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Neontological and paleontological congruence in the evolution of Podocarpaceae (coniferales) reproductive morphology

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Introduction: Podocarpaceae are a diverse, primarily tropical conifer family that commonly produce large leaves and highly reduced, fleshy seed cones bearing large seeds. These features may result from relatively recent adaptation to closed-canopy angiosperm forests and bird-mediated seed dispersal, although determining precisely when shifts in leaf and seed cone morphology occurred is difficult due to a sparse fossil record and relatively few surviving deep lineages.

Methods: We compare the fossil record of Podocarpaceae with results from ancestral state reconstruction methods and correlated character models using neontological data and a previously published molecular time-tree.

Results: Ancestral state reconstructions suggest that small leaves, small seeds, and multi-seeded cones are ancestral in crown Podocarpaceae, with reduced cones bearing few seeds appearing in the Early Cretaceous and the correlated evolution of large leaves and large seeds occurring from the Late Cretaceous onwards. The exact timing of these shifts based on neontological data alone are poorly constrained, however, and estimates of leaf and seed size are imprecise.

Discussion: The fossil record is largely congruent with results based on the molecular time-tree, but provide important constraints on the range of leaf and seed sizes that were present in Cretaceous Podocarpaceae and the time by which changes in cone morphology and seed size likely occurred. We suggest in particular that reduced seed cones appeared in the Early Cretaceous and are linked to the contemporaneous diversification of small bodied avialans (birds), with shifts to larger seed sizes occurring after the Cretaceous in association with the spread of closed-canopy angiosperm forests.

KEYWORDS

conifers, seed cone, birds, plant-animal interactions, Early Cretaceous

Introduction

Molecular phylogenies are crucial tools for studying major patterns in plant evolution, thanks to the tremendous growth in sequence data over the past several decades and increasingly powerful ways to analyze it. Although molecular phylogenies necessarily include only living (or subfossil) plants, they are nevertheless widely used to understand much deeper evolutionary processes through statistical methods that estimate divergence ages among extant clades (Sanderson, 2003; Drummond and Rambaut, 2007), reconstruct ancestral states (Cunningham et al., 1998; Pagel et al., 2004; Revell, 2014), and detect changes in diversification rate through time (Morlon, 2014; Rabosky et al., 2014; Condamine et al., 2019). Fossils are a crucial component of these analyses, as they provide the means to calibrate divergence times (see Parham et al., 2012) and can improve the accuracy of ancestral state reconstructions (Slater et al., 2012). The importance of fossil data has driven the development of increasingly sophisticated methods for incorporating them into molecular analyses (e.g., Slater et al., 2012; Heath et al., 2014; Zhang et al., 2016; O'Reilly and Donoghue, 2020). Nevertheless, the ages of deep divergences inferred from molecular clock analyses often differ from those expected based on the fossil record (Smith et al., 2010; Wilf and Escapa, 2015; Herendeen et al., 2017; Morris et al., 2018; Sauquet et al., 2022) and molecular phylogenies can give rise to unexpected or ambiguous ancestral state estimates (e.g., Harris et al., 2020). Recent studies also suggest that the ability of molecular phylogenies to unambiguously record past diversification histories may be inherently limited (Louca and Pennell, 2020). Reconciling results based on molecular phylogenies with the fossil record therefore remains an ongoing challenge for evolutionary biology.

Conifers, the most species-rich group of extant non-flowering seed plants, are a useful system in which to evaluate congruence between the fossil record and molecular analyses because they combine a well-resolved extant phylogeny with a temporally extensive fossil record (Leslie et al., 2018). Although most extant conifer species likely diverged relatively recently (Leslie et al., 2012), the earliest probable stem conifers date back ~310 million years (Scott and Chaloner, 1983) and crown conifer clades have been present since at least the Early Jurassic (~200 million years ago; Axsmith et al., 2008; Contreras et al., 2019). Because crown conifer clades were a dominant component of many Mesozoic terrestrial ecosystems, the rich fossil record of their leaves, cones, and seeds provides many opportunities to evaluate divergence ages, ancestral state reconstructions, and diversification shifts inferred from extant phylogenies. In this study, we focus on one major clade of living conifers, the Podocarpaceae, and explore congruence between the fossil record and reconstructions of trait evolution based on a time-calibrated molecular phylogeny.

The Podocarpaceae are the second most speciose major clade among extant conifers, with ~180 species (Farjon, 2010). The clade has a deep evolutionary history; widely acknowledged fossil representatives of the group occur in the Jurassic and Cretaceous (Harris, 1979; Hill and Brodribb, 1999; Taylor et al., 2009) with possible stem members as far back as the Late Permian (Blomenkemper et al., 2018). Among living conifers, the clade is distinctive for its primarily tropical distribution (Cernusak et al., 2011), the occurrence of large, flattened needle leaves in many taxa (Biffin et al., 2012), and small fleshy reproductive structures specialized for seed dispersal by animals (Contreras et al., 2017; Leslie et al., 2017), particularly birds (Geldenhuis, 1993; Willson et al., 1996; Klaus and Matzke, 2020). Despite the age of the clade, these distinctive leaf and reproductive traits may reflect adaptations to relatively recent ecological conditions associated with the Cenozoic: large, broad leaves for more efficient light reception in closed-canopy, angiosperm-dominated forests (Brodribb and Feild, 2008; Biffin et al., 2012) and reduced, fleshy seed cones that link seed dispersal with crown bird groups radiating over the last 65 million years (Leslie et al., 2017; Klaus and Matzke, 2020). Several Podocarpaceae lineages have also evolved notably large seeds (Leslie et al., 2017), a further possible adaptation to angiosperm-dominated forests where closed canopies favor substantial nutritional reserves for seedlings growing in low light (Tiffney, 1984; Eriksson et al., 2000; Eriksson, 2016). These hypotheses remain incompletely tested, however, and the exact evolutionary history of leaf and reproductive traits in Podocarpaceae is uncertain due to long phylogenetic branches and a limited fossil record.

Podocarpaceae have been the focus of many molecular phylogenetic studies (e.g., Kelch, 1998; Biffin et al., 2012; Knopf et al., 2012; Quiroga et al., 2016; Chen et al., 2022) which have generally split the group into a large clade of species-rich, primarily tropical taxa (e.g., *Dacrydium*, *Dacrycarpus*, *Podocarpus*) sister to a more depauperate, primarily temperate “prumnopityoid” clade (Chen et al., 2022; Figure 2A), and both clades contain long-branched lineages often regarded as relictual (e.g., *Lagarostrobos*, *Microstrobos*, and *Saxegothaea*; Khan and Hill, 2021). However, most extant Podocarpaceae richness is inferred by dated phylogenies to be relatively recent, with species divergences concentrated in the Neogene (Leslie et al., 2012, 2018). The number of lineages that span the deeper Jurassic and Cretaceous history of the group is therefore limited, although previous studies have nevertheless been able to use dated phylogenies to link the evolution of large leaves with the appearance of closed-canopy tropical angiosperm forests (Biffin et al., 2012). Using neontological data and molecular phylogenies to understand the deep evolution of reproductive traits is challenging, however; Podocarpaceae appear to have repeatedly evolved simplified, fleshy reproductive structures whose specific morphology and anatomy differ in detail (Contreras et al., 2017; Khan and Hill, 2021; Khan et al., 2022).

With such disparate morphologies on long phylogenetic branches, the exact sequence of evolutionary events leading to Podocarpaceae reproductive diversity is unclear.

Podocarpaceae fossils provide a direct view of past leaf and reproductive morphologies, but their record is sparse compared to other species-rich conifer clades like Cupressaceae and Pinaceae (Hill and Brodribb, 1999; Leslie et al., 2018). Widely acknowledged Mesozoic Podocarpaceae with preserved reproductive structures are restricted to a few representatives from the Jurassic (Harris, 1979; Reymanowna, 1987) and a handful of taxa from the Early Cretaceous of Antarctica (Cantrill and Falcon-Lang, 2001), Argentina (Archangelsky, 1966; Archangelsky and Del Fueyo, 1989), and India (Vishnu-Mittre, 1959; Banerji and Ghosh, 2006). None of these taxa have been directly linked to modern Podocarpaceae clades, either qualitatively or through an explicit phylogenetic analysis, and fossils unambiguously belonging to extant genera appear only in the early Cenozoic (Greenwood, 1987; Hill and Brodribb, 1999; Wilf, 2012; Andruchow-Colombo et al., 2019a,b) or the latest Cretaceous (Wilf et al., 2017). Podocarpaceae leaves are more common in the fossil record than reproductive structures, but older examples are difficult to unambiguously interpret. For example, large multi-veined fossil leaves are often assigned to the Podocarpaceae because some living members of the group (e.g., *Nageia*) produce them, but detailed whole plant reconstructions of fossil taxa suggest that at least some of them belong to unrelated conifer clades (e.g., the Triassic conifer leaf *Heidiphyllum* and seed cone *Telemachus*; Escapa et al., 2010; Bomfleur et al., 2013). Overall, both the Podocarpaceae fossil record and inferences from molecular trees suggest a deep Mesozoic history for the group, but neither source of information provides a detailed understanding of leaf and seed cone evolution and thus the extent to which changes in these traits relate to ecological shifts in the Cretaceous and Cenozoic.

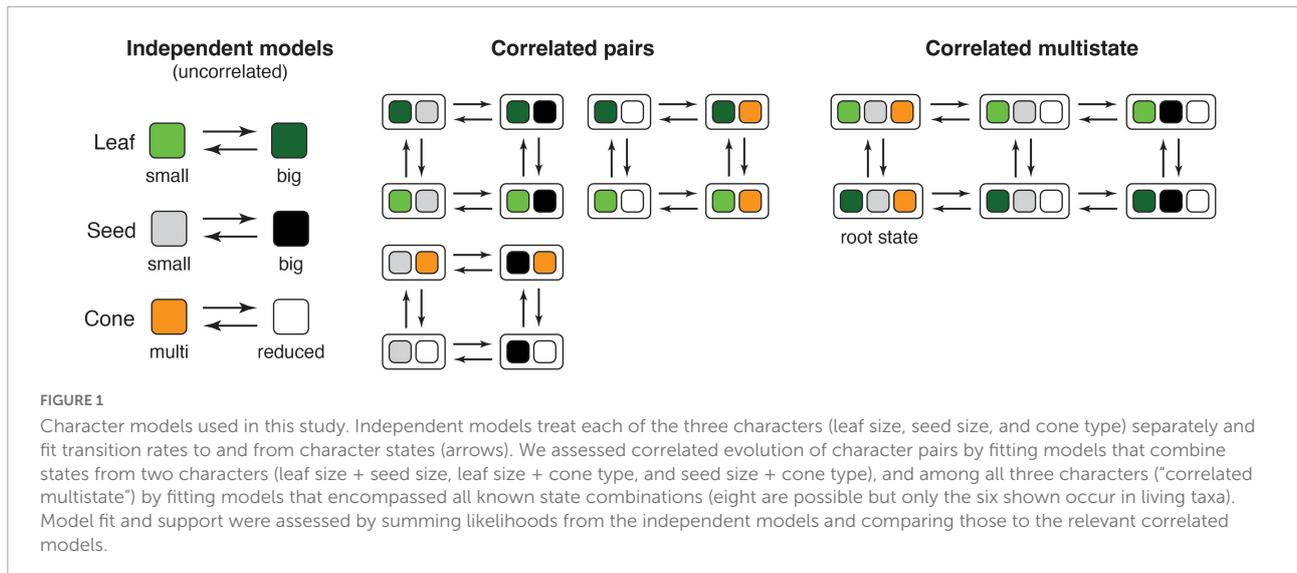
Here we compare the fossil record of Podocarpaceae reproductive structures and their associated leaves to ancestral state reconstructions from a molecular phylogeny to ask if combining these two sources of evolutionary information generates a clearer picture of trait evolution in the group. Although Podocarpaceae fossils and reconstructions based on the molecular phylogeny are both sparse sources of data, we find that they are nonetheless consistent and complementary: both suggest that shifts to highly reduced seed cones bearing few seeds began in the Early Cretaceous and pre-date the ecological dominance of angiosperms, while the evolution of large leaves and seeds is correlated and begins in the Late Cretaceous through the early Cenozoic. The suite of traits that makes Podocarpaceae distinctive among living conifers (large leaves, reduced seed cones, and large seeds) therefore appears to have evolved in a stepwise manner over a prolonged period of the Cretaceous. We use vertebrate fossil data to further suggest that these changes began due to interactions with small-bodied stem avialans (birds), which also underwent an important

diversification event in the Early Cretaceous (O'Connor et al., 2011; Benson and Choiniere, 2013; Yu et al., 2021).

Materials and methods

We compiled leaf size measurements from 177 extant Podocarpaceae species and seed length measurements from 163 extant species based on data from Farjon (2010). Where ranges in leaf length and width were provided, we used midpoint values. In taxa with strongly dimorphic foliage (e.g., some *Dacrycarpus* species), we used measurements from the largest leaves. For *Phyllocladus*, we treated the phylloclades as “leaves”, given that they are the photosynthetic units of the plant. For all taxa, we estimated leaf (or phylloclade) area as an ellipse with major and minor axes corresponding to provided length and width measurements, respectively. Seeds are difficult to measure in some Podocarpaceae genera [e.g., *Podocarpus*, *Prumnopitys* sensu lato (*Pectinopitys*, *Prumnopitys*, *Sundacarpus*)] because they are enveloped by an organ called the epimatium that creates a combined diaspore larger than the seed itself; seed size is only directly accessible if the diaspore is broken or sectioned. To be consistent across all Podocarpaceae, including taxa that lack an enclosing epimatium, we analyzed the true length of the seed. For taxa where only total diaspore length was provided, we estimated seed length using a scaling factor based on comparing diaspore to seed length in the subset of taxa for which both measurements were given in Farjon (2010). This subset includes 27 species from *Afrocarpus*, *Podocarpus*, *Retrophyllum*, and *Sundacarpus*. On average, seed length in these taxa was 75% of total diaspore length (median 75%, mean 75.1%, interquartile range 70.4–80.9%). For taxa with only diaspore length given (93 species in total), we therefore estimated seed length as 75% of diaspore length.

In addition to continuous leaf and seed size characters, we analyzed several discrete or binned characters. We binned leaf size into “small” and “large” states based on a threshold value of 10 mm²; this represents a natural break in the distribution of estimated leaf areas for extant taxa (see Figure 3A) and generally corresponds to a distinction between taxa with imbricated scale leaves and those with larger needles. We also binned seed length, although here there was no clear break in the distribution of sizes. We used three schemes to assess the potential effects of binning: we divided seed length into “small” and “large” states based on whether length was greater or less than the median value (50th percentile) of extant taxa (6 mm; see Figure 3B), the lower 25th percentile (4.5 mm), or the lower 10th percentile values (3.375 mm; Supplementary Figure 2). Lastly, we scored the typical number of fertile (potentially seed-producing) units produced in Podocarpaceae reproductive structures, using the average or midpoint value when a range was given (Molloy, 1995; Farjon, 2010). We then used these data to score whether Podocarpaceae taxa produce “multi-seeded

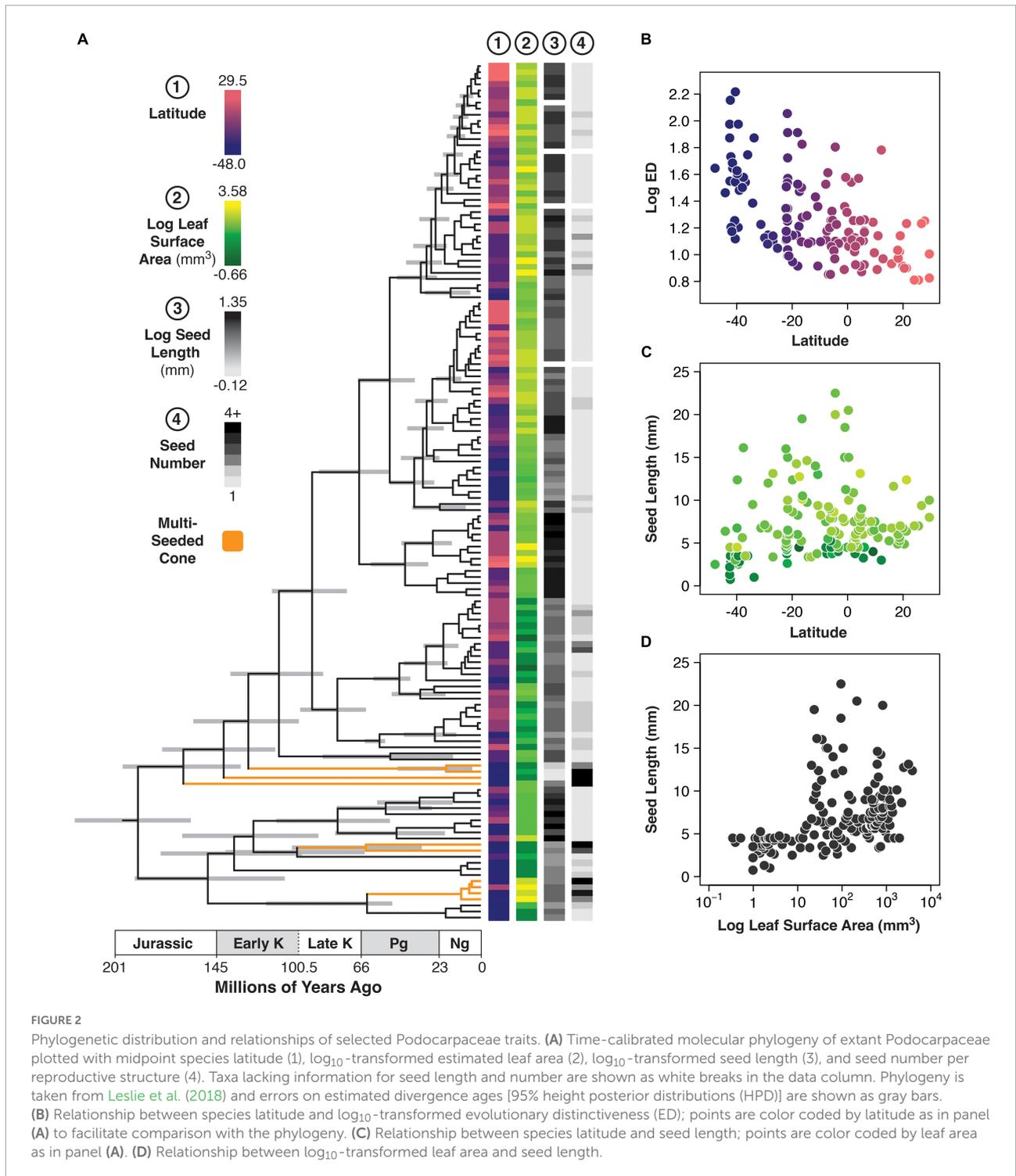


cones” or “reduced” cones; multi-seeded cones are defined here as having three or more fertile scales (or sterile scales that are morphologically equivalent to fertile scales) arranged in a packed or imbricated configuration. In contrast, many extant Podocarpaceae (e.g., *Afrocarpus*, *Podocarpus*, and *Prumnopitys* sensu lato) produce ovulate structures consisting of just one or two seeds enclosed or subtended by fleshy organs (Contreras et al., 2017; Leslie et al., 2017). Taxa with multi-seeded cones here include *Lagarostrobos*, *Manoao*, *Microcachrys*, *Pherosphaera*, *Phyllocladus*, and *Saxegothaea*. *Manoao* cones often have only two mature seeds, but they generally produce more fertile scales initially and are known to produce three or more seeds occasionally (Molloy, 1995).

To reconstruct the evolution of leaf size, seed size, and the presence of multi-seeded cones based on extant taxa alone, we used phylogenetic analyses of both continuous and discrete/binning characters. We used a time-calibrated phylogeny of extant Podocarpaceae taken from a larger, previously published conifer phylogeny (Leslie et al., 2018) based on three loci (*rbcL*, *matK*, 18S). This phylogeny was dated using the Bayesian software BEAST (Drummond and Rambaut, 2007) with 26 conifer calibration fossils, including five from the Podocarpaceae (dating splits of crown *Dacrycarpus*, stem *Dacrydium*, stem *Phyllocladus*, crown *Podocarpus*, and stem *Retrophyllum*; see Leslie et al., 2018 for details). The phylogeny includes 141 taxa, from which leaf measurements were available for all and seed measurements from 137. To reconstruct ancestral states for continuous characters (using \log_{10} -transformed leaf area and seed length), we used the maximum likelihood-based “fastAnc” function in the R package “phytools” (Revell, 2012); this method provides estimates of values with associated confidence intervals for internal nodes of the tree. To reconstruct ancestral states for discrete/binning characters, we used two likelihood-based approaches that

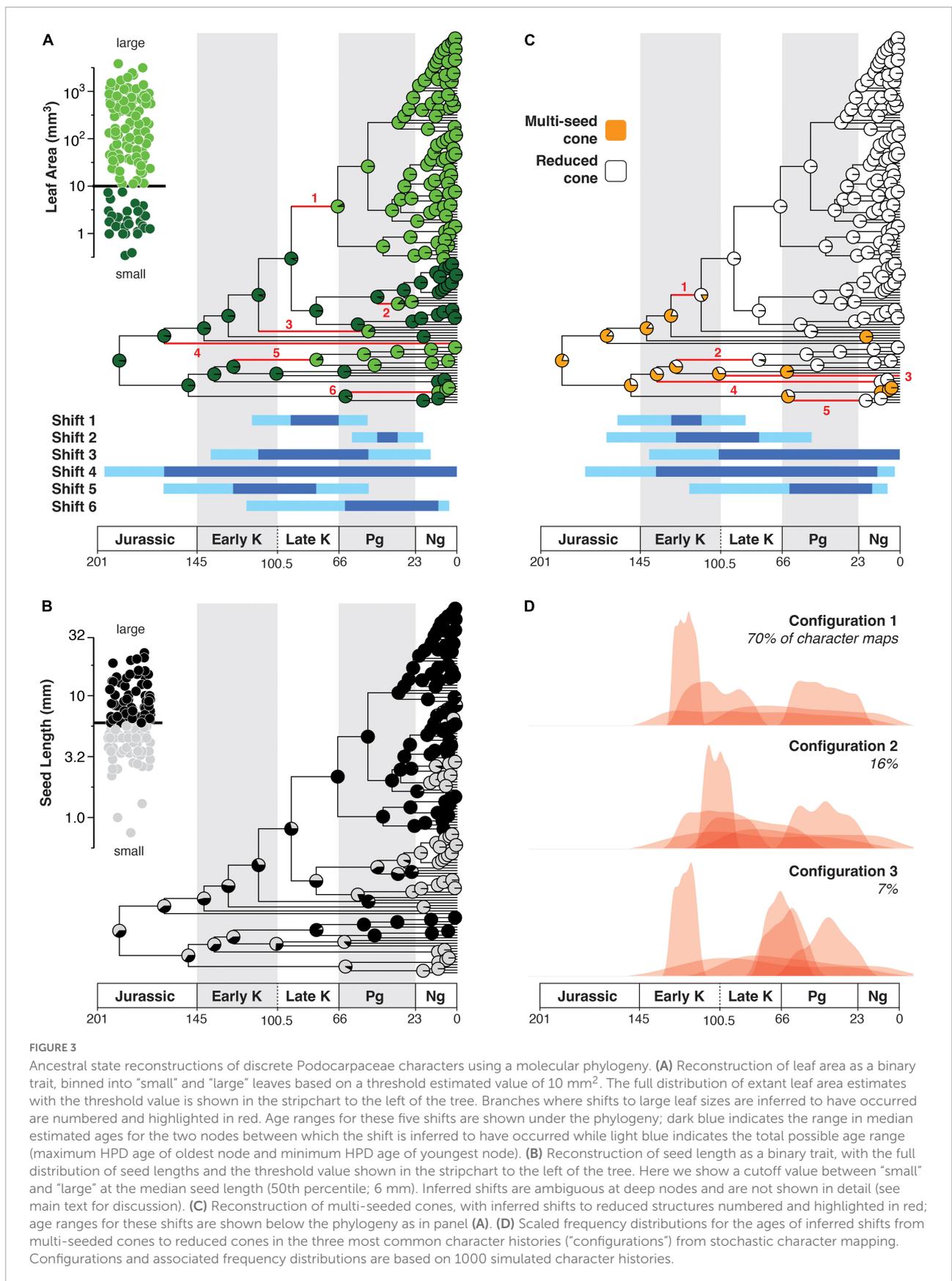
estimate transition rates among character states (e.g., between “small” and “large” leaves) given tree topology and branch lengths, and then used these parameters to estimate character state probabilities for internal nodes within the tree. One approach implemented in the R package “corHMM” (version 2.8; Beaulieu et al., 2021) calculates the marginal likelihood of character states at internal nodes. The other, stochastic character mapping (Huelsenbeck et al., 2003), uses transition rates to simulate character histories. For each character we ran 1000 simulations using the R package “phytools” (Revell, 2012), which were then summed in order to calculate state probabilities at internal nodes and the most likely locations of state shifts along branches. To explore the potential effect of outgroups on reconstructions of deep nodes, we also ran analyses in “corHMM” with a phylogeny that includes three members of the Podocarpaceae sister lineage (the Araucariaceae, including *Agathis australis*, *Araucaria bidwillii*, and *Wollemia nobilis*). These taxa were all scored as having large leaves, large seeds, and multi-seeded cones.

We also used likelihood-based transition rate models implemented in “corHMM” to test whether leaf size, seed size, and cone reduction evolved in a correlated manner (see Pagel, 1994). To test whether characters evolved independently, we fit separate models for binned leaf size (“small” versus “large”), binned seed size (“small” versus “large” using the 50th and 25th percentile cutoffs), and cone type (“multi-seeded” versus “reduced”). For each character, we fit two types of models whose parameters were transition rates to and from each character state: one in which transition rates were constrained to be the same (equal rates; ER) and one in which they were allowed to vary (all rates different; ARD). We then summed the likelihoods of these models and calculated an aggregate AIC score for the ER and ARD models, respectively; this score can be thought of as representing a single evolutionary



model with three completely independent (uncorrelated) traits (**Figure 1**). We then compared these AIC scores to those of models where characters were scored as combined states, implying that they evolve in a correlated fashion and cannot be analyzed separately ([Pagel, 1994](#)). We first tested whether pairs of characters evolve in a correlated manner by fitting transition

rate models with up to eight parameters (for an ARD model) between possible character state combinations (**Figure 1**). We then fit full character state models consisting of either five or six combinations of leaf size, seed size, and the presence of multi-seeded cones; five combinations occur in extant Podocarpaceae using the 50% percentile binning scheme and six in the 25%



scheme, resulting in either 10 or 14 possible transition rate parameters among the combined states (Figures 1, 4). We also tested versions of these models where the root (ancestral state) was fixed as having small leaves, small seeds, and multi-seeded cones. For all these analyses, we included only taxa that were sampled in the phylogeny and for which leaf size, seed size, and cone morphology were known (137 taxa). All statistical analyses were performed using R version 4.1.3 (R Core Team, 2022).

To place extant leaf and seed sizes in a broader macroecological and macroevolutionary context, we compiled selected data from living Podocarpaceae species generated by several previous studies of conifers (Sundaram et al., 2019; Sundaram and Leslie, 2021). Specifically, these data include midpoint species latitude and several climatic variables that could potentially influence the evolution of leaf and seed size: mean annual temperature, temperature seasonality, annual precipitation, and precipitation seasonality. Climatic variables were derived from WorldClim bioclimatic variables (BIO1,4,12, and15, respectively; Fick and Hijmans, 2017) and calculated for a given taxon as the average value for each variable across all 100×100 km grid cells in which the taxon occurs. We also analyzed evolutionary distinctiveness (ED) for each extant taxon; this is based on the “fair proportion” metric where ED is the sum of branch lengths from root to tip weighted by the inverse of the number of taxa sharing each portion of branch length (Isaac et al., 2007; Jetz et al., 2014). Values used here are based on the full podocarp phylogeny of 141 taxa and taken from Sundaram et al. (2019). We used phylogenetic least squares (PGLS) regression to analyze potential relationships among latitude, climatic variables, ED, and leaf/reproductive characters; our analyses were conducted with scripts described in Leslie et al. (2014) that incorporate phylogenetic covariance into regression calculations.

To compare ancestral state reconstructions based on the extant Podocarpaceae phylogeny with fossil data, we compiled leaf and seed size measurements from published studies of fossil taxa (see Supplementary Appendix I for sources). We included leaf measurements from shoots that were either in organic connection with fossil reproductive structures or were interpreted to belong to the same plant based on strong and consistent association; we did not compile a general database of possible fossil Podocarpaceae leaves due to uncertainty about whether all such leaves belong to the group. We focused on Mesozoic seed and leaf sizes for this study, as Cenozoic Podocarpaceae reproductive structures are rare and similar to extant genera where known (Hill and Carpenter, 1991; Wilf, 2012). We also compared the timing of shifts in Podocarpaceae reproductive structures to the avian fossil record using a compilation of avian body sizes spanning the Late Jurassic through to the recent. This compilation was assembled by combining body mass estimates for Mesozoic birds from Benson et al. (2014) and Benson et al. (2018) with measurements of Cenozoic and extant birds from Mitchell (2015). Body masses

of Cenozoic birds were estimated using a linear model resulting from regression of \log_{10} body mass on \log_{10} femoral length in extant birds, using phylogenetic regression (Grafen, 1989) implemented in the R packages nlme 3.1–159 (Pinheiro et al., 2022) and ape version 5.6.2 (Paradis and Schliep, 2019). We note that most data on extinct birds comes from a handful of exceptional fossil-bearing deposits (e.g., Mayr, 2009; Zhou, 2014), and so does not represent a continuously-sampled time series. Nevertheless, these data allowed us to constrain the timing of the appearance and diversification of small-bodied stem or crown birds (Avialae). Small-bodied birds are involved in podocarp seed dispersal today (Geldenhuijs, 1993; Willson et al., 1996) and have the potential to have done so since their early evolutionary history (Tiffney, 2004; Hu et al., 2022).

Results

Leaf area, seed length, and cone morphology are variable across extant Podocarpaceae (Figure 2A). Deep-branching, species-poor lineages (i.e., those with high ED) tend to occur at higher southern latitudes (Figure 2B; $\beta = -0.001$, $R^2 = 0.04$, $p = 0.01$ using PGLS with a Brownian motion model of phylogenetic trait covariance) and often produce multi-seeded cones (Figure 2A). Temperate taxa superficially appear to produce smaller seeds and leaves compared to tropical ones (Figure 2C), but these relationships are not statistically significant using PGLS ($\beta = -0.012$, $R^2 = 0.005$, $p = 0.40$ and $\beta = -2.23$, $R^2 = 0.003$, $p = 0.49$ for seed length and leaf area regressed on latitude, respectively). Small seeds are associated with small leaves in extant Podocarpaceae (Figure 2D; $\beta = 1.74$, $R^2 = 0.12$, $p < 0.001$ using PGLS regression of seed length on \log_{10} -transformed leaf area; also significant if using untransformed leaf area) although the relationship is not linear; large seeds occur only in taxa with relatively large leaves while small seeds occur in large-leaved taxa as well. Of the climate variables that we included for analysis, only precipitation seasonality (BIO15) was significantly correlated with seed length using a PGLS regression ($p < 0.001$), where precipitation seasonality was positively associated with larger seeds, albeit weakly ($\beta = 0.042$, $R^2 = 0.10$).

Ancestral state reconstructions based on neontological data suggest that small leaves, small seeds, and multi-seeded cones were ancestral in crown Podocarpaceae with varying degrees of uncertainty (Figure 3; Supplementary Figures 1, 2). Including an outgroup in the analysis does not appreciably change ancestral state reconstructions for deep nodes in crown Podocarpaceae (Supplementary Figure 1). Reconstructions of binned leaf area strongly support small leaves as ancestral (Figure 3A; Supplementary Figure 1A) with six shifts to larger leaves: on the lineage leading to the *Podocarpus+Retrophyllum+Afrocarpus+Nageia* clade (1), along

TABLE 1 Comparison of trait evolution models.

Model type	Seed size cutoff at 50th percentile			Seed size cutoff at 25th percentile		
	Trait	LogLik	AIC	Trait	LogLik	AIC
Equal Rates (ER)	Leaf Size	-33.93		Leaf Size	-33.93	
	Seed Size	-65.66		Seed Size	-41.86	
	Multi-seeded cone	-15.13		Multi-seeded cone	-15.13	
	Independent Sum	-114.72	235.44	Independent Sum	-90.92	187.84
	Leaf+Seed+MSC	-100.52	203.04	Leaf+Seed+MSC	-85.53	173.06
	Leaf+Seed+MSC*	-100.70	203.40	Leaf+Seed+MSC*	-85.34	172.68
All Rates Different (ARD)	Leaf Size	-31.52		Leaf Size	-31.52	
	Seed Size	-63.90		Seed Size	-41.86	
	Multi-seeded cone	-14.61		Multi-seeded cone	-14.61	
	Independent Sum	-110.03	232.06	Independent Sum	-87.99	187.98
	Leaf+Seed+MSC	-88.23	196.46	Leaf+Seed+MSC	-67.22	154.44
	Leaf+Seed+MSC*	-84.98	189.96	Leaf+Seed+MSC*	-62.40	144.80

The best supported models (in bold) by the Akaike information criterion (AIC) are all rates different (ARD) combined character state (correlated) models with root states of small leaves, small seeds, and multi-seeded cones (MSC). These are the best supported models regardless of whether a 50th or 25th percentile cutoff is used to define “small” and “large” seeds. *Indicates a model where the root state was fixed.

stem *Falcatifolium* (2), along stem *Acmopyle* (3), along the lineage leading to *Saxegothaea* (4), along stem *Prumnopitys* sensu lato (the *Pectinopitys+Prumnopitys+Sundacarpus*; 5), and along stem *Phyllocladus* (6). Dating these shifts is imprecise due to wide age uncertainties [given as 95% height posterior density (HPD)] on nodes in the dated phylogeny; we show the range in median estimated ages for the two nodes along which each shift in leaf size was inferred to have occurred and the full age uncertainty associated with these pairs of nodes (i.e., the maximum HPD age for the older node and the minimum HPD age for the younger node; **Figure 3A**). Some of these estimated dates are uninformative; for example, the shift to larger leaves in extant monotypic *Saxegothaea* could have occurred at any point between the Jurassic and the present (**Figure 3A**; shift 4). But potential age distributions for more informative shifts are centered in the Late Cretaceous and Cenozoic.

In contrast to leaf size, the ancestral state for binned seed size is uncertain, with nearly equal probabilities inferred for most deep nodes in the Early Cretaceous if the “small” and “large” distinction is made at the 50th percentile seed length (**Figure 3B**). Adding to this uncertainty, different binning schemes produce different results; “small” seeds are reconstructed as the ancestral condition with higher probability if the 25th length percentile (4.5 mm) is used as the cutoff (**Supplementary Figure 2B**). In contrast, a very strict “small” seed cutoff at the 10th percentile (3.375 mm) results in strong support for “large” seeds as ancestral (**Supplementary Figure 2C**), although many of the “large” seeds here are objectively quite small. Given this ambiguity, we did not attempt to quantify or summarize possible ages for state shifts, although the distribution of “small” and “large” seeds at both

the 50th or 25th percentiles across the Podocarpaceae tree does qualitatively mirror shifts inferred for leaf size (**Figures 3A,B**; **Supplementary Figures 2A,B**).

Ancestral state reconstruction provides a more robust estimate for multi-seeded cones as ancestral (**Figure 3C**), with five likely shifts to reduced (1–2 seeded) structures in crown Podocarpaceae: along the lineage leading to the last common ancestor of *Acmopyle* and the *Podocarpus+Dacrydium* clade (1), along stem *Prumnopitys* sensu lato (2), along the lineage leading to *Parasitaxus* (3), along stem *Halocarpus* (4), and along stem *Lepidothamnus* (5). The inferred dates for these shifts are again highly imprecise, but several of them are older than inferred shifts in leaf morphology with age distributions centered around the Early Cretaceous (**Figure 3C**). Similar results are found by stochastic character mapping, with five shifts to reduced seed cones in the most common inferred pathway (**Figure 3D**; “Configuration 1”: 70% of simulated histories). A smaller fraction of simulated histories reconstruct six shifts, either separate shifts in *Acmopyle* and the *Podocarpus+Dacrydium* clade (“Configuration 2”) or separate shifts in stem *Prumnopitys* sensu lato (“Configuration 3”). In all common character histories there is a high probability of shifts to reduced seed cones first occurring in the Early Cretaceous, with additional shifts in the Cenozoic (**Figure 3D**).

Leaf size, seed size, and cone type also appear to evolve in concert, as combined character state models of leaf area, seed length, and cone type were better supported than independent models (**Table 1**; **Supplementary Table 1**). Combined models of character pairs (leaf size + seed size, leaf size + cone type, seed size + cone type) are all better supported than independent models for the evolution of these character pairs, regardless of whether a 50th or 25th percentile length cutoff

was used to define “small” and “large” seeds (Supplementary Table 1). Among the three-character combined states, an all rates different (ARD) model where small leaves, small seeds, and multi-seeded cones was fixed as the root or ancestral condition was the best supported, regardless of seed size binning (Table 1 and Figure 4). These models suggest the ancestral condition evolved along separate pathways, one path reversibly leading to large-leaved taxa with small, multi-seeded cones (e.g., *Saxegothaea*) and the other unidirectional path leading to small-leaved taxa with small-seeded, reduced cones (e.g., *Dacrydium*). This latter state is then inferred to unidirectionally shift to larger leaves, which in turn ultimately gives rise to the combination of large leaves, large seeds, and reduced cones (Figure 4A) that is most abundant among extant Podocarpaceae (e.g., *Podocarpus*). Results are generally similar for a 25th percentile seed size cutoff, although here shifts to and from an additional combined state occur (Figure 4B).

For continuous measurements of leaf area and seed length, ancestral state reconstruction is largely uninformative for nodes with Mesozoic estimated divergence times (Figure 5). For these nodes, leaf area is reconstructed as intermediate between that of extant “small” and “large” leaves, with median values close to our binning threshold size of 10 mm² (Figure 5A). These estimates are associated with extremely wide confidence intervals, however, spanning nearly the entire range of extant leaf sizes. Leaves from Mesozoic fossil Podocarpaceae are considerably smaller than reconstructed median values and similar in size to extant taxa that produce small, imbricated scale leaves (e.g., *Dacrydium*, *Halocarpus*, and *Lepidothamnus*), apart from one taxon (*Mataia*) whose Podocarpaceae affinities are not definitive given its seed cone (Figure 5A). Confidence intervals on estimated seed lengths for Mesozoic nodes are likewise wide (Figure 5B), and known fossil seeds are considerably smaller than reconstructed median values for contemporaneous nodes in the molecular tree (Figure 5B). The total number of fertile scales (for fossils this was assessed as the total number of seeds) produced per reproductive structure changes through time (Figure 5B), with highly reduced cones producing 1–3 seeds appearing by around 120 Ma. Although the Cenozoic record of Podocarpaceae reproductive structures is extremely sparse, available representatives (*Dacrydium puertae*, *Podocarpus witherdenensis*) are nearly identical in morphology to extant taxa and fall within typical modern seed sizes (Figure 4B).

Body mass data show that small-bodied birds weighing less than 100 grams, reflecting the modal body size of extant birds (~30 grams; e.g. Maurer, 1998), first appeared by the mid Early Cretaceous, approximately 125 million years ago (Figure 5C). These body masses are much smaller than any other theropod dinosaurs (which have minimum adult body masses of several hundred grams) and are much more similar to body masses of extant and early Cenozoic birds (Figure 5C). It is also noteworthy that the initial appearance of extremely small birds

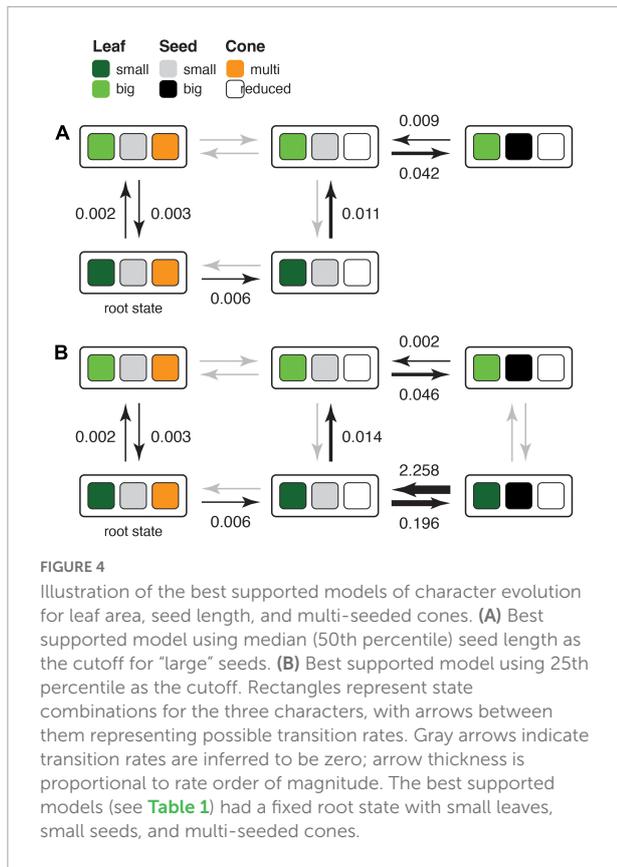
closely matches the initial appearance of reduced Podocarpaceae seed cones (Figure 5B).

Discussion

Congruence between molecular analyses and the fossil record

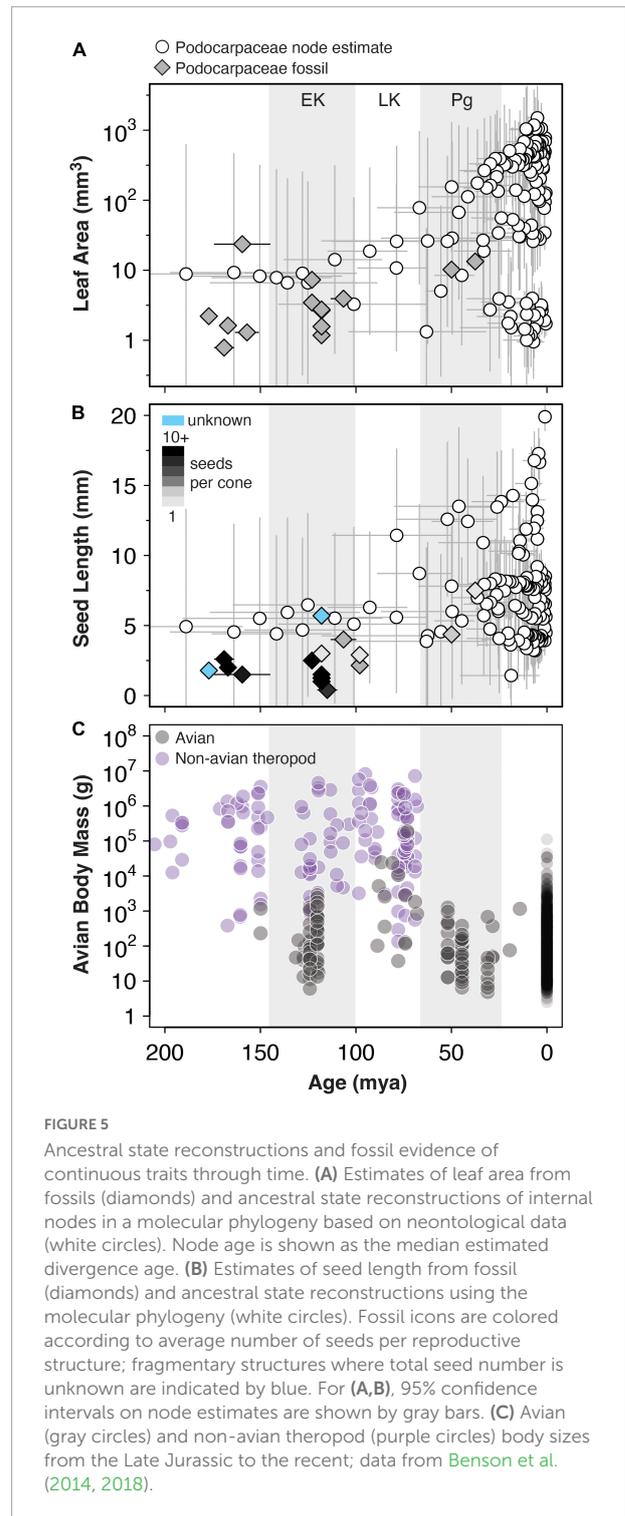
Both the phylogenetic structure and the fossil record of Podocarpaceae provide information about the evolutionary history of the group. Both have important limitations, however: ancestral state reconstructions of deep nodes and estimated ages of character transitions based on molecular phylogenies are imprecise, while the fossil record is poorly sampled and the phylogenetic affinities of most fossils are unknown. Nevertheless, both data sources tell a congruent story for reproductive evolution in the group: the molecular phylogeny suggests the ancestral state was multi-seeded cones bearing small seeds, consistent with the morphology of Jurassic and many Cretaceous fossil taxa (Vishnu-Mittre, 1959; Harris, 1979). Ancestral state reconstructions and transition rate models suggest that Podocarpaceae clades first evolved reduced cones with one or two seeds in the Early Cretaceous, and that seeds in these lineages remained relatively small. This inference is consistent with the fossil record, where reduced podocarp cones with one or two seeds first appeared as fossils during the middle of the Early Cretaceous, around 120 Ma (Figure 5B). Although confidence intervals are extremely wide for reconstructions of specific leaf and seed sizes, mean estimates for Jurassic and Early Cretaceous nodes are not radically different from fossil data, particularly for seeds (Figures 5A,B). Comparing the Late Cretaceous and Cenozoic history of the group is more challenging given the lack of reproductive fossils (Figure 5B), but the few that do exist are consistent with patterns inferred from the molecular tree; the largest known podocarp seed in the fossil record comes from the Late Eocene and was tentatively placed in crown *Podocarpus* (Hill and Carpenter, 1991), which is inferred by these molecular analyses to have been diversifying around this time.

Whether the temporal congruence between our inferences from molecular phylogenies and the fossil record is coincidental is difficult to assess; a single new Jurassic Podocarpaceae fossil with a reduced seed cone would complicate the concordance between molecular reconstructions and the fossil record with regards to seed number, as would molecular trees with different estimated divergence ages. Indeed, inferred Podocarpaceae divergence dates do vary considerably among studies; several (Quiroga et al., 2016; Chen et al., 2022) estimate older ages than our analysis while others estimate younger dates (Biffin et al., 2012; Klaus and Matzke, 2020). In these studies, the earliest shifts to reduced cones (corresponding to shifts 1 and 2 in Figure 3C) would have most likely occurred either between the



Early to Late Jurassic (older inferred ages) or the middle to Late Cretaceous (younger ages). Taken at face value, the fossil record suggests that Jurassic splits between crown genera (e.g., [Quiroga et al., 2016](#); [Chen et al., 2022](#)) are too old; with the exception of an undescribed possible *Lepidothamnus* from the mid-Cretaceous ([Khan et al., 2022](#)), the first fossils unambiguously assigned to extant genera appear only in the latest Cretaceous to Paleogene ([Hill and Brodribb, 1999](#); [Wilf, 2012](#); [Wilf et al., 2017](#)). On the other hand, the phylogenetic position of Jurassic and Cretaceous Podocarpaceae (e.g., [Vishnu-Mittre, 1959](#); [Archangelsky and Del Fueyo, 1989](#)) is unresolved; they could in theory represent stem members of extant genera and it could then be argued that the Mesozoic fossil record is simply uninformative as to when deep divergences among extant clades and genera occurred. Determining the timing of major splits within crown Podocarpaceae therefore remains an ongoing challenge in understanding their evolution.

But beyond challenges with dating, the fossil record is congruent with reconstructions based on the molecular phylogeny regarding the sequence of character state transitions in Podocarpaceae cone evolution, from initially multi-seeded cones with small seeds to reduced cones with large seeds. From the molecular perspective, this sequence of events is based primarily on topology, which varies less among the phylogenetic studies. Small multi-seeded cones are also the



most likely ancestral state combination from a fossil perspective, as all Mesozoic fossil taxa produce small seeds and multi-seeded cones are undoubtedly more common among early taxa. A general taphonomic bias against the preservation of small, reduced seed cones is likely because they are fragile, but such

structures nonetheless do occur in the fossil record and there is no reason to expect that this bias would suddenly change halfway through the Early Cretaceous to favor the preservation of reduced cones, which are the only type known in the subsequent fossil record (Figure 5B). The fossil record then suggests that extant Podocarpaceae clades do accurately record aspects of the Cretaceous reproductive evolution of the group, with deep divergences among living lineages sampling major reproductive character state changes occurring in the Mesozoic, especially among currently depauperate lineages that appear to show long-term stasis in reproductive morphology.

Seed cones, birds, and forests in Podocarpaceae reproductive evolution

If the fossil record and our molecular reconstructions tell a congruent story of Cretaceous reproductive evolution in Podocarpaceae, what factors might have favored a shift toward reduced cones, and later, larger seeds? Our findings raise the possibility that the diversification of stem-birds acted as an initial driver of reproductive changes in the Early Cretaceous. Birds are regarded as the primary seed dispersal agents for Podocarpaceae today (e.g., Geldenhuys, 1993; Willson et al., 1996; Klaus and Matzke, 2020) and there is a general correlation between the size of plant reproductive structures and the size of vertebrates that consume them (Wheelwright, 1985; Burns, 2013; Chen and Moles, 2015). The diversification of a new group of small vertebrates with the potential for frugivory should then be highly relevant for Podocarpaceae cone size evolution. We suggest in particular that early birds favored the evolution of reduced cones, consisting of a few seeds and adhering fleshy tissues, that could function as small, easily ingestible disseminules. The reduction in seed number may have been favored in order to maintain relatively small disseminule sizes regardless of seed size (Leslie et al., 2017). For example, multi-seeded cones that can be ingested whole by small animals such as birds will necessarily contain very small seeds (e.g., extant *Microcachrys*). Evolving larger seeds (even if they were still “small” based on the thresholds used in this analysis) while minimizing total disseminule size could be accommodated by reducing the number of seeds per cone, a trade-off that appears across gymnosperms (Leslie et al., 2017). The shift toward fewer seeds per cone in Early Cretaceous Podocarpaceae may then reflect a new selective regime dominated by small-bodied animal dispersal agents like birds.

The timing of this change in cone morphology is consistent with the avian fossil record. The bird crown-group evolved slightly more than 70 million years ago, during the latest Cretaceous (Prum et al., 2015; Field et al., 2020), but was preceded by the origins of highly bird-like stem lineages during the Early Cretaceous (e.g., O’Connor et al., 2011; Zhou, 2014; Benson, 2018). These taxa, belonging to the group Pygostylia

and its subclade Ornithoraces (which includes the diverse Mesozoic group Enantiornithes) had high species richness from shortly after their origin (Benson, 2018). They also possessed many bird-like flight-related and dietary features (O’Connor et al., 2011; O’Connor, 2019; Miller and Pittman, 2021) as well as small body sizes similar to those of extant birds (Figure 5C; Benson et al., 2018). Pygostylians were therefore equivalent to extant birds in at least some important aspects of their ecology and diversity, and unlike any previous groups of non-avian dinosaurs. Moreover, several Early Cretaceous stem-birds include seeds in their fossilized gut contents (Zhou and Zhang, 2002; Zheng et al., 2011, 2018). The affinity of these seeds is unknown, but there is strong evidence in at least one taxon (Hu et al., 2022; see also Ksepka et al., 2019; Mayr et al., 2020) that their presence reflects frugivory and mutualism rather than seed predation. Together, the observations above are consistent with the potential for bird-plant mutualisms as an important driver of plant evolution from the Early Cretaceous onwards (and see Tiffney, 2004).

Klaus and Matzke (2020) likewise suggested that bird diversification in the Cretaceous drove the evolution of seed cone morphology in Podocarpaceae, although they focused on fleshiness rather than seed number. We found that the evolution of fleshiness was more difficult to reconstruct and interpret than seed number. If treated as a single trait, fleshiness was the likely ancestral state for crown Podocarpaceae (results not shown; see also Herting et al., 2020; Khan et al., 2022). But specific fleshy tissues across Podocarpaceae are variable (including epimatia, arils, and different bracts; see Contreras et al., 2017; Khan et al., 2022), suggesting either that the ability to produce fleshy cone tissues evolved once and has been expressed in many organs around the cone or that fleshiness evolved independently many times. Podocarpaceae cones that were apparently fleshy do occur throughout the fossil record, including Jurassic *Scarburgia* and *Harrisocarpus* (Harris, 1979; Reymanowna, 1987) and Cretaceous *Trisaccocladus* and *Bellingshausium* (Archangelsky, 1966; Cantrill and Falcon-Lang, 2001), but these taxa have not been placed phylogenetically and therefore shed little light on possible ancestral states. Nevertheless, these fossils suggest that fleshiness was a feature of podocarp reproductive structures prior to the reduction in seed number. To the extent that fleshiness is correlated with biotic seed dispersal (but see Fountain et al., 1989 for an alternative explanation), Podocarpaceae may have utilized animals prior to the diversification of stem birds, but the rapid radiation of a new lineage of small-bodied arboreal taxa would likely have expanded opportunities for animal seed dispersal syndromes and the range of successful dispersal morphologies, and with these the potential for long-distance seed dispersal in the group. The nearly synchronous appearance of small-bodied birds and reduced Podocarpaceae cones (Figures 5B,C) is suggestive that this is the case.

Following the evolution of reduced seed cones, the fossil record is too sparse to pinpoint when a shift toward larger seed sizes occurred. However, a general Paleogene increase in average seed size among plant communities has long been noted (Tiffney, 1984) and would be consistent with molecular-based reconstructions for Podocarpaceae. This increase has been attributed to the spread of closed-canopy angiosperm forests after the K-Pg extinction, which are thought to have favored increased investment in seeds as nutrient reserves for seedlings growing under low light (Eriksson et al., 2000; Eriksson, 2008) and/or promoted the diversification of frugivorous seed dispersers (Wing and Tiffney, 1987). As noted in the Introduction, the spread of Cenozoic angiosperm forests has been proposed as a driver of leaf evolution in Podocarpaceae (Biffin et al., 2012) and may explain the correlation between large leaves and large seeds (Figures 2D, 4); low-light ecosystems that favor large leaves would presumably also favor investment in large seeds. Although such a mechanism is intuitive, extant species do not provide much support for it; Podocarpaceae seed size shows no significant relationship with tropical latitudes (Figure 2C) and is correlated only with high precipitation seasonality, a feature not typically associated with warm and wet early Cenozoic forests. The data used in this study may not be appropriate to tease apart these patterns, however; latitude does not necessarily indicate whether a species lives in a closed-canopy forest and our climatic data is of low resolution in general (species-wide averages of 100×100 km grid cells). More finely resolved Podocarpaceae data sets would be needed in order to assess current climatic and ecological correlates of seed size more fully. Furthermore, current species climatic tolerances and dispersal strategies may be far removed from conditions early in the history of modern genera; a focus on reproductive material, seed size, and paleoclimate from Paleogene Podocarpaceae (e.g., Hill and Carpenter, 1991; Wilf, 2012) may therefore provide a more accurate ecological context for understanding their early Cenozoic evolution.

Conclusion

Although molecular and fossil data regarding the deep evolution of Podocarpaceae reproductive structures are sparse, they are complementary. Both suggest that cones, seeds, and leaves evolved as a suite of traits in a stepwise manner in response to changing ecological conditions from the Early Cretaceous through the early Cenozoic. First, cones evolved a reduced number of seeds in conjunction with a major radiation of stem birds in the middle Early Cretaceous. Next, large seeds then evolved in lineages with large leaves, most likely as forest ecosystems were reorganized in the wake of angiosperm dominance from the Late Cretaceous through the early Cenozoic. Given the poorly sampled Podocarpaceae fossil record and nature of inferences made from molecular phylogenies of extant taxa, this apparent congruence in

evolutionary history should be taken more as a hypothesis than a complete picture of Podocarpaceae evolution. Further testing this scenario requires better phylogenetic integration of fossil and living Podocarpaceae; incorporating more fossil taxa into total evidence analyses (e.g., fossilized birth-death; Heath et al., 2014) may help to refine estimated divergence ages, while focused phylogenetic studies of fossil Podocarpaceae should help to unravel the polarity of reproductive character shifts within the group.

Data availability statement

The original contributions presented in this study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

AL conceived of the study. AL and RB designed and conducted the analyses, contributed to write the manuscript. Both the authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2022.1058746/full#supplementary-material>

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