Resolution of ray-finned fish phylogeny and timing of diversification

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Ray-finned fishes make up half of all living vertebrate species. Nearly all ray-finned fishes are teleosts, which include most commercially important fish species, several model organisms for genomics and developmental biology, and the dominant component of marine and freshwater vertebrate faunas. Despite the economic and scientific importance of ray-finned fishes, the lack of a single comprehensive phylogeny with corresponding divergence-time estimates has limited our understanding of the evolution and diversification of this radiation. Our analyses, which use multiple nuclear gene sequences in conjunction with 36 fossil age constraints, result in a well-supported phylogeny of all major ray-finned fish lineages and molecular age estimates that are generally consistent with the fossil record. This phylogeny informs three long-standing problems: specifically identifying elopomorphs (eels and tarpons) as the sister lineage of all other teleosts, providing a unique hypothesis on the radiation of early euteleosts, and offering a promising strategy for resolution of the "bush at the top of the tree" that includes percomorphs and other spiny-finned teleosts. Contrasting our divergence time estimates with studies using a single nuclear gene or whole mitochondrial genomes, we find that the former underestimates ages of the oldest ray-finned fish divergences, but the latter dramatically overestimates ages for derived teleost lineages. Our time-calibrated phylogeny reveals that much of the diversification leading to extant groups of teleosts occurred between the late Mesozoic and early Cenozoic, identifying this period as the "Second Age of Fishes."

Actinopterygii | molecular clock | species tree | Teleostei | Percomorpha

Ray-finned fishes (Actinopterygii) are one of the most successful radiations in the long evolutionary history of vertebrates, yet despite the rapid progress toward reconstructing the Vertebrate Tree of Life, only 5% of the ray-finned fish phylogeny is resolved with strong support (1). Actinopterygii contains more than 30,000 species (2), with all but 50 being teleosts (3). Compared with other large vertebrate radiations, such as mammals (4) or birds (5), a general consensus on the phylogenetic relationships and timing of diversification among the major actinopterygian and teleost lineages is lacking (3, 6, 7). This uncertainty about relationships has prevented the development of a comprehensive time-calibrated phylogeny of ray-finned fishes, which is necessary to understand macroevolutionary processes that underlie their diversity.

Most working concepts of actinopterygian relationships are based on morphological data (6, 8), and unlike other clades of vertebrates, there has been no comprehensive effort to resolve the phylogeny of actinopterygians and teleosts using molecular data that sample multiple nuclear genes and include taxa that span the major lineages. Despite the long history of using morphological data in the phylogenetics of ray-finned fishes, there are several areas of uncertainty and disagreement regarding some of the most fundamental relationships. First, there are two competing hypotheses on the phylogenetic relationships that reflect the earliest diversification of teleosts: either the Osteoglossomorpha [bony tongues (9, 10)] or Elopomorpha [eels, tarpons, and bonefish (11, 12)] are the sister lineage of all other teleosts. Second, the relationships of lower euteleosts (e.g., salmons, smelts, pikes, slickheads, and galaxiids), or "protacanthopterygians," has changed frequently as a result of phylogenetic analyses of different morphological datasets (13–15). Third, with at least 16,950 species (2), the staggering diversity of spiny-rayed fishes, and particularly percomorphs, has impeded phylogenetic resolution of this lineage, prompting Nelson (16) to label the Percomorpha as the "bush at the top of the [teleost] tree."

Applications of molecular data to these three long-standing questions in teleost phylogenetics have yielded mixed results. For example, analyses of nuclear and mtDNA gene sequences have supported all three possible relationships among osteoglossomorphs, elopomorphs, and all other teleosts [i.e., clupeocephalans (17–20)]. Molecular phylogenies have agreed with morphological inferences that "protacanthopterygians" are not monophyletic (8, 13, 14, 19, 21, 22); however, molecular inferences resolve relationships, such as a clade containing salmonids (salmon and trouts) and esociforms (pikes and mudminnows) (21–23), which are not supported in analyses of most morphological datasets (13, 14). Investigations of percomorph phylogeny using molecular data have resulted in the exciting discovery of new clades, such as monophyly of tetraodontiforms (pufferfishes) plus lophiiforms (anglerfishes) (19, 24), and the resolution of an inclusive clade of more than 4,800 species, containing cichlids, atherinomorphs (silversides), blennioids (blennies), pomacentrids (damselfishes), embiotocids (surfperches), mugilids (mullets), and other less known lineages (25). However, molecular phylogenetic analyses that have sampled the most broadly among the disparate lineages of Percomorpha have not resulted in strongly supported resolution of the deepest nodes in the clade (19, 26, 27).

Resolution of phylogenetic relationships of teleosts is critical to understanding the timing of their diversification. Currently there is discordance between the estimated age of divergence for teleosts, as inferred from the fossil record and molecular studies. Fossils of four of the earliest teleost lineages (Elopomorpha, Osteoglossomorpha, Clupeiformes, and Ostariophysi), as well as stem-lineage euteleosts (e.g., †*Leptolepides*, † = an extinct taxon) appear in a very short time interval between the Late Jurassic and Early Cretaceous (11). In contrast, molecular and genomic inferences consistently indicate that there may be a gap in the fossil record of crown-lineage teleosts, as the age estimates for

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the most recent common ancestor of living teleosts range from 310 to 350 Ma based on whole mtDNA genome sequences (28), ~320 Ma based on comparisons of paralogous gene copies resulting from the teleost whole-genome duplication (WGD) event (29), and 173–260 Ma based on fossil-calibrated nuclear gene phylogenies (7, 19, 20). Although these studies estimated ages for the crown teleost clade that are older than the fossil record, molecular age estimates across ray-finned fish lineages include those that are older, as well as younger, than fossil-based estimates. For example, the fossil record implies an origin of crown-lineage actinopterygians in the Devonian, ~385 Ma (30). However, relaxed-molecular clock analyses of a single nuclear gene resulted in an age that is younger (299 Ma) than the so-called Devonian "Age of Fishes" [416–359 Ma (19, 20)]. Discordance between these molecular and fossil age estimates, along with uncertainty in the phylogeny, contribute to a lack of understanding of this fundamental aspect of vertebrate evolution.

We investigated phylogenetic relationships and divergence times of all major lineages of Actinopterygii and Teleostei using DNA sequences of nine unlinked protein-coding nuclear genes sampled from 232 species. We used 36 well-justified absolute time calibrations from the fossil record of ray-finned fishes in relaxed-molecular clock analyses to estimate divergence times. Phylogenies resulting from these analyses were well resolved, the majority of phylogenetic inferences were supported with strong node support values, were robust to inferences using new "species tree" methods, and provide a comprehensive molecular perspective on areas of long-standing disagreement and uncertainty in the relationships of teleost fishes. Divergence times estimated from relaxed-molecular clock analyses yield a comprehensive time-scale of actinopterygian diversification that is remarkably close to ages inferred from the fossil record.

Results and Discussion

Maximum-likelihood analyses of the nine nuclear gene dataset resolved 89% of the 232 nodes in the actinopterygian phylogeny with bootstrap replicate scores (BS) ≥70% and the phylogenies inferred using the Bayesian method had 91% of the nodes strongly supported posterior probabilities (BPP) ≥ 0.95 (Fig. 1, and Figs. S1 and S2). Relationships of nonteleostean actinopterygians were consistent with traditional morphologically-based inferences (6) with polypterids (bichirs and ropefish) resolved as the sister lineage of all other actinopterygians (Actinopteri) in the relaxed-clock analysis (Fig. 1). In addition, Acipenseriformes (sturgeons and paddlefishes) were the sister lineage of Neopterygii with strong support (BS = 100%, BPP = 1.00), and Holostei (bowfin and gars) was resolved as the sister lineage of teleosts [BS = 100%, BPP = 1.00 (Fig. 1, and Figs. S1 and S2)]. These results contrast with earlier molecular studies that either resolved acipenseriforms and holosteans as an "ancient-fish" clade (31) or acipenseriforms and polypteriforms as a weakly supported clade (32).

Our results provide resolution to three of the most compelling questions in teleost phylogenetics. The molecular phylogeny resulted in the strongly supported position (BS = 97%, BPP = 1.00) of elopomorphs as the sister lineage of all other teleosts (Fig. 1, and Figs. S1 and S2). This result is also strongly supported in a species tree analysis, which accounts for potential discordance among individual gene histories, with a bootstrap proportion of 100% (Fig. S3). Evidence for Osteoglossomorpha as the sister lineage of all other teleosts was based on the presence of a single character state in the caudal fin skeleton (9, 10). On the other hand, the hypothesis that Elopomorpha is the sister lineage of all other teleosts was based on eight derived character-state changes identified from optimization of a matrix containing 135 discretely coded morphological characters (11). Our results strongly support the latter hypothesis, illustrating agreement between phylogenetic inferences from a robust morphological data matrix and our densely sampled nuclear gene DNA sequence dataset.

With regard to the relationships of early euteleosts, our phylogenetic analyses support several results from previous molecular studies and a new result that places Galaxiidae as the sister lineage of Neoteleostei (without stomiiforms) [BS = 95%, BPP = 1.00 (Fig. 1, and Figs. S1 and S2)]. Lineages previously treated as "protacanthopterygians" (3) are polyphyletic in the molecular phylogeny because the alepocephaliforms (slickheads) are resolved in a clade containing clupeomorphs (anchovies and herrings) and ostariophysians (catfish and minnows) [BS = 94%, BPP = 1.00 (21, 33)], the enigmatic freshwater Australian species *Lepidogalaxias salamandroides* is the sister lineage to all other Euteleostei (15, 23) [BS = 100%, BPP = 1.00 (Fig. 1, and Figs. S1 and S2)], salmonids (trouts and salmon) and esociforms (pikes and mudminnows) are resolved as a clade [BS = 100%, BPP = 1.00 (21, 23)], and there is strong support for a clade containing stomiiforms (dragonfishes), osmeriforms (smelts), and retropinnids (southern smelts) [BS = 100%, BPP = 1.00 (23)]. Although most of these relationships were reflected in the species tree, *Lepidogalaxias* was resolved as the sister lineage of Galaxiidae (Fig. S3). However, only one of the two gene trees (*rag1*) that sampled both *Lepidogalaxias* and Galaxiidae resolved these lineages as sharing a common ancestor. The phylogenetic resolution of these early euteleost lineages using morphology is thought to have been hampered by a mosaic of highly modified and ancestral character states (3, 13). The relationships inferred in our trees provide a phylogenetic framework to investigate the evolution of morphological character state changes, which have proven difficult to use in the inference of relationships among early diverging euteleost lineages (e.g., ref. 34).

One of the most important problems in vertebrate phylogenetics is the resolution of the major lineages of Percomorpha. The phylogeny confirms several results presented in previous molecular analyses, including the resolution of ophidiiforms (cusk eels) and batrachoidids (toadfish) as early diverging percomorphs (25, 26), a clade containing tetraodontiforms and lophiiforms (19, 24), a clade dominated by percomorphs with demersal eggs that includes cichlids, pomacentrids, blennies, ricefishes, and silversides (Atherinomorpha) (25), and the revised placement of sticklebacks with scorpionfishes, eelpouts, and perches (Perciformes) rather than their historical placement with seahorses (24–27, 35). Our molecular phylogeny provides substantial resolution and node support for the deepest percomorph relationships (Fig. 1, and Figs. S1 and S2). The degree of resolution in our phylogeny among the earliest diverging percomorphs is encouraging, and holds promise that increased taxon sampling for these molecular markers will result in the phylogenetic resolution of both the deepest and the most apical nodes in the "bush on the top of the tree" that has long vexed vertebrate biologists (6).

The phylogenetic resolution offered by the nine nuclear gene dataset not only has broad implications for understanding the evolutionary history of actinopterygians, but also provide the necessary basis for estimating their divergence times. Molecular age estimates from the nine nuclear genes agree with published analyses using whole mtDNA genomes for older nodes and with the *rag1* nuclear gene for younger nodes (Fig. 2*A* and Table S1), which is reflected in the proportion of fossil calibrations shared between those studies and our relaxed-clock analyses (Fig. 2*B*). This finding offers an explanation and reconciliation for several points of disagreement observed between molecular age estimates for ray-finned fishes and the fossil record. For example, we estimate a Silurian-Devonian origin of extant Actinopterygii, between 438.9 and 383.4 Ma (Fig. 2*A* and Table S1), which is consistent with the first occurrences of crown actinopterygian fishes (e.g., †*Mimipiscis toombsi*) in the fossil record (30). This finding contrasts with previous efforts using *rag1* that estimated the age of living ray-finned fishes between 337 and 284 Ma

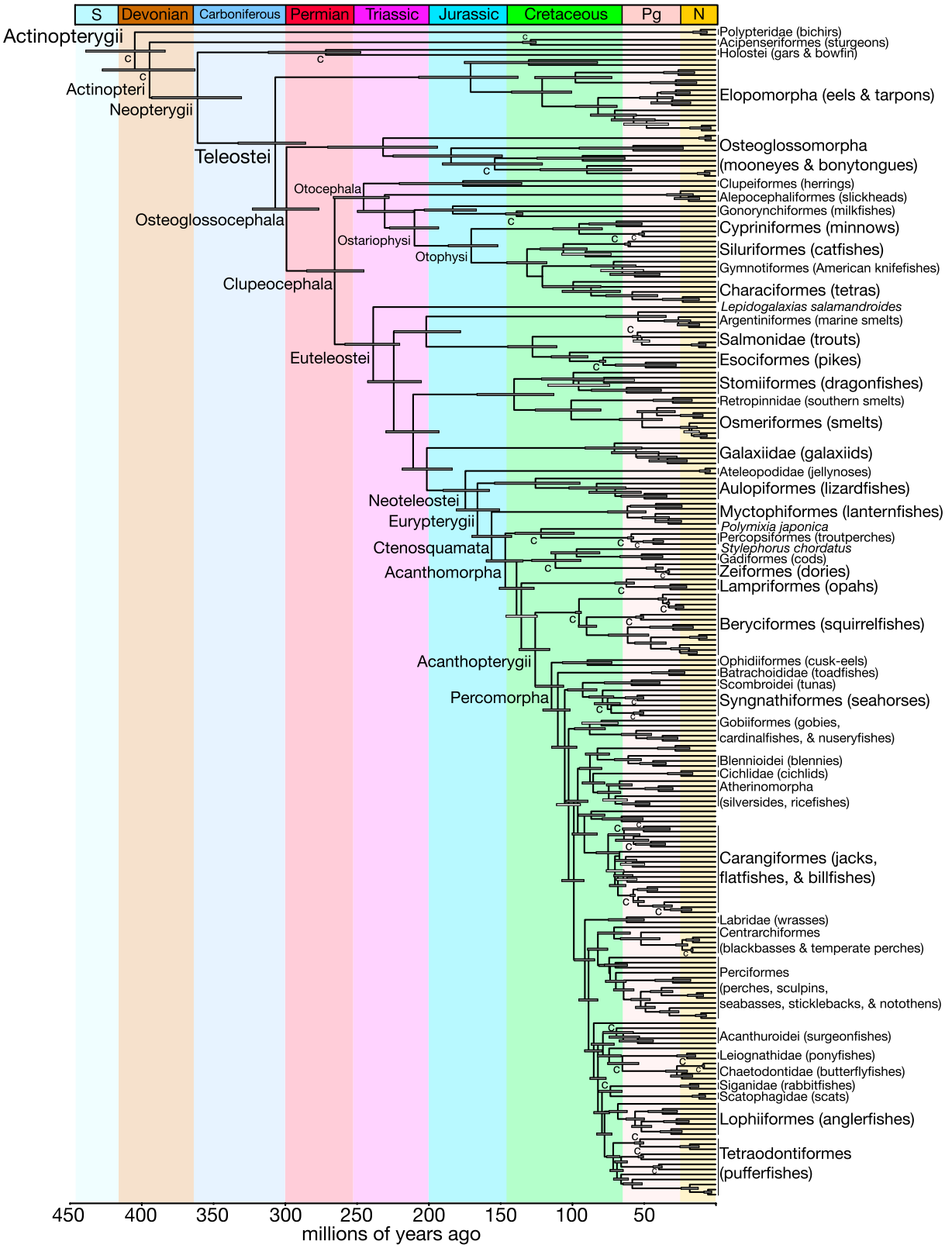

Fig. 1. Actinopterygian time-calibrated phylogeny based on nine nuclear genes and 36 fossil age constraints. Bars represent the posterior distribution of divergence-time estimates. Gray bars identify nodes supported with BPP $\geq$ 0.95, and white bars mark nodes with BPP $<$ 0.95. Nodes with age priors taken from the fossil record are marked with a "c." For full details on calibration see *Materials and Methods* and Fig. S2. The time-calibrated tree is scaled to the geological time scale with absolute time given in millions of years.

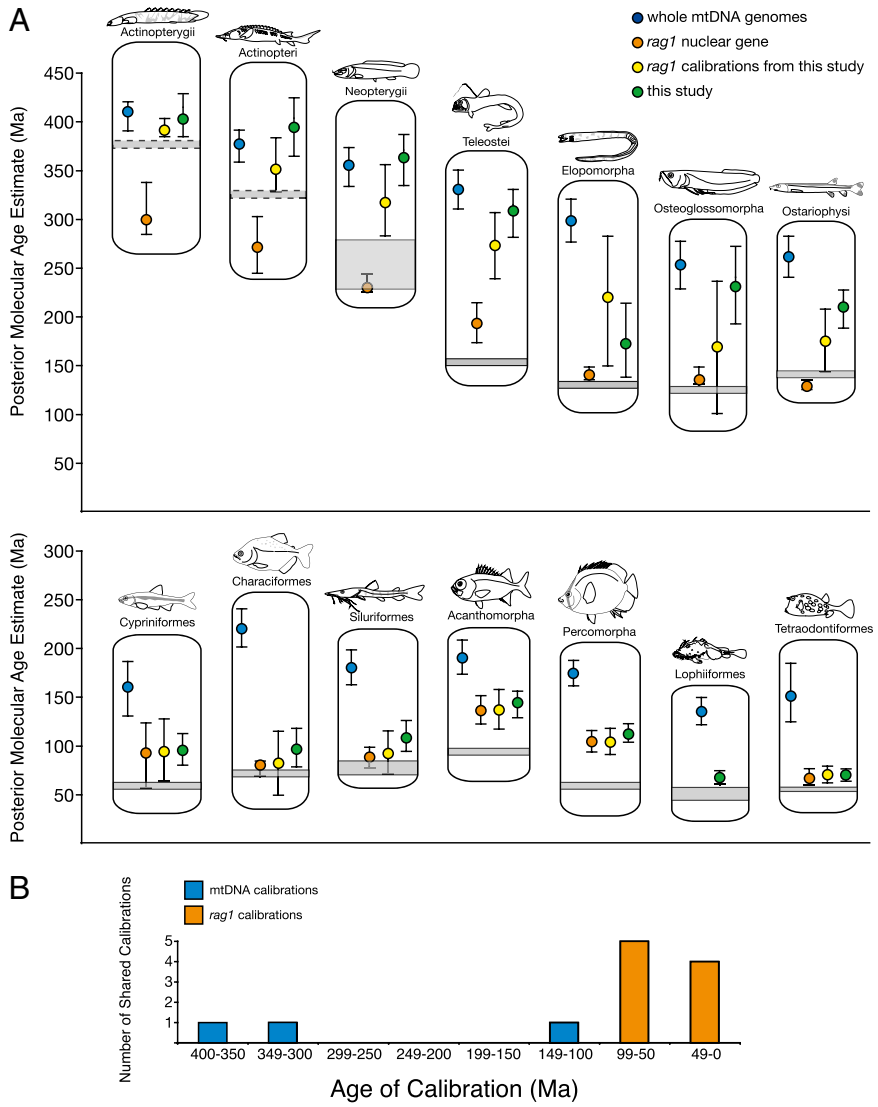

Fig. 2. Posterior distribution of molecular age estimates and patterns of calibration sharing across studies of ray-finned fish phylogeny. (*A*) Posterior distribution of molecular age estimates, in millions of years, for 14 actinopterygian lineages, resulting from analyses of whole mtDNA genomes (blue), the *rag1* nuclear gene (orange), the *rag1* nuclear gene using the calibrations from this study (yellow), and the nine nuclear gene dataset presented in this study (green). The circle represents the mean of the posterior estimate and the whiskers mark the upper and lower 95% highest posterior density of the age estimates. Gray boxes mark the oldest fossils for a given lineage, those with dashed lines were used as calibration age priors (see *Materials and Methods*) and those with solid black lines were not used as age calibrations. Line drawings of ray-finned fish species are based on photographs of specimens housed at the Peabody Museum of Natural History, Yale University, New Haven, CT. (*B*) Frequency of calibrations shared between this study and those using whole mtDNA genomes (blue) and the *rag1* nuclear gene (orange) binned by the age of the fossil calibration in millions of years (Ma).

in the Carboniferous-Permian [Fig. 2*A* (19, 20)]. This discrepancy is likely because of the use of the putative neopterygian †*Brachydegma caelatum*, which dates to the Early Permian (Artinskian-Sakmarian boundary) ∼284 Ma (7), as a minimal age calibration for the crown actinopterygian clade in the *rag1*-based studies (19, 20). Using the same *rag1* gene with our calibration strategy, we estimated the age of ray-finned fishes between 402.3 and 384.3 Ma in the Devonian (Fig. 2*A*). Similarly, discrepancies between our molecular age estimates and those obtained from whole mtDNA genome analyses may be because of the use of entirely different sets of fossil calibrations that are younger than 100 Ma (Fig. 2*B*), and their application of biogeographic calibrations that constrain the ages of the derived percomorph lineage Cichlidae to correspond with specific events in the fragmentation of Gondwana that range between the Late Jurassic and Late Cretaceous [145–85 Ma (28)].

In general, published molecular age estimates for derived teleost lineages using whole mtDNA genomes are much older than the known fossils for these clades, implying the existence of substantial gaps in the fossil record that often exceed 100 Ma [Fig. 2*A* (28, 36)]. However, our molecular age estimates reject the necessity for invoking such temporally large gaps in the geological record, as our age inferences are much closer to the fossil age estimates (Fig. 2*A* and Table S1). For example, the published mtDNA age estimate for crown tetraodontiforms (pufferfishes and relatives) span the Cretaceous and Jurassic between 184 and 124 Ma (37), but the earliest definitive fossils assigned to this lineage are from Paleogene deposits in the late Paleocene (Thanetian) ∼59–56 Ma (38). The lower bound of our age estimate for crown tetraodontiforms is less than 10 Ma older than these earliest fossils (Fig. 2*A* and Table S1). The same pattern of substantial difference between our age estimates and those using whole

mtDNA genome sequences was observed for the most recent common ancestors of Cypriniformes (minnows), Characiformes (piranhas and tetras), Siluriformes (catfishes), Acanthomorpha (spiny-rayed fishes), Percomorpha (perch-like fishes), and Lophiiformes (anglerfishes), with our estimates being much closer to the oldest known fossils of these lineages (Fig. 2*A* and Table S1). We obtained these results without using any of the fossil ages for these younger lineages as calibrations in our study.

The reconciliation of molecular divergence time estimates with ages implied by the fossil record allows us to investigate the age of teleosts, which has proven difficult to infer using paleontological information (11). We estimated that crown lineage teleosts first diverged during the Carboniferous to early Permian (Fig. 2*A*) (333.0–285.8 Ma), following the Devonian Age of Fishes. This estimate agrees with analyses of whole mtDNA genomes (28) and the assessment of a WGD event occurring in teleosts (29). The credibility of teleosts diversifying in the Paleozoic was challenged by analyses of the *rag1* nuclear gene that estimated teleosts diversified during the Late Triassic to Middle Jurassic (20). However, when we analyzed the *rag1* locus using the set of calibrations presented in this study, the age of teleosts shifted nearly 100 Ma, ranging from the Carboniferous to Early Triassic (305.6–237.3 Ma) (Fig. 2*A*). A Paleozoic origin for crown teleosts differs considerably from estimates based on paleontological data. The earliest fossil representatives of the teleost crown are Late Jurassic elopomorphs and ostariophysians, and these are preceded by a series of stem-teleost clades that appear between the Late Triassic and Middle Jurassic, and in roughly the temporal sequence dictated by phylogeny (11). If our molecular age estimates are accurate, then the first 100 million years of crown-teleost history is absent from the fossil record. This "teleost gap" has been noted in previous relaxed-molecular clock studies, which have attributed this discrepancy to a relatively poor record of ray-finned fishes in the latest Paleozoic (7). When taken together, our molecular age estimates, those of mtDNA based inferences, as well as the "genomic fossils" in the form of the WGD event, imply a missing record of crown teleost fossils from the Permo-Carboniferous to Middle Jurassic. We suggest that additional systematic work is needed on fossil fishes from this stratigraphic interval. If this gap in the teleost fossil record is genuine, it may be a direct consequence of a lack of suitable fossil deposits. The nearly 70-million-year span between the mid-Carboniferous and earliest Triassic is characterized by a paucity of species-rich fish *Lagerstätten* (exceptional fossil deposits yielding abundant articulated material), with existing sites of this age subject to comparatively little research (39). We hope that the recurring disagreement between timescales for the emergence of crown teleosts based on molecular and fossil datasets will encourage renewed paleontological research on this critical stratigraphic interval.

Despite the apparent gap in the fossil record for early crown–group teleosts, we find that most major teleost lineages originated in a period spanning the late Mesozoic into the early Cenozoic (Figs. 1 and 2*A*), which corresponds to patterns apparent in the fossil record (39). We identify this interval as the "Second Age of Fishes." The Devonian Age of Fishes is characterized by the presence of all major vertebrate lineages referred to as "fishes," both living and extinct [e.g., ostracoderms, placoderms, acanthodians, chondrichthyans, and so forth (40)]. Although this period in time appears to mark the origin of crown Actinopterygii (Figs. 1 and 2*A*), it does not correspond to the divergence of the major lineages that comprise the bulk of living actinopterygian biodiversity. Instead, the Second Age of Fishes represents the interval in geologic time where these species-rich lineages (e.g., otophysians and acanthomorphs) originated and eventually flourished, becoming the dominant vertebrate component of marine and freshwater habitats.

Ray-finned fishes include half of the entire species richness of vertebrates (2, 3), but had ranked last, by a wide margin, in the degree of phylogenetic resolution offered by available DNA sequence and genomic resources (1). Our phylogeny, based on a multilocus dataset, provides robust resolution and strong support across all major lineages of ray-finned fishes and teleosts. Additionally, our divergence time estimates reconcile inferences from paleontology with those obtained from other studies that used molecular methods, providing a molecular time scale that is more consistent with ages implied by the fossil record. This comprehensive molecular perspective on the evolutionary diversification of one-half of all vertebrate species provides DNA sequence data and calibration information from which to integrate resolution of clades at lower taxonomic levels (e.g., families) and estimate ages of actinopterygian lineages that lack a fossil record.

Materials and Methods

Collection of DNA Sequence Data and Phylogenetic Analyses. Standard phenol-chloroform extraction protocol or Qiagen DNeasy Blood and Tissue kits were used to isolate DNA from tissue biopsies sampled from 232 ray-finned fish species (Table S2). Previously published PCR primers were used to amplify and sequence an exon from each of nine nuclear genes [*Glyt*, *myh6*, *plagl2*, *Ptr*, *rag1*, *SH3PX3*, *sreb2*, *tbr1*, and *zic1* (22, 41)]. The genes were aligned by eye using the inferred amino acid sequences. No frame mutations or DNA substitutions that resulted in stop codons were observed in the aligned DNA sequences. The combined nine-gene dataset contained 7,587 base pairs.

Twenty-seven data partitions were designated that corresponded to the three separate codon positions for each of the nine genes. A GTR+G substitution model was used in a portioned maximum-likelihood analysis using the computer program RAxML 7.2.6 (42) run with the –D option. Support for nodes in the RAxML tree was assessed with a thorough bootstrap analysis (option –f i) with 1,000 replicates.

A species tree was inferred using gene tree parsimony implemented in the computer program iGTP (43). Individual gene trees estimated using RAxML were used as input files. Several rooting strategies were used. The individual gene trees were rooted using *Erpetoichthys calabaricus* or *Polypterus ornatipinnis*, except in three cases when these species were not sampled for a specific gene. In these cases the individual gene trees were rooted using *Scaphirhynchus platorynchus*, *Amia calva*, or *Atractosteus spatula*. A heuristic search using randomized hill climbing was performed to find the species tree that minimized the reconciliation cost for deep coalescence. This search was bootstrapped by performing it 100 times and bootstrap proportions for the resulting species trees were calculated using SumTrees in the DendroPy package (44).

Relaxed-Molecular Clock Analyses. Divergence times of ray-finned fish lineages were estimated using an uncorrelated lognormal (UCLN) model of molecular evolutionary rate heterogeneity implemented in the computer program BEAST v1.6.1 (45, 46). The nucleotide substitution models for the nine-gene dataset were partitioned by gene and codon as in the RAxML analysis above, but the UCLN molecular clock models were partitioned by gene. Thirty-six lognormal calibration priors from the fossil record of ray-finned fishes were used in the UCLN analyses (*SI Text*). To assess the rooting of the ray-finned fish phylogeny, the node representing the most recent common ancestor of Actinopteri was assigned a lognormal age prior and the monophyly of this clade was not enforced. Preliminary analyses resulted in monophyly of Actinopteri with a Bayesian posterior support = 1.0. A birth-death speciation prior was used for branching rates in the phylogeny. The BEAST analyses were run four times with each run consisting of 2.0×10^8 generations, sampling at every 5,000 generations. The resulting trees and log files from each of the five runs were combined using the computer program LogCombiner v1.6.1 (http://beast.bio.ed.ac.uk/LogCombiner). Convergence of model parameter values and estimated node-heights to their optimal posterior distributions was assessed by plotting the marginal posterior probabilities versus the generation state in the computer program Tracer v1.5 (http://beast.bio.ed.ac.uk/Tracer). Effective sample size (ESS) values were calculated for each parameter to ensure adequate mixing of the Markov chain Monte Carlo (ESS > 200). The posterior probability density of the combined tree and log files was summarized as a maximum clade credibility tree using TreeAnnotator v1.6.1 (http://beast.bio.ed.ac.uk/TreeAnnotator). The mean and 95% highest posterior density estimates of divergence times and the posterior probabilities of inferred clades were

visualized on the using the computer program FigTree v1.3.1 (http://beast.bio.ed.ac.uk/FigTree).

Fossil Calibration Age Priors. For each fossil calibration prior, we identify the calibrated node in the ray-fin fish phylogeny, list the taxa that represent the first occurrence of the lineage in the fossil record, describe the character states that justify the phylogenetic placement of the fossil taxon, provide information on the stratigraphy of the rock formations bearing the fossil, give the absolute age estimate for the fossil, outline the prior age setting in the BEAST relaxed-clock analysis, and provide any additional notes on the calibration (*SI Text*). Each calibration is numbered and the phylogenetic placement of the calibration is highlighted in Fig. S2.

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