting of the extant genera *Agathis* and *Wollemia*, but *Wollemia*-like leaves do not appear until the beginning of the Late Cretaceous (Chambers et al., 1998; Cantrill and Raine, 2006), and *Agathis* leaves and reproductive structures are not known until the Eocene, ~50 mya (Wilf et al., 2014). In general, almost no aspect of the Araucariaceae molecular tree, whether its topology or estimated divergence dates, fits very well with the fossil record of the group. These discrepancies imply that the current molecular topologies are inaccurate, that many important fossil Araucariaceae have been assigned to the wrong clades, and/or that some key fossil taxa remain to be discovered.

Such difficulties highlight the need for continuing work to understand phylogenetic relationships among living and extinct Araucariaceae, particularly through studies that integrate morphological and molecular data. For example, a recent analysis by Escapa and Catalano (2013) resolved some Jurassic fossils traditionally assigned to Section *Bunya* (e.g., *Araucaria mirabilis* and *A. sphaerocarpa*) as stem representatives of *Araucaria* and therefore only distantly related to the modern section. This analysis also resolved the Late Cretaceous fossil genera *Emwadea* Dettman, Clifford, et Peters (Dettman et al., 2012) and *Wairarapaia* Cantrill et Raine (Cantrill and Raine, 2006) as stem members of the agathoid clade.

Although these taxa do not bridge the considerable temporal gap between the inferred divergence time of agathoids and their first fossil appearance, they do show the existence of potential stem members in the fossil record. These types of analyses thus provide a way forward by focusing attention on specific taxa that are key to resolving conflicts between molecular and fossil data, as well as directly testing whether particular fossil taxa are have been properly placed.

**Podocarpaceae: Limited fossil record, unclear congruence**—Like the Cupressaceae, extant Podocarpaceae include many lineages that probably diverged over the Jurassic and Cretaceous (Fig. 1; Appendices S3 and S5). Although the group should therefore provide a good opportunity to compare molecular results with fossil data, the pre-Cenozoic record of Podocarpaceae is unfortunately neither extensive nor easy to interpret, particularly with regard to informative reproductive structures. Possible Podocarpaceae have been described from the Middle Triassic to the Late Cretaceous (e.g., Townrow, 1967; Zhou, 1983; Archangelsky and Del Fueyo, 1989; Axsmith et al., 1998; Cantrill and Falcon-Lang, 2001; Bannerji and Ghosh, 2006), but none of them can be assigned to modern lineages, and some early taxa may not be closely related to Podocarpaceae at all (e.g., the *Telemachus-Heidiphyllum* plant of Bomfleur et al., 2013). At the most general level, the appearance of various lineages of Podocarpaceae over the Jurassic is consistent with our inferred mean divergence ages for major extant podocarp subclades (Early Jurassic to Late Cretaceous), although HPD intervals on these nodes are wide (see Appendix S5). But as in Pinaceae, it is difficult to use the pre-Cenozoic fossil record of Podocarpaceae to evaluate specific molecular results because fossils cannot be clearly placed with respect to extant clades.

The first unambiguous representatives of extant lineages of Podocarpaceae, based on a combination of reproductive and vegetative morphology, date to the Paleocene and Eocene of Australia and South America (Brodribb and Hill, 1999; Wilf, 2012). This time frame generally fits with our mean estimated divergence dates for crown taxa in the most species-rich and ecologically important extant clades, such as *Podocarpus*, *Dacrydium*, and *Dacrycarpus* (all found in the “tropical” clade; see Fig. 1 and Appendices S3 and S5). Recently, the derived genus *Retrophyllum* has been found in Patagonia at 51 mya (and may extend to the latest Cretaceous; Wilf et al., 2017), which predates our mean estimated stem age (41 mya) and potentially even the range of the HPD interval (28–60 mya). Our divergence ages for extant genera of Podocarpaceae may therefore still be underestimated to some extent (see Wilf and Escapa, 2015), even with the addition of a less restricted set of calibration fossils. It is important to note, however, that latest Cretaceous *Retrophyllum* would not fundamentally change the age structure of our tree; even if the mean age of generic splits were consistently underestimated by 10–20 million years, many of the most important modern genera (*Podocarpus*, *Dacrydium*, *Dacrycarpus*) would still have mean estimated divergence ages in the Late Cretaceous (see Appendices S3 and S5). Such a time frame would be consistent with the lack of clear fossil evidence for extant genera prior to the latest Cretaceous.

On the other hand, the lack of pre-Cenozoic examples of extant lineages of Podocarpaceae could be due to the nature of their ovulate structures. Most extant seed cones of Podocarpaceae are adapted for animal dispersal; they are reduced in size and show a variety of fleshy tissues in different parts of the cone (see Contreras

et al., 2017; Leslie et al., 2017). Small size and fleshiness appear to have been common in the clade since at least the Cretaceous, limiting their preservation potential and making it difficult to interpret the structure of the relatively few cones that are known (e.g., Cantrill and Falcon-Lang, 2001). It is therefore difficult to place fossil Podocarpaceae in relation to extant taxa, not only because they typically lack interpretable reproductive structures, but also because the seed cones of Podocarpaceae in general have been modified to the point that they contain few meaningful synapomorphies.

One possible way forward in the group is to focus on their more commonly preserved vegetative remains. The cuticle and stomatal anatomy of Podocarpaceae have already proved useful for systematics at the genus level (e.g., Stockey and Ko, 1990; Hill and Pole, 1992), but to our knowledge, no large-scale study has explicitly mapped and analyzed the distribution of major cuticular features (e.g., the presence of Florin rings, epidermal cell outlines, and stomatal distributions) across a detailed phylogeny of the family. Such an analysis could potentially identify novel cuticular synapomorphies, particularly for clades above the genus level, which may prove useful in linking fossil leaf material to extant lineages.

### Advantages and limitations of molecular approaches to understanding conifer evolution

The effects of extinction may be limited in groups of recent origin, but in conifers they are likely pervasive because the group is old and is characterized by long time intervals between the divergence of clades and the bulk of their crown diversity (a pattern that is common in gymnosperms generally; see Won and Renner, 2006; Nagalingum et al., 2011). This “stemminess” in the phylogeny may reflect high extinction rates (Crisp and Cook, 2011) and/or high turnover rates (Leslie et al., 2012) that eliminate many of the deeper branching lineages within the major extant clades, even though the major clades themselves persist through time.

But whatever its ultimate causes, the preponderance of recent diversification in extant conifer phylogeny means that the current distribution of character states is strongly biased by Cenozoic species and Cenozoic ecology. This bias can make it challenging to reconstruct deeper patterns of character evolution. For example, the spruce lineage (*Picea*) first appears in the fossil record ~130 mya (Klymiuk and Stockey, 2012), but the deepest extant branch (*Picea breweriana* S. Watson) is estimated to have diverged ~30 mya (Leslie et al., 2012; Lockwood et al., 2013). The ~100 million years separating the origin of the genus and the origin of its crown diversity preclude any meaningful assessment of its early ecology or biogeography based on living species alone, *contra* Ran et al. (2006), who used the location of early-diverging extant *Picea* lineages to suggest that the genus originated in North America.

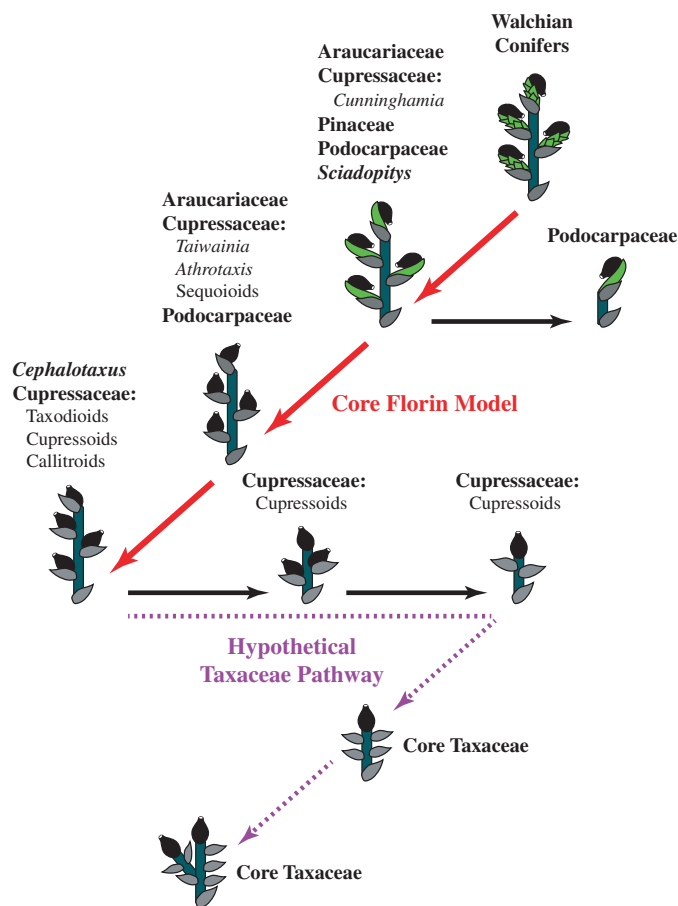
In general, studies that infer ancestral states using only extant conifer traits and phylogenetic relationships (e.g., He et al., 2012; Leslie et al., 2013a) could easily be misled by the current distribution of traits unless the characters under study are highly conserved. But despite this limitation, molecular phylogenies can offer important insights into patterns of character evolution in conifers, provided that questions are asked at the right scale and inferred ancestral states are treated with an appropriate degree of caution. To illustrate some of the advantages and limitations of molecular phylogenies for understanding character evolution, we conclude with one of the oldest questions in the evolution of conifers, the origin of their seed cones.

### A case study: Ovulate cone evolution

The evolutionary history of conifer cones has long occupied botanists (see discussion in Florin, 1954), but by the middle of the 20th century, Rudolf Florin had used Paleozoic conifers to demonstrate that they are almost certainly modified shoot systems (Florin, 1938, 1951, 1954). Specifically, he noted that reproductive structures of the so-called “walchian” conifers of the Permo-Carboniferous consisted of reiterated leafy shoots, each subtended by a bract, which bore ovules on their adaxial surfaces. These cones were also fundamentally similar to those of an earlier Paleozoic group, the Cordaitales, except that cordaitalean ovules were borne on stalks distributed radially around a leafy fertile shoot. Florin proposed that modern conifer cones evolved by reduction of such an ancestral shoot system: cordaitalean-like ovules lost their stalks and were then borne directly on the adaxial surface of leafy shoots in early conifers, which in turn became flattened and lost their sterile leaves to form the structures that we call the ovuliferous scale in many extant conifer clades (e.g., *Araucaria*, early Cupressaceae, Pinaceae, *Sciadopitys*). This reduction process continued further within some of the major extant clades (e.g., cupressoids and calitroids in Cupressaceae, agathoids in Araucariaceae, and some Podocarpaceae), resulting in the eventual loss of the entire fertile shoot/ovuliferous scale complex (Fig. 5).

Florin’s model has remained the standard explanation of conifer cone evolution (Rothwell, 1982; Rothwell et al., 2005), although not all conifers neatly fit it. For example, even some of the earliest conifers completely lack fertile shoots and show no known intermediate or transitional forms (e.g., the Ferugliocladaeae from Patagonia; Archangelsky and Cuneo, 1987). Among living conifers, Taxaceae are even more difficult to explain because all genera except *Cephalotaxus* have terminal ovules borne on fertile axes (Stützel and Rökewamp, 1999; Tomlinson and Takaso, 2002). Such an arrangement is difficult to derive from a Florin model because even if ovuliferous shoots were completely eliminated, ovules should still be axillary to a bract (an arrangement seen in many Cupressaceae and the Ferugliocladaeae; see Fig. 5). Florin was thus unable to place Taxaceae into this evolutionary framework and instead proposed that they represented an independent evolutionary line (Florin, 1948, 1954).

Molecular phylogenies help resolve the origin of ovulate structures in the Taxaceae because they clearly show that the group is related to other conifers, specifically the Cupressaceae and Sciadopityaceae. Their ovulate structures must therefore be derived from compound shoot systems, just like the more typical cones of their relatives. But we can also use molecular phylogenies to identify and test possible evolutionary pathways through which these structures evolved, by taking advantage of variation shown within extant conifer clades (Fig. 5). For example, terminal ovules are actually present in a few derived species in the Cupressaceae (some *Juniperus* and *Microbiota* Komarov) that have highly reduced cones (Farjon and Ortiz Garcia, 2003). Ovules in these taxa appear to have become dissociated from their corresponding cone scales and then reduced in number, resulting in a single ovule in a terminal position (Fig. 5; see Farjon and Ortiz Garcia, 2003). Ovulate structures in the Taxaceae could conceivably have originated through such a process, although the earliest fossils are already highly reduced (see Florin, 1938; Harris, 1979), with no direct evidence of intermediate states. The molecular tree, however, provides some qualitative evidence for such a pathway: the cone-like ovulate structures of *Cephalotaxus*,



**FIGURE 5.** Potential pathways of evolutionary change in conifer seed cones given the phylogenetic relationships suggested by molecular data. Schematic cartoons at each node illustrate basic cone structure. The putative ancestral cone (“Walchian Conifers”) consists of an axis and helically arranged bracts (gray triangles) that subtend leafy fertile shoots (green branches) bearing ovules (black ovoids). The “Core Florin Model” shown by red arrows describes the reduction and ultimate loss of the fertile shoot. First, the sterile leaves are lost and the fertile shoot becomes the pad of tissue referred to as the ovuliferous scale or epimatium. Next, the fertile shoot may be further reduced in prominence and even fully fused to the bract, as in agathoid Araucariaceae, some Podocarpaceae, and some early-diverging Cupressaceae. Finally, the visible fertile shoot may be lost entirely, resulting in axillary ovules seen in *Cephalotaxus* and some Cupressaceae. Note that taxodioids have axillary ovules but also have an ovuliferous scale that is not shown in the diagram for simplicity. Some clades show additional reduction of the cone, either by a decrease in the number of fertile units (Podocarpaceae) or by the dissociation of terminal ovules from subtending bracts (*Tetraclinis*, *Microbiota*, and some *Juniperus* within the cupressoids) and reduction to a single ovule (in *Microbiota* and some *Juniperus*). The dashed purple line indicates a hypothetical pathway that may explain the origin of the highly reduced seed cones of the Taxaceae.

the sister clade to other Taxaceae, have axillary ovules subtended by bracts (Fig. 5). The likelihood that ovulate structures evolved in the Taxaceae through the reduction of a typical cone structure could also be directly evaluated using the molecular tree coupled

with models of discrete character evolution (e.g., Beaulieu and Donoghue, 2013; Zanne et al., 2014).

Although molecular phylogenies can provide insights into deep evolutionary patterns like basic cone structure, they are less useful for understanding the history of specific ovulate characters, in large part because the major surviving conifer clades are so distinct from each other. For example, the sister relationship between the Araucariaceae and Podocarpaceae reveals little about the specific morphology of their ancestor, because their extant reproductive structures are very different in detail (large woody cones vs. reduced fleshy “berries”) and any potential outgroups are also only distantly related. Molecular phylogenies thus provide little help in unraveling one of the more difficult aspects of conifer paleobotany: understanding the relationships among the “transition” or “voltzialean” conifers, a likely paraphyletic group that was abundant from the Permian through the Jurassic (Taylor et al., 2009) and which almost certainly includes early representatives of most extant lineages. In general, ancestral states for conifer nodes deeper than the Late Cretaceous are difficult to reconstruct with any degree of confidence because there are relatively few of these nodes and because the inferred states are heavily biased by lineages that originated in the Cenozoic. The basic shape and branching structure of the extant conifer tree then fundamentally limits what it can tell us about specific seed cone character states in Paleozoic and most Mesozoic conifers.

## CONCLUSIONS

Molecular phylogenies of conifers may or may not conflict with fossil data, depending on the specific group in question. The topology and estimated divergence dates in some clades, such as the Cupressaceae, are highly congruent with the fossil record, while those of other clades like the Araucariaceae have major conflicts. In other cases, including the Pinaceae and Podocarpaceae, it is difficult to assess the degree of congruence because of the difficulty in placing fossil taxa. Molecular phylogenies in a general sense can be powerful tools for understanding conifer evolution and diversification, but primarily as backbone constraints on tree topology and as a means to generate hypotheses that require further testing against the fossil record. The abundance of recent diversification within otherwise widely divergent conifer lineages also limits their utility for reconstructing specific patterns of trait evolution, because large morphological branch lengths separate the surviving lineages. Paleobotany is therefore essential to understanding conifer phenotypic evolution, because it offers the potential to break up long morphological branches by identifying and characterizing extinct taxa with unusual traits (e.g., Herrera et al., 2015; Pacyna et al., 2017) and by testing the phylogenetic placement of such taxa (Escapa et al., 2012).

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## DATA ACCESSIBILITY

The phylogenetic trees used by this article is deposited into figshare at 10.6084/m9.figshare.6948152.

## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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