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An evaluation of fossil tip-dating versus node-age calibrations in tetraodontiform fishes (Teleostei: Percomorphaceae)

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ABSTRACT

Time-calibrated phylogenies based on molecular data provide a framework for comparative studies. Calibration methods to combine fossil information with molecular phylogenies are, however, under active development, often generating disagreement about the best way to incorporate paleontological data into these analyses. This study provides an empirical comparison of the most widely used approach based on node-dating priors for relaxed clocks implemented in the programs BEAST and MrBayes, with two recently proposed improvements: one using a new fossilized birth–death process model for node dating (implemented in the program DPPDiv), and the other using a total-evidence or tip-dating method (implemented in MrBayes and BEAST). These methods are applied herein to tetraodontiform fishes, a diverse group of living and extinct taxa that features one of the most extensive fossil records among teleosts. Previous estimates of time-calibrated phylogenies of tetraodontiforms using node-dating methods reported disparate estimates for their age of origin, ranging from the late Jurassic to the early Paleocene (ca. 150–59 Ma). We analyzed a comprehensive dataset with 16 loci and 210 morphological characters, including 131 taxa (95 extant and 36 fossil species) representing all families of fossil and extant tetraodontiforms, under different molecular clock calibration approaches. Results from node-dating methods produced consistently younger ages than the tip-dating approaches. The older ages inferred by tip dating imply an unlikely early-late Jurassic (ca. 185–119 Ma) origin for this order and the existence of extended ghost lineages in their fossil record. Node-based methods, by contrast, produce time estimates that are more consistent with the stratigraphic record, suggesting a late Cretaceous (ca. 86–96 Ma) origin. We show that the precision of clade age estimates using tip dating increases with the number of fossils analyzed and with the proximity of fossil taxa to the node under assessment. This study suggests that current implementations of tip dating may overestimate ages of divergence in calibrated phylogenies. It also provides a comprehensive phylogenetic framework for tetraodontiform systematics and future comparative studies.

1. Introduction

Accurate calibration of molecular clocks to estimate divergence times relies critically on the interpretation of paleontological information, particularly on the inferred ages of fossils and their phylogenetic placement. Fossil taxa are typically used in morphological phylogenies as terminals and in molecular phylogenetics as constraints or priors for minimum or maximum ages on the divergence times of internal nodes (Asher et al., 2002, 2003; Donoghue, 1989; Kumar and Hedges, 1998; Zuckerkandl, 1987). Systematic studies of fossils present limitations arising from the variable nature of material preservation, which poses technical difficulties in character scoring and often results in morphological datasets with large proportions of missing data (Wiens, 2005). In spite of these challenges, including fossils in the analysis expands taxonomic sampling, ultimately improving phylogenetic accuracy by subdividing long branches, introducing ancestral character states, and arbitrating among hypotheses (molecular or morphological) based on extant taxa (Forey, 2004; Gauthier et al., 1988). But the best way to use fossils to inform tempo of evolution in molecular phylogenetic analyses continues to be a topic of debate (Benton and Donoghue, 2007; Ho, 2009; Parham et al., 2012).

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There has been significant discussion about the appropriate way to use temporal data from fossils for divergence time estimation (Parham et al., 2012; Pyron, 2011; Ronquist et al., 2012). The commonly used node-dating approach (e.g., BEAST; Drummond et al., 2012) requires identifying the oldest fossil that can be assigned with confidence to the youngest internal node, thus imposing the age of the fossil as a minimum age constraint. Occasionally, fossils are also used to derive maximum ages. Although the node-dating method is widely used in molecular phylogenetic studies, arguably it introduces several shortcomings. By only using the oldest fossils assignable to circumscribed groups, node dating discards potentially useful temporal and topological information from younger fossils. Underestimation of divergence times may erroneously result when clades are considerably older than their oldest known fossil (Heads, 2012). Misidentification of fossils or their incorrect placement in the phylogeny can lead to inaccurate age estimates (e.g., Parham and Irmis, 2008), and fossil assignment to crown versus stem lineages is not always straightforward (Lukoschek et al., 2012; Parham et al., 2012). The definition of maximum age bounds and other prior settings (e.g., parametric distributions) is usually based on subjective or arbitrary criteria (Benton and Donoghue, 2007; Heads, 2012; Hedges and Kumar, 2004; Ho and Phillips, 2009; Marshall, 2008; Müller and Reisz, 2005). Finally, use of fixed calibration points may result in artifacts such as exaggerated confidence in the tree topology and inferred ages of divergence (Ronquist et al., 2012).

Recently, a total-evidence or (hereafter) tip-dating method to use fossil calibrations for molecular clock analyses has been proposed by Pyron (2011) and Ronquist et al. (2012). This approach requires morphological data to simultaneously infer the placement of the fossil in the phylogeny and to calibrate the tree. Tip dating can include all available paleontological information (rather than just the oldest fossil for a given node), and it integrates over fossil-placement uncertainty while simultaneously incorporating fossil ages into the analysis. A major challenge in applying this method is the requisite to compile morphological character data for both extant and fossil taxa, which is complicated by the fact that for many groups the fossil record is extremely scarce and fragmentary. A somewhat contentious assumption of a "morphological clock" based on the Mk model (Lewis, 2001) to infer branch lengths for fossil taxa is at the core of tip-dating methods. In spite of these challenges, tip dating is a conceptually promising approach in phylogenetics, yet only a handful of empirical studies have used it (Near et al., 2014; Pyron, 2011; Ronquist et al., 2012; Slater, 2013; Wood et al., 2013). None of these studies has explored the impact of fossil placement in the accuracy and precision of divergence time estimates. It has been suggested that increasing the number of fossils included in the analysis may result in reduced uncertainty for age estimates and may help reduce dating error (Pyron, 2011; Ronquist et al., 2012; Wiens, 2009). But the effect on dating precision of adding or removing fossils has not been quantified with empirical data.

Most recently, another method has been proposed to calibrate molecular phylogenies based on the Fossilized Birth–Death Process (FBD) model (Heath et al., 2014). The FBD is a diversification model derived from a serially sampled birth–death process (Didier et al., 2012; Stadler, 2010). It has four major parameters (speciation rate, extinction rate, fossil recovery rate, and proportion of sampled extant species) to account for temporal uncertainty associated with the speciation events on the tree. In a strict sense, the FBD is a node-dating method (and treated as such hereafter) because it does not estimate fossil placement in the phylogeny using morphological data. However, it is similar to tip dating in the sense that it eliminates the specification of *ad hoc* calibration densities and it can use all fossils available for a given clade, not just the oldest taxon. The performance of the FBD has been evaluated using simulations and tested so far on a single empirical dataset for bears (Ursidae), showing that the method is accurate, precise, and robust to model assumption violations, such as unbiased sampling of fossils and extant taxa (Heath et al., 2014).

Here, we use tetraodontiform fishes as a case study to compare node-dating and tip-dating approaches for calibrating molecular phylogenies and estimating divergence times. The order Tetraodontiformes is a well-known group of mostly marine taxa found in temperate and tropical waters worldwide; it includes puffers (fugu), ocean sunfishes (molas), porcupinefishes, boxfishes, triggerfishes, filefishes, and their allies. Tetraodontiforms are spectacularly diverse, both taxonomically (ca. 450 species; Eschmeyer and Fong, 2013) and morphologically, and the group includes one of the best studied fossil records among teleosts (summarized in Santini and Tyler, 2003; Tyler and Santini, 2002; Tyler and Sorbini, 1996). The monophyly of Tetraodontiformes is supported on the basis of 28 morphological synapomorphies (Santini and Tyler, 2003) and the group has often been divided into three suborders: Triacanthodoidei (Triacanthodidae and Triacanthidae), Balistoidei (Balistidae, Monacanthidae, Aracanidae and Ostraciidae), and Tetraodontoidei (Tetraodontidae, Triodontidae, Diodontidae and Molidae). Recently, an alternative classification with six tetraodontiform suborders has been proposed on the basis of molecular data (Betancur-R. et al., 2013a; Betancur-R. et al., 2014), which we follow herein (see Table S1 for a comparison of classifications).

Because of its considerable familial diversity, several key relationships among tetraodontiform lineages remain controversial, including the higher-level relationships and the placement of some fossils in extant families (Alfaro et al., 2007; Holcroft, 2005; Lauder and Liem, 1983; Santini et al., 2013b; Santini and Tyler, 2003; Tyler, 1980; Tyler and Sorbini, 1996; Winterbottom, 1974; Yamanoue et al., 2008a, 2007). Previous studies that addressed relationships among tetraodontiforms and included morphological characters of extant and fossil taxa mostly agree on the circumscription of extant families (Santini and Tyler, 2003; Tyler, 1980; Tyler and Sorbini, 1996). However, molecular studies using RAG1, mtDNA, or mitogenomics presented strong discrepancies in the relationships among the families with regard to previous morphological hypotheses (Alfaro et al., 2007; Holcroft, 2005; Yamanoue et al., 2008a, 2007).

Time-calibrated phylogenies of tetraodontiform fishes using node-dating methods produced disparate estimates for their age of origin. Analyses of mitogenomic data (Yamanoue et al., 2011, 2008a) imply much older ages (159 Ma) than studies based on nuclear genes (78–55 Ma), or a combination of nuclear and mitochondrial genes (68–67 Ma; Alfaro et al., 2007). Part of this discrepancy may be a consequence of mtDNA saturation, a known cause that leads to underestimation of branch lengths for older lineages (Dornburg et al., 2014; Lukoschek et al., 2012; Mulcahy et al., 2012), but such disagreements in age seem too large to be explained by this factor alone. Recent multi-locus analysis of higher level relationships among bony fishes using many fossil calibration points, both inside and outside Tetraodontiformes, have estimated even younger dates for the origin of tetraodontiforms (Betancur-R. et al., 2013a; Near et al., 2013, 2012), suggesting that methodological issues and interpretation of fossil evidence are in need of a critical revision.

The goals of this paper are to (1) estimate a multi-locus phylogeny of tetraodontiform fishes complemented with morphological data of extant and fossil taxa in order to reassess the time scale of tetraodontiform evolution; (2) compare divergence times estimated via node- and tip-dating approaches, providing a direct empirical comparison of the most important phylogenetic-dating methods currently in use; and (3) explore some of the factors that can influence the accuracy and precision of tip dating in order to gain a better understanding of this new method.

2. Materials and methods

2.1. Taxonomic sampling

Tetraodontiform diversity is represented in this study by a sample of 132 species, comprising 95 extant and 36 fossil taxa (plus one outgroup, the caproid *Antigonia capros*). Of these, 75 taxa (57.2%) have only molecular data, 36 taxa (27.5%) have only morphological data, and 20 taxa (15.3%) have both molecular and morphological data (Fig. 1). The sample includes all ten families of extant and nine families of fossil tetraodontiforms (including † Plectocretacidae; see below). Extant families are represented by 5 species of Aracanidae (out of 13), 21 of Balistidae (out of 43), 7 of Diodontidae (out of 22), 3 of Molidae (out of 4), 12 of Monacanthidae (out of 107), 12 of Ostraciidae (out of 24), 25 of Tetraodontidae (out of 185), 5 of Triacanthodidae (out of 22), 4 of Triacanthidae (out of 7), and by *Triodon macropterus* as the only extant species of Triodontidae. Our analyses do not include a few fossils of relatively young age assigned to extant genera or species with rather incomplete material (not coded by Santini and Tyler, 2003). Subsequent to 2003, an additional 14 fossil tetraodontiform species with relatively entire skeletons have been described (e.g., Bannikov and Tyler, 2008a,b; Miyajima et al., 2014; Sorbini and Tyler, 2004; Tyler and Križnar, 2013), but have not been coded in the matrix and thus are not included herein. Fossil families follow the names in Tyler and Sorbini (1996), Tyler and Santini (2002), and Santini and Tyler (2003). A complete list of included material (132 taxa) and catalog numbers is given in Table S2 and Appendix 1.

2.2. Molecular and morphological data

DNA sequence data were compiled for one mitochondrial locus (16S ribosomal DNA), and 15 nuclear markers (EGR1, ENC1, GLYT, IRBP, MLL, MYH6, PLAGL2, PTR, RAG1, RH, SH3PX3, SIDKEY, SREB2, TBR1, and ZIC1). Table S3 provides detailed information on markers examined and abbreviations used throughout the text. The sequences examined herein were previously published in other studies (e.g., Betancur-R. et al., 2013a; Dornburg et al., 2008; Holcroft, 2005; Miya et al., 2003; Near et al., 2013; Santini et al., 2013a,b,c; Wainwright et al., 2012; see Appendix 1). Exon markers were aligned individually based on their underlying reading frame in Geneious Pro vR6 (Biomatters Ltd.) using the MAFFT aligner (Katoh and Standley, 2013). The 16S sequences were aligned with SATé II (Liu et al., 2012) using MAFFT and 25 iterations. The molecular dataset consisted of 14,648 sites for 95 species. In terms of coverage, 70% of the cells of the molecular data matrix have sequence data. All supplementary files listed herein, including figures, tables, and aligned data sets are available from http://datadryad.org, doi:10.5061/dryad.82k0q.

The morphological data were taken from the matrix compiled by Santini and Tyler (2003) that contains 210 characters, mostly

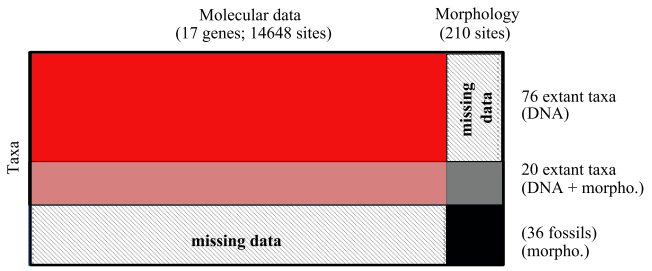

Fig. 1. Schematic representation of the combined data matrix used for the tip-dating (and other) analyses. Cells with missing data also exist in the molecular (red) and the morphological (black) partitions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

internal osteological and some external, for 20 extant and 36 fossil tetraodontiform taxa plus two outgroups (including the percomorph caproid *Antigonia capros* as the primary outgroup). The dataset is 50.3% complete, accounting for both missing data and ambiguities. No changes are introduced herein to the matrix of Santini and Tyler (2003). †*Plectocretacicus clarae* from the Cenomanian (ca. 97–95 Ma) of Lebanon is recognized as the oldest stem tetraodontiform fossil, and the sole representative of the family † Plectocretacicidae (Santini and Tyler, 2003; Tyler and Sorbini, 1996). The phylogenetic evidence presented by Santini and Tyler (2003) places the †Plectocretacicidae and †Protriacanthidae (whose sole representative is †*Protriacanthus gortanii*) as the sister group of the †Cretatriacanthidae (whose sole representative at that time was †*Cretatriacanthus guidottii*; an additional new genus and species was described by Tyler and Križnar (2013) from the Santonian of Italy; *ca.* 85 Ma). Together, these three families comprise the superfamily †Plectocretacicoidea (Tyler and Sorbini, 1996), which forms the most basal branch (stem lineage) of the Tetraodontiformes (Santini and Tyler, 2003). The placement of †*Plectocretacicus* within Tetraodontiformes is thus of major interest for phylogenetic studies, and it has been widely used for molecular clock calibrations of tetraodontiforms (Alfaro et al., 2007) and other groups (Benton and Donoghue, 2007; Betancur-R. et al., 2013a; Broughton et al., 2013).

More recently, some authors have questioned the placement of *Plectocretacicus clarae* within Tetraodontiformes based on unpublished morphological reassessments that suggest similarities with non-percomorph groups (Dornburg et al., 2014; Friedman et al., 2013; Santini et al., 2013a). While inclusion or exclusion of †*Plectocretacicus clarae* may impact divergence time estimates for the root of the Tetraodontiformes and its subtending nodes (up to 11 Ma), we note that all of our topologies place †*Plectocretacicus* with high confidence within both the †Plectocretacicoidea and the Tetraodontiformes stem (see below). We thus validate the traditional placement of †*Plectocretacicus* until new evidence and new specimens (which we would welcome) become available.

2.3. Phylogenetic analyses

To assess the impact of morphological data and the inclusion of extinct taxa on molecular phylogenetic inference, two separate analyses were conducted: (1) using morphological and molecular data alone; and (2) using a concatenated dataset with molecular and morphological data (total evidence). These datasets were analyzed under parsimony using TNT v1.1 (Goloboff et al., 2008) and under maximum likelihood (ML) in RAxML v7.3 (Stamatakis, 2006). Bayesian analyses of the molecular and the total evidence datasets were conducted using BEAST v1.7 (Drummond et al., 2012) and MrBayes v3.2.2 (Ronquist et al., 2012), described in detailed in the following sections. Details for the TNT and RAxML analyses are presented in online Supplementary File 1.

2.4. Comparison of dating approaches

2.4.1. Node dating (ND)

The following analyses were conducted to compare two types of node-dating analyses, one based on the popular calibration-density method (ND-CD), and one implementing the FBD model (ND-FBD) of Heath et al. (2014). The ND-CD analyses were conducted in MrBayes and BEAST. A total of 9 or 12 calibration points were selected for the ND-CD runs, based on the fossil placements obtained with analyses of the combined data (see details in online Supplementary File 2). Following the recommendations of Parham et al. (2012), the youngest fossil date from a biostratigraphic interval was used as the absolute age for minimum age constraint. The 95% soft maxima are based on non-restrictive calibration densities

(i.e., relatively old maxima). Root calibrations in the ND-CD analyses used two alternative distribution priors (exponential and lognormal) for direct comparison with the TD analyses (see below); other calibrations were set assuming exponential distributions (online Supplementary File 2). Many of these calibrations have been implemented in previous studies (Alfaro et al., 2007; Betancur-R. et al., 2013a; Dornburg et al., 2008; Near et al., 2013; Santini et al., 2013a).

Settings for the MrBayes ND-CD analysis (e.g., clock model, partitions, MCMC operators, etc.) are given below, under tip dating. For the BEAST analyses, a starting chronogram that satisfied all priors (e.g., monophyly and initial divergence times) was generated under penalized likelihood in r8s v1.7.1 (Sanderson, 2003) using the molecular tree produced by RAxML. To model branching rates on the tree, a birth–death process was used for the tree prior with an initial growth rate of 1.0 and a death rate of 0.1. The substitution model was GTR+Γ, with five rate classes. The data were partitioned into four categories with independent parameter estimation: three codon positions across exons of protein-coding genes and 16S. Clock and tree priors were linked across partitions. Four replicates of the Markov chain Monte Carlo (MCMC) analyses were run for 150–200 million generations. To assess the impact of using multiple fossil calibrations in the estimation of marginal branch-length densities in BEAST (Heled and Drummond, 2012), the same analyses were repeated using the root calibration only (with data but without non-root priors), and the influence of all priors was explored by performing MCMC runs with priors but without data.

The ND-FBD analyses were run in a Bayesian framework using the program DPPDiv v1.1 (Heath, 2012; Heath et al., 2012). The ND-FBD runs used 36 fossil calibrations based on the fossil placement obtained with the combined analyses (see online Supplementary File 3). The DPPDiv program requires an input topology consisting of extant taxa, for which the ND-CD trees (BEAST and MrBayes) were used. An attempt to run DPPDiv with the tip-dating topology from the MrBayes analyses failed because this tree had many polytomies (see below) that conflicted with the program's requirements. The ND-FBD approach requires the specification of absolute dates for fossils, and the current recommendation is to sample their ages from an uniform distribution given by their stratigraphic ranges (Table S2) in order to approximate random recovery (future implementations of the program will treat the ages of fossils as random variables by placing prior densities on occurrence times; Heath et al., 2014). For comparison purposes, however, we used the minimum ages of fossils as in other analyses.

For ND-FBD analyses, the default Dirichlet process prior model (DPP) was implemented to draw lineage-specific substitution rates from an underlying distribution. Under DPP, rate parameters are allowed to vary according to discretized rate categories, which are random variables of the model (the UCLN model is a special case of the DPP; Heath et al., 2012). However, because analyses under DPP were computationally intensive, a second approach was used implementing the uncorrelated gamma-distributed rates model (UGR). Performance of MCMC chains under UGR was approximately an order of magnitude faster than DPP. For each of these two analyses, five independent chains were run for 14 days in a computer cluster using the parallel implementation of the DPPDiv program.

Log files from BEAST, MrBayes, and DPPDiv (MCMC) analyses were analyzed using Tracer in order to obtain parameter estimates, as well as to evaluate effective sample sizes (ESS) and convergence plots. Tree files from the multiple runs were combined using either LogCombiner v1.7.4 (BEAST and MrBayes analyses) (Drummond and Rambaut, 2007) or custom Unix code (DPPDiv), with the first 10% of the trees saved by each run discarded as burn-in. The maximum clade credibility trees, with means and 95% highest posterior density (HPD) of divergence times, were summarized with TreeAnnotator v1.6.1 (Drummond and Rambaut, 2007). In summary, six different node-dating analyses were conducted: MrBayes-ND exponential, MrBayes-ND lognormal, BEAST-ND exponential, BEAST-ND lognormal, ND-FBD using the DPP model, and ND-FBD using the UGR model.

2.4.2. Tip dating (TD)

Topology and divergence times were simultaneously estimated using TD in MrBayes and BEAST. Data for the 36 terminal fossils (Table S3) and a single calibration for the root were used as priors for age parameterization. For both MrBayes and BEAST, the sensitivity of analyses to calibration density was explored by comparing both exponential and lognormal distributions for root priors (as in the ND-CD analyses; see online Supplementary File 2). Lognormal distributions are defined by two parameters (mean [$\boldsymbol{\mu}$] and standard deviation [$\boldsymbol{\sigma}$]), which can result in multiple distribution shapes for the intended hard lower and soft upper bounds. By contrast, exponential distributions are convenient because they are characterized by a single rate parameter (λ), but small increments of λ can place much higher probabilities on excessively old divergences in exponential versus normal distributions (i.e., exponential distributions have a "fatter tail") (Heath, 2012). For MrBayes, the molecular clock was modeled under the independent gamma rates (IGR) model. The IGR is a continuous uncorrelated model (also similar to the UCLN model) in which branch rates are drawn independently from the same gamma distribution (Lepage et al., 2007; Ronquist et al., 2012).

The same five partitions defined for the RAxML searches (online Supplementary File 1) were used for this analysis (i.e., a separate partition for each codon position of exon markers, 16S mtDNA, and morphology). The first four molecular partitions were analyzed under the GTR+Γ model and allowed separate rates with a rate multiplier for all parameters except for branch lengths and topology, which were linked across partitions. The morphological partition was analyzed using the Mk model with a gamma-distributed rate variation (Lewis, 2001). The rate variation and morphological coding priors were both set as variable. The clock rate prior was set based on marginal estimations of the "meanRate" parameter obtained with a pilot run in BEAST (based on node dating). Eight independent runs were conducted for 70 million generations each and sampling every 1000 generations. Although it is theoretically possible to unlink the clock rates across the morphological and molecular partitions (i.e., 2-clock analysis; Pyron, 2011), this feature is not currently implemented in MrBayes (it is only possible with BEAST); thus, all TD analyses used a single clock rate. To obtain a resolved topology and to simplify detailed comparisons for all nodes, a summary phylogeny was estimated using the allcompat consensus (50% consensus majority rule plus compatible elements) rather than the default "halfcompat" majority rule consensus.

Tip-dating analyses were also performed in BEAST v1.8. Because this option is not formally implemented in the program, it required substantial modification of the XML code. The morphological characters were separated into ordered and unordered partitions and given Mk model elements with the appropriate number of states for each character (e.g., a partition for unordered three-state characters, ordered five-state characters, and polymorphic characters). Models for the DNA data remained as specified above. Parameters were unlinked across partitions, with a single UCLN clock model. Unlike the MrBayes-TD runs, which can only handle uniform tree priors, the BEAST-TD analyses assumed a birth–death process for speciation. As in the MrBayes-TD analyses, age parameterization used both exponential and lognormal root priors (online Supplementary File 2) as well as the temporal data of the 36 fossils included in the analysis. Twenty independent chains were run for 100 million generations each, sampling every 1000 generations. Analyses were also run with empty data matrices (all characters

changed to "?") in order to sample from the prior distribution for comparisons with the posterior distribution. Trees sampled before achieving stationarity were discarded as relative burn-in (10%). Convergence of the MCMC was verified using the ESS criterion for each parameter in Tracer v1.7 (Drummond and Rambaut, 2007). In summary, four different tip-dating analyses were conducted: MrBayes-TD exponential, MrBayes lognormal, BEAST-TD exponential, and BEAST-TD lognormal.

2.5. Assessment of accuracy and precision of tip dating

Given the empirical nature of this study, accuracy of divergence time estimates cannot be evaluated directly. However, it is possible to assess the extent of ghost lineages implied by the tip-dating results. For groups with an extensive fossil record, such as Tetraodontiformes, it may be reasonable to assume that fossil ages of the oldest fossils are close in time to the speciation events that gave origin to these lineages (Hedges and Kumar, 2004; Ho and Phillips, 2009; Norrel, 1992; Smith, 1994). Under this assumption, an accurately calibrated tip-dating phylogeny would reveal relatively short ghost lineages, if any. To quantify the extent of ghost lineages at the family level in the TD trees, all extant tips were pruned from the chronograms and the branch lengths (in Ma) were measured from the nodes where each stem family originates to each fossil tip. Also, if the prevalence of ghost lineages is minimal, terminal branch lengths for fossil tips should be either shorter than or similar to those in extant tips, but not longer. The length of terminal branches were thus extracted from each of the tip-dating trees and the mean values were compared for fossil and extant taxa using whisker plots and the non-parametric Mann–Whitney U test in R. Finally, the lengths of ghost lineages estimated under TD were compared with those obtained using the ND-FBD method. For eight fossils that attach to extant clades in the ND-FBD trees, ghost lineage lengths were calculated by subtracting the fossil ages from their inferred attachment times (mean posterior probability values). The remaining fossils were not compared because they cluster with other fossils in the TD trees, making the comparisons difficult.

Precision of the divergence time estimates is approximated by the width of the 95% HPD intervals. Because interval width varies as a function of clade age (e.g., Dos Reis and Yang, 2013; Heath, 2012; Rabosky et al., 2012), the relative uncertainty was calculated by correcting for the node's mean age (relative uncertainty = width of the 95% HPD interval/mean age). An empirical test for how fossil placement may affect the precision of tip dating was then developed based on the expectation that nodes phylogenetically closer to fossil taxa should exhibit smaller uncertainty relative to more distant nodes. For this test, the results obtained from the four alternative TD analyses outlined above using tetraodontiform fishes (132 terminals, 36 of which are fossils) were compared with those obtained by other tip-dating studies using lissamphibians (75 terminals, 41 fossils; Pyron, 2011), hymenopterans (113 terminals, 45 fossils; Ronquist et al., 2012), and palpimanoid spiders (23 terminals, 5 fossils; Wood et al., 2013). For each node in these tip-dating trees the distance (in Myr) to the closest fossil was measured and the relative uncertainty was estimated as explained above. The putative influence of fossil proximity on dating precision was explored using linear regression analyses between these two variables.

3. Results

3.1. Phylogenetic analyses

Analyses of morphological, molecular, and concatenated datasets resolve the monophyly of all extant tetraodontiform families as well as the subordinal clades (sensu Betancur-R. et al., 2013a; see Table S1). These results are also largely congruent with those obtained by previous molecular studies (Alfaro et al., 2007; Holcroft, 2005; Santini et al., 2013b; Yamanoue et al., 2008b), at least with respect to well-supported groups. By contrast, the backbone branching pattern close to the base of the intraordinal relationships are often weakly supported and incongruent among different analyses. The addition of molecules to the morphological datasets influences the placement of some fossils, in particular †*Eomola*, †*Eotetraodon*, †*Zignodon*, †*Moclaybalistes*, †*Eoplectus*, †*Eospinus*, and †*Bolcabalistes*. In most cases, however, missing data in fossil taxa do not affect their placement in the expected familial and subordinal clades. Finally, bootstrap support values are significantly higher in the analyses of the molecular datasets alone (68.2–86.4%), relative to those based on morphological data (38.6–53.1%) or the combined dataset (35.6–57.9%). Additional details of the phylogenetic results are presented in online Supplementary File 1, Table S4, and Figs. S1–S3.

3.2. Comparison of dating approaches

Examination of log files and traces from the MrBayes, BEAST, and DPPDiv analyses in Tracer suggested MCMC convergence, with ESS values >200 obtained for all parameters and for age estimates of all nodes (except for the ND-FBD runs using the DPP model; see below). Clade ages derived from the ND-CD analyses using either exponential or lognormal root priors (MrBayes and BEAST) were highly similar and only the former results are thoroughly discussed (but see Table S5 for full comparisons). The BEAST analyses resulted in moderate amounts of rate heterogeneity (standard deviation of the uncorrelated log-normal relaxed clock or ucld.stdev parameter = 0.763), suggesting that sequence evolution is not clock-like. This dataset was also run on an empty alignment as well as with the root calibration only, to compare it with the analyses using 12 calibration priors. The Tracer output from this comparison shows that priors impact the marginal distributions of age estimates in five out of 11 cases (i.e., 12 calibrations minus the root constraint, which is common to all analyses; see details in Fig. S4).

The ND-CD Bayesian analyses under different program implementations (MrBayes and BEAST) yielded highly similar topologies (Fig. 2; online Supplementary File 1; Table S4). Mean age estimates were also similar, but for some clades the divergence times obtained with MrBayes ND were somewhat older (Fig. 3). The most striking age differences between the two trees were obtained for Aracanidae (27.5 Ma versus 12.3 Ma, respectively), Diodontidae (31.7 Ma versus 20.8 Ma), Tetraodontidae (64.2 Ma versus 52.3 Ma), and Monacanthidae (66.8 Ma versus 47.5 Ma) (all crown-group comparisons). In all cases, however, the 95% HPD intervals overlap (Fig. 3; Table S5).

After 14 days of running time, the MCMC chains of the ND-FBD analyses reached 6 and 30 million generations each for the five independent runs under the DPP and UGR models, respectively. All marginal parameters obtained from the combined analyses applying the UGR model had ESS values greater than 200, suggesting appropriate mixing of the MCMC chains. However, the ND-FBD analyses applying the DPP model failed to converge, with several ESS values <200. In spite of the lack of MCMC convergence of the DPP runs, age estimates for most nodes between the two clock models were similar, although slightly older with DPP than with UGR (Table 1; Fig. 3). Finally, a comparison of the ages inferred with different ND methods shows higher similarity among values obtained with ND-CD in BEAST and ND-FBD (both UGR and FBD models) relative to those obtained with ND-CD in MrBayes, which resulted in relatively older estimates for many tetraodontiform clades (Fig. 3; Table S5). ND analyses in BEAST and MrBayes are

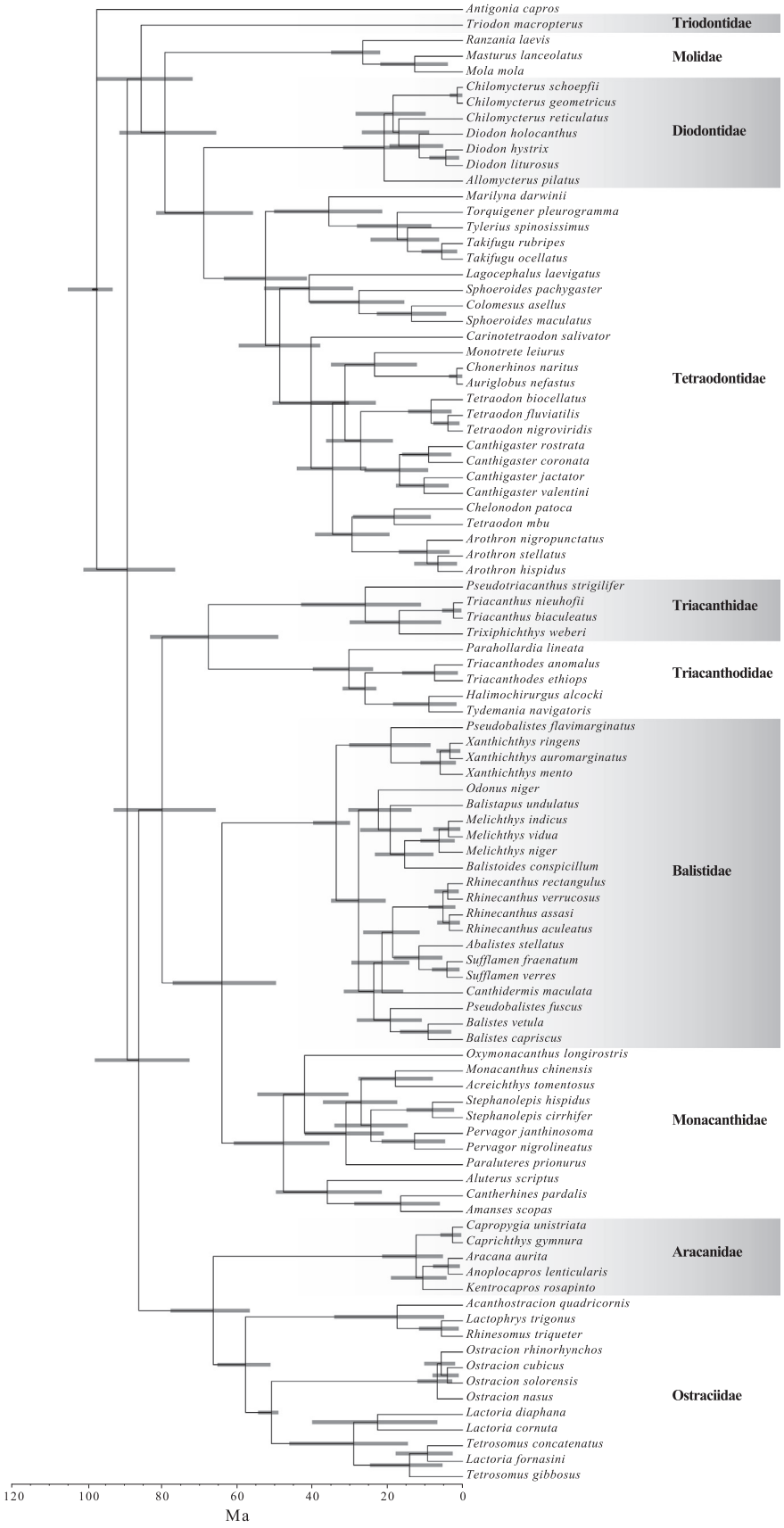

Fig. 2. Chronogram for the radiation of Tetraodontiformes based on node-dating (ND) analyses, as implemented in BEAST (exponential root prior). Only the most informative fossils (12) were used in this analysis (i.e., oldest fossil assignable to youngest clades). Additional details, including calibration points and 95% HPD are given in Fig. S5. Other chronograms estimated with node-dating approaches (MrBayes-ND) are shown in Fig. S5; a comparison of ages obtained with ND is given in Fig. 3 and Table S5.

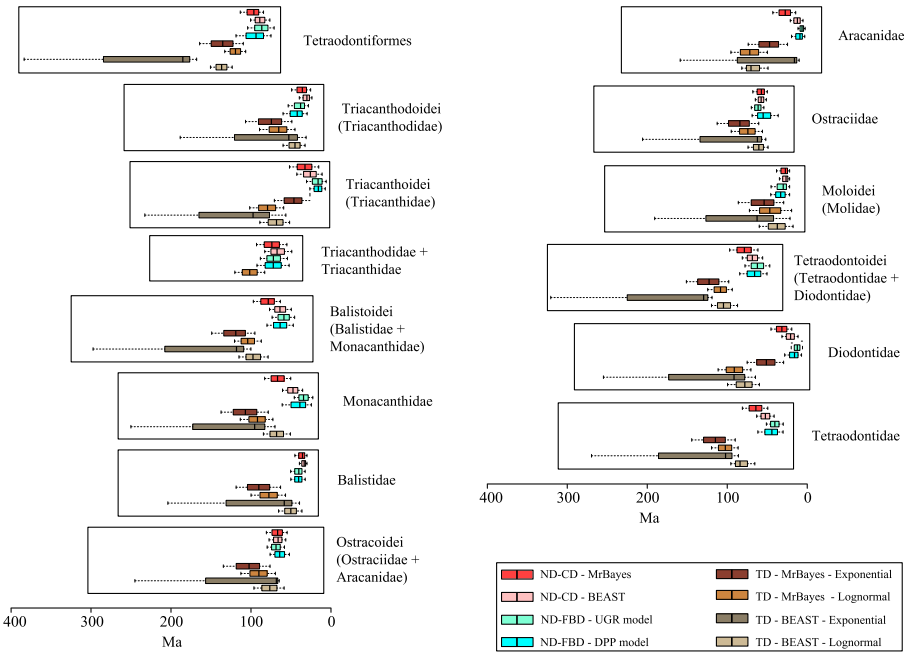

Fig. 3. Comparison of divergence times for major tetraodontiform clades estimated from alternative analyses based on node- and tip-dating approaches (bars indicate mean and 95% HPD ages). ND-CD, node-dating calibration-density approach; ND-FBD (estimated with DPPDiv), node-dating fossilized birth–death process (tree model); UGR, uncorrelated gamma-distributed rates (clock) model; DPP, Dirichlet process prior (clock) model; TD, tip dating. Results from the ND-CD using lognormal root priors are reported in Table S5.

not sensitive to root distribution priors (exponential or lognormal, Table S5).

Unlike ND, the TD analyses are highly sensitive to root distribution priors (exponential or lognormal). The TD trees using exponential root priors resulted in considerably older divergences relative to those based on lognormal distributions, and the oldest and least precise (see below) estimates were obtained with the BEAST exponential analysis. There are also minor topological differences resulting from the different priors (see online Supplementary File 1). The preferred TD tree obtained with MrBayes lognormal analysis is reported in Fig. 4.

The TD ages are consistently and significantly older than the ND results reported above (Figs. 2–4; Tables S5–S6), with little or no overlap in their 95% HPD intervals (Fig. 3). Some extreme examples include: crown Tetraodontiformes (mean 96.4–89.0 Ma with ND versus 185.2–119.0 Ma with TD), Balistidae (mean 40.4–33.2 Ma with ND versus 90.2–50.2 Ma with TD), and Tetraodontidae (mean 64.2–44.2 Ma with ND versus 114.8–84.9 Ma with TD). The TD and ND chronograms also differ markedly in the relative length of the stem branch leading to the tetraodontiform crown group, which is close to zero in the TD tree (Figs. 2 and 4).

3.3. Assessment of accuracy and precision of tip dating

Tip-dating results imply the existence of considerable ghost lineages for all major groups of tetraodontiforms, against the expectation that the oldest fossils for each lineage would be close in age to the speciation events that gave rise to them. In most cases, the estimated divergence times for clades obtained with MrBayes TD are substantially older than the oldest fossils they contain (Table 2). For instance, the oldest possible occurrence of three tetraodontid fossils (†*Archaeotetraodon jamestyleri*, †*Archaeotetraodon winterbottomi*, †*Sphoeroides hyperostosus*) is 34 Ma, but the basal familial divergence is dated at 133–101 Ma, implying a 99–

Table 1
Comparisons of divergence times for major tetraodontiform (crown) clades inferred by previous studies (in Ma; mean and 95% HPD). In addition to these, the inferred age for crown Balistidae in Dornburg et al. (2011) is ca. 10 Ma (95% HPD = 7–14).

Taxon/clade	Alfaro et al. (2007)	Near et al. (2013)	Betancur-R. et al. (2013)	Santini et al. (2013b)
Tetraodontiformes	68 (78–60)	59 (62–56)	78 (85–69)	–
Triacanthodoidei (Triacanthodidae)	–	11 (17–7)	–	–
Triacanthoidei (Triacanthidae)	21 (38–7)	–	–	–
Triacanthodidae + Triacanthidae	–	–	–	–
Balistoidei (Monacanthidae + Balistidae)	40 (44–35)	40 (43–37)	43.8	–
Monacanthidae	24.6 (31–18)	27 (29–24)	25.8	–
Balistidae	22.9 (30–16)	17 (20–14)	19 (33–5)	–
Ostracioidei (Aracanidae + Ostraciidae)	52 (56–50)	27 (34–21)	54.8 (62–50)	63 (71–59)
Aracanidae	6.7 (13–1)	–	8.4	26 (36–18)
Ostraciidae	20.8 (36–9)	14 (26–18)	40.9	56 (67–45)
Moloidei (Molidae)	25.9 (37–17)	24 (26–22)	20 (36–5)	–
Molidae + Tetraodontoidei	–	–	–	–
Tetraodontoidei (Diodon. + Tetraodon.)	55 (60–50)	51 (53–50)	55.9	–
Diodontidae	10.9 (16–5)	18 (21–13)	55.6	–
Tetraodontidae	38.3 (41–35)	35 (40–31)	35.6 (41–32)	–

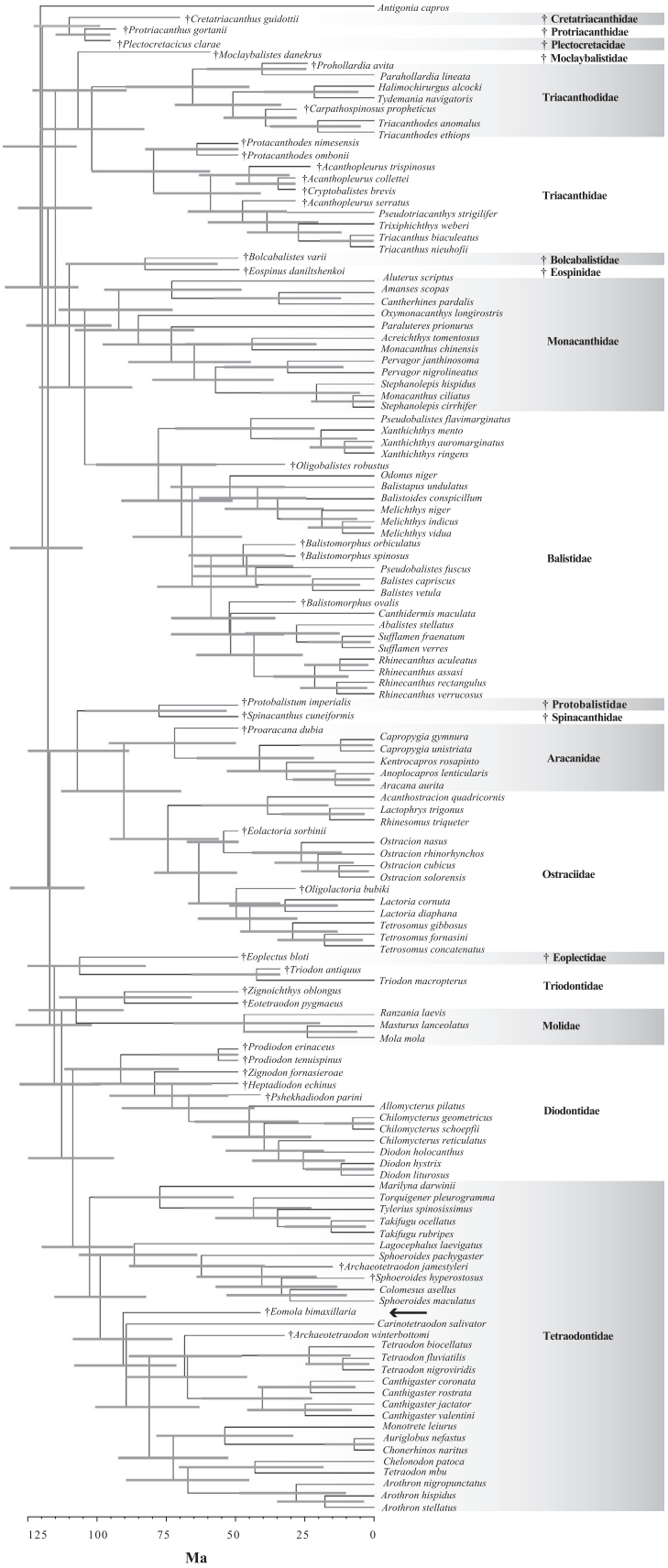

Fig. 4. Chronogram of tetraodontiform diversification obtained with tip dating (TD) using MrBayes with lognormal root priors (see Fig. 3 and Table S6 for results based on other TD analyses). Names of extinct fossil taxa are preceded by a dagger symbol ('†') and placed at their inferred chronological horizon. Gray bars represent 95% HPD intervals. Except for Molidae, the monophyly of the remaining tetraodontiform families (both extant and fossil) is supported by this analysis (apparent misplacement of † *Eomola* is indicated by black arrow).

Table 2
Length of ghost lineages implied by the tip-dating trees (in Myr), measured from the nodes where each stem family originates to each fossil tip.

Families	BEAST – Exponential	BEAST – Lognormal	MrBayes – Exponential	MrBayes – Lognormal
†Cretatriacanthidae	97.9	50.9	39.1	39.8
†Plectocretacicidae	35.7	15.9	7.8	9.1
†Protriacanthidae	36.5	17.9	18.4	11.1
†Eoplectidae	91.1	76.7	78	57
†Moclaybalistidae	56.6	54.2	54.7	47.6
†Bolcabalistidae	64.8	54.2	39.7	33.9
†Eospinidae	62.6	36.9	39.5	33.9
†Spinacanthidae	49.9	28.4	32.9	28.4
†Protobalistidae	27.4	28.4	33.1	28.4
Triacanthodidae	44.5	19	38.5	30.3
Triacanthidae	65.9	16.8	59	50.9
Balistidae	16.5	21.7	60.3	40.8
Monacanthidae	19.3	18	60.4	37.1
Aracanidae	14.8	27.5	50.9	41.1
Ostraciidae	36.3	27.4	51.1	41.1
Tetraodontidae	99.2	89.3	93.4	93.4
Diodontidae	41.4	55.6	70.1	59.3
Molidae	48.1	36.9	69.7	49.3

67 Myr gap in the fossil record (Table 2). Consistent with this pattern, all other families also are characterized by extended gaps, most notably †Eoplectidae (91.1–57.0 Myr), Triacanthidae (65.9–16.8 Myr), and †Cretatriacanthidae (97.9–39.1 Myr). The terminal branch lengths for fossils are also, on average, longer than those in extant taxa (although only 3 of 5 are significant; Fig. 5A), suggesting that TD age overestimation is more striking in fossil than in extant lineages. Finally, the lengths of ghost lineages for eight selected fossils estimated with ND-FBD are significantly shorter than those inferred with TD (Fig. 5B).

Precision of divergence time estimates using the TD method is positively influenced by the proximity of fossil taxa to the dated nodes. Scatter plots of relative uncertainty (width of the 95% HPD intervals/mean age) against distance from the closest fossil (in Myr) yielded significantly positive correlations in all previous TD studies as well as in 3 (of 4) of the TD trees inferred herein ($P < 0.00001$ unless otherwise indicated; Fig. 6). The fit of the data to the linear model is strongest for amphibians (Pyron (2011) ($R^2 = 0.80$), followed by one of the tetraodontiform trees (MrBayes exponential; $R^2 = 0.38$), and by hymenopterans (Ronquist et al. (2012) ($R^2 = 0.35$). The MrBayes TD tetraodontiform trees have a much closer linear fit relative to the BEAST TD trees, which in turn resulted in a single non-significant correlation (BEAST exponential; $R^2 < 0.001$; $P = 0.35$). The study on spiders (Wood et al., 2013) also had a weak linear fit ($R^2 = 0.14$; $P = 0.02$), presumably due to the low number of fossils included (i.e., 5 fossil spiders accounting for 22% of the terminals versus 36–45 fossils or 33%–45% of the terminals in the remaining studies).

4. Discussion

4.1. Comparison of dating approaches and accuracy and precision of tip dating

This empirical study shows that current implementations of the tip-dating method can estimate substantially older divergences than would otherwise be obtained with the most widely used node-dating approaches. In spite of differences among node-dating methods (ND-CD and ND-FBD), all analyses tested yielded similar results. The times of divergence estimated with tip dating are, however, significantly older and more sensitive to priors or method of choice. Notably, the use of exponential root priors resulted in significantly older TD age estimates (especially with BEAST-TD) relative to ND. Taken at face value, these TD estimates imply ghost lineages up to five times older than the clade's oldest fossil (Table 2) and the inferred age of the root (185.2–119.4 Ma, mean

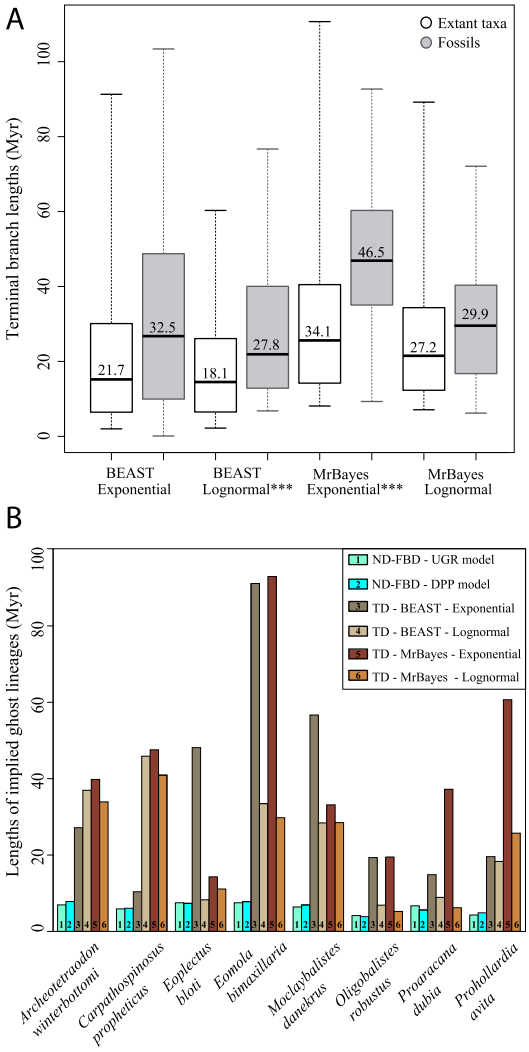

Fig. 5. (A) Comparisons of terminal branch lengths for extant (open boxes) and fossil (gray boxes) taxa based on the alternative tip-dating (TD) analyses. Values and thick lines inside boxes indicate data means and medians, respectively. ***U-test $P < 0.001$. (B) Comparisons of ghost lineage lengths estimated with ND-FBD versus TD (eight selected fossils).

144.1 Ma) leaves a stratigraphic gap of *ca.* 88.3–22.5 (mean 47.3) Myr to the first fossil appearance (i.e., †*Plectocretacicus clarae*, 96.9–95.0 Ma). Terminal branches for fossil taxa in the TD trees

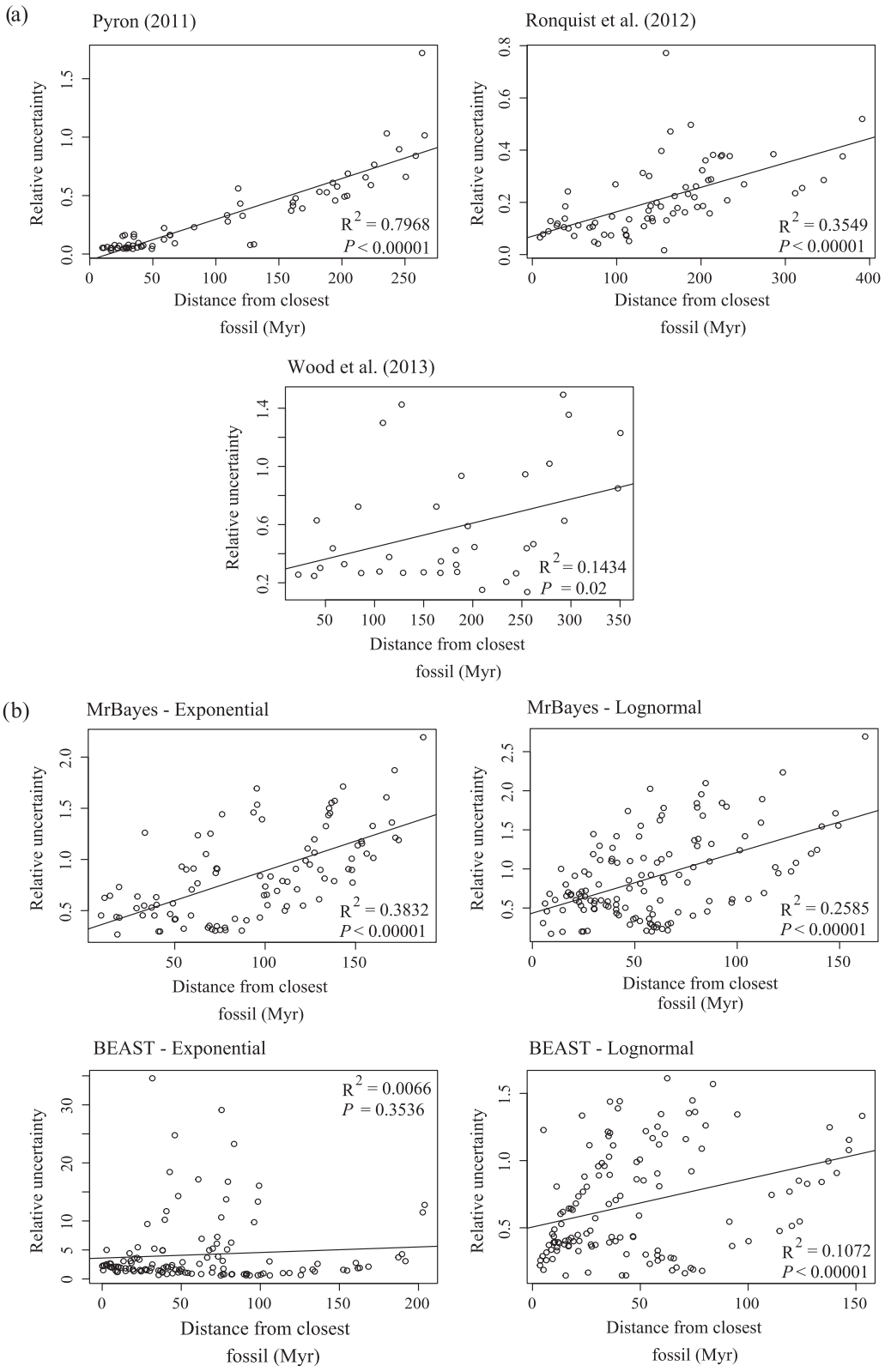

Fig. 6. Precision of divergence-time estimates (relative uncertainty) obtained with TD analyses in previous studies (a) and with different implementations of TD analyses in this study (b). In all cases, relative uncertainty for all nodes on the tree (each dot represents one node) is plotted as a function of the distance between the node of interest and its closest fossil in the tree. Relative uncertainty is measured as width of 95% HPD interval/node's mean age. The relationship between relative uncertainty and fossil proximity is assessed using linear regressions. In all but one comparison, there is a significant positive correlation among these variables.

are also, on average, longer than those in extant tips (Fig. 5A), and the ghost lineages are substantially longer than those inferred with ND-FBD (Fig. 5B), further demonstrating the prevalence of large paleontological gaps implied by the TD results. Such long paleontological gaps seem surprising in light of the diversity and prevalence of the tetraodontiform fossil record (see Section 4.2).

Ronquist et al. (2012) compared the results of tip and node dating using the Hymenoptera for empirical testing in MrBayes. These authors showed that when fossils are used as terminal tips, divergence time estimates are less sensitive to prior assumptions than node-dating analyses. Contrary to those findings, this study shows that the selection of root prior distributions (exponential or lognormal) impacts more severely the TD than the ND inferences in tetraodontiforms.

Consistent with the trend reported herein, Ronquist et al. (2012) estimated the origin of Hymenoptera to be much older (ca. 310 Ma) than the oldest fossil (235 Ma), implying a stratigraphic gap of *ca.* 75 Myr. They did not report major differences for the age of the root obtained with the TD and ND methods, but it is important to note that their older estimate for ND is likely the result of their calibration rationale. These authors used the upper bound of their calibration points as the mean of the exponential distribution, whereas this value is most commonly used to reflect the 95% upper distribution bound. For instance, their root calibration was based on an exponential distribution with offset of 315 Ma (†*Katerinka*, oldest Neoptera) and mean 396 Ma (†*Rhyniognatha*, oldest insect). Based on these parameters, the 95% upper bound of the distribution becomes 557.7 Ma. Conversely, using † *Rhyniognatha* as the soft upper bound for the calibration, the mean is much smaller (342.03 Ma). This artifact produces a 162 Myr difference between their prior choice and the commonly used 95% upper bounds, effectively resulting in much older age estimates than implied by the fossil record. Another recent TD study assessing the timing of gonorynchiform fish diversification (Near et al., 2014) also estimated substantially older (>50 Ma) root divergences relative to previous ND efforts (Davis et al., 2013), although the TD estimates for some internal clades were younger.

Tip dating has desirable theoretical properties that make it an appealing method for estimating divergence times, at least with respect to the popular node-dating calibration-density approach (Pyron, 2011; Ronquist et al., 2012). For instance, it allows direct incorporation of the fossil information instead of simply relying on *ad hoc* age constraints. It also utilizes all the fossils for which there are morphological data available, not just the oldest members of a given clade. Nevertheless, the current implementations of tip dating seem to produce biases toward overly old divergences in Tetraodontiformes (and possibly Hymenoptera and Gonorynchiformes; see above). It is noteworthy that the node-dating analyses of tetraodontiform ages based on the FBD model, using all available fossils without requiring *ad hoc* calibration densities, resulted in similar estimates of divergence times than those using ND-CD, especially when compared with the BEAST analyses (the MrBayes ND-CD analyses resulted in slightly older ages; Fig. 3; Table S5).

Factors potentially biasing the tip-dating results remain to be explored, including: (1) the implementation of a single relaxed clock model shared by the molecular and morphological sites (Pyron, 2011; Ronquist et al., 2012); (2) a lack of the method's ability to account for fossil sampling (Hedges and Kumar, 2004; Ho and Phillips, 2009; Kumar and Hedges, 1998) or fossilization potential (Heath et al., 2014); (3) the implementation of unrealistic tree priors in MrBayes (uniform) and BEAST (birth–death serially sampled, proposed for rapidly-evolving viruses); (4) overestimation of branch lengths on fossil clades owing to properties of the Mk model (e.g., failure to properly account for ascertain bias) (Lewis, 2001); or a combination of these factors. In spite of these potential pitfalls, tip dating remains a promising approach, and future implementations may benefit from penalizing extended ghost lineages (Nowak et al., 2013; Ronquist et al., 2012) and/or by applying the FBD model for tip dating (T. Heath pers. comm.) to obtain more consistent results.

Finally, the majority of tip-dating trees assessed herein show a significant positive correlation between relative uncertainty and node distance from the closest fossil (Fig. 6). Ronquist et al. (2012) also noted that the 95% HPD intervals were generally wider on a tip-dating tree derived from the analysis of a hymenopteran dataset with reduced fossil representation, particularly for clades from which fossils were removed. Taken together, these results suggest that the inclusion of more fossils with placement scattered throughout the tree can lead to an increase in precision of tip-dating time estimates (Dos Reis and Yang, 2013; Pyron, 2011). In addition, possible spurious effects caused by missing data in fossil taxa are probably outweighed by the benefit of including more fossils (Ronquist et al., 2012).

4.2. Origin and diversification of tetraodontiformes

Tetraodontiformes have one of the best-known and most extensive fossil records among teleost fishes. Thirty-six fossil species have been described, relatively thoroughly revized, and phylogenetically analyzed as of 2003. These extinct taxa represent nine fossil families and all ten extant families (Santini and Tyler, 2003; see Table S2; Tyler and Santini, 2002; Tyler and Sorbini, 1996). The great majority of these 36 fossil species are known from ten or fewer specimens (12 of these species are known only from the holotype), whereas several species are known from dozens of specimens, and one species (the Pliocene †*Sphoeroides hyperostosus*) is known from a relatively complete skull, a cranium, and several thousand disarticulated bones (mostly hyperostotic postcleithra and opercular elements). The stratigraphic occurrences of tetraodontiforms date back to the late Cretaceous (stem plectocretacicoids), with fossil genera extending into the Pliocene and Pleistocene (e.g., the monacanthid †*Frigocanthus*; Sorbini and Tyler, 2004). Diversity peaks in the middle Eocene (50 Ma) of the Monte Bolca formation in northern Italy, which has yielded some of the best-preserved specimens of numerous species. Most importantly, the stem plectocretacicoids are one of the few extant percomorph lineages with known representatives in the Cretaceous, suggesting that tetraodontiforms had an important fossilization potential throughout their history (Forey et al., 2003; Friedman, 2010; see Discussion; Patterson, 1993).

The results of this study yield two remarkably different time scales for tetraodontiform evolution, depending upon the dating method applied (Fig. 3). The ages obtained with node dating (BEAST ND-CD and DPPDiv dates combined; Table S5) place the origin of the crown group during the Late Cretaceous (94–87 Ma), a date estimate that differs from most previous reports (Table 1). Several studies using nuclear markers inferred a much younger tetraodontiform divergence, dated between the end of the Cretaceous (ca. 70 Ma; Alfaro et al., 2007; Betancur-R. et al., 2013a) and the Paleocene (62–59 Ma; Near et al., 2013, 2012). Although the majority of these studies included a rich sample of percomorph species and calibrations outside of Tetraodontiformes, they had a smaller taxonomic representation and fewer calibration points within that order. One of the most striking differences between this and previous studies includes the divergence of crown balistids, which is dated at Eocene (40–33 Ma) herein, whereas previous studies suggest a Late Oligocene-Middle Miocene origin of the group (25–10 Ma; Alfaro et al., 2007; Dornburg et al., 2008, 2011). These discrepancies may be due to the fewer markers examined by previous studies or by differences in the placement (crown versus stem) of the oldest balistid fossils (†*Balistomorphus ovalis*, †*B. orbiculatus*, and †*B. spinosus*; see comments under calibration 5 in online Supplementary File 2). Other conflicts in the observed dates concern the families Ostraciidae and Monacanthidae. Whereas our estimates place the origin of the crown Ostraciidae during the Paleocene (61–55 Ma), previous studies

have inferred a much younger split (41–14 Ma; Alfaro et al., 2007; Betancur-R. et al., 2013a; Near et al., 2013). Likewise, the age of the crown Monacanthidae is 48–34 Ma in this study, whereas a Late Oligocene/Early Miocene origin for the group (ca. 27–24 Ma) was previously proposed.

Despite these conflicts, some similarities are worth mentioning. For instance, the inferred crown group ages for Aracanidae (12–6 Ma) and Tetraodontidae (ca. 52–35 Ma) are all largely concordant with some previous studies (Alfaro et al., 2007; Santini et al., 2013c); see Table 1 for additional details. A Late Cretaceous origin of crown Tetraodontiformes obtained with ND methods is perhaps more consistent with their known stratigraphic record and that of their associated stem groups (the plectocretacicoids; see below). Other studies using mitogenomic evidence arrived at older estimates, proposing a Late Jurassic origin for tertaodontiform fishes (ca. 150–140 Ma; Yamanoue et al., 2011, 2006), but these results may be overestimates owing to mitochondrial saturation (Lukoschek et al., 2012; Mulcahy et al., 2012). The ages inferred by the mitogenomic studies are (most likely erroneously) consistent with the estimates obtained herein with the TD approach, which imply the existence of unexpectedly long ghost lineages in the rich fossil record of Tetraodontiformes.

4.3. Placement of the Plectocretacicoids and implications for the timing of acanthomorph origin

The stem plectocretacicoids are arguably the most informative acanthomorph and percomorph fossils that can be utilized for molecular dating (Benton and Donoghue, 2007). They comprise some of the oldest fossils in these groups (otherwise assignable to rather shallow clades; e.g., Marshall, 2008), bearing important implications for the timing of the origin of acanthomorphs. Two Upper Cretaceous plectocretacicoids (†*Protriacanthus* and †*Plectocretacious*) were originally described as tetraodontiforms, but their position in the order was not widely accepted until Tyler and Sorbini (1996) redescribed the taxa and presented a formal description of the superfamily †Plectocretacicoidea (including the therein described †*Cretatriacanthus*). Their assessment showed that members in this superfamily possess all 12 of the unequivocal morphological synapomorphies that diagnose the Tetraodontiformes. Subsequently, Santini and Tyler (2003) coded a large morphological dataset (used herein) consisting of 210 characters for 20 extant and 36 fossil taxa of tetraodontiforms. Using the caproid *Antigonia capros* as the main outgroup, the monophyly of the total group Tetraodontiformes was supported by 28 synapomorphies, with the plectocretacicoids as the most basal clade (plectocretacicoid monophyly was supported by six synapomorphies; monophyly of the crown tetraodontiform clade was supported by 19 synapomorphies).

Despite this evidence, some authors have recently questioned the placement of †*Plectocretacicus* (and perhaps other plectocretacicoids) within Tetraodontiformes based on a projected morphological reassessment (Santini et al., 2013a) and/or possible similarities to other non-tetraodontiform groups (Dornburg et al., 2014; Friedman et al., 2013). However, no significant morphological data was offered in support of this conjecture. Both Friedman et al. (2013) and Dornburg et al. (2014) utilized †*Cretatriacanthus*, the youngest plectocretacicoid (ca. 70 Ma, Late Campanian-Early Maastrichian, Nardò, Italy), for the purpose of earliest tetraodontiform dating in their studies.

Dornburg et al. (2014) also suggested that there is a similarity between plectocretacicoids and some armored beryciforms recently described by González-Rodríguez et al. (2013) from the Albian/Cenomanian of Mexico. We point out that González-Rodríguez et al. (2013) compared their monocentrid-like fossils (new family †Pseudomonocentrididae) with numerous representatives of percomorphs (using cleared and stained specimens, dry skeletons, and data in the literature, including that on plectocretacicoids and other tetraodontiforms) and found no relationships of the pseudomonocentridids with them. Dermal body armor has arisen independently in many acanthomorphs, but the shields of the pseudomonocentridids are overlapping (presumably movable) with curvaceous outlines, whereas the carapaces of protriacanthids and plectocretacicoids have much larger, mostly hexagonal, flat plates that are sutured immovably to one another (similar to those of Eocene to extant ostracioids and of the Eocene of Bolca †*Protobalistum*). The body is apparently scaleless in cretatriacanthids, but †*Cretatriacanthus* has a few small hexagonal plates at the rear of the head and guard plates at the base of the pelvic fin. A recently described second genus of cretatriacanthids, †*Slovenitriacanthus* (Upper Santonian/Lower Campanian of Slovenia, *ca.* 84–82 Ma), also is apparently scaleless except for the guard plates at the base of the pelvic fin; the single specimen is poorly preserved and it cannot be determined whether small hexagonal plates are present at the rear of the skull like those of †*Cretatriacanthus* (Tyler and Križnar, 2013). Thus, the single species known of †*Plectocretacicus* and of †*Protriacanthus* are armored by a carapace of large, sutured, hexagonal plates, whereas the two genera of †Cretatriacanthidae lack body armor.

As the rationale for not using *Plectocretacicus* for molecular dating, Friedman et al. (2013: supplementary material) state that the taxa of the three families of plectocretacicoids "...differ strikingly in their morphology, and some are linked to tetraodontiforms by features present in other groups of fishes." Indeed, these three Upper Cretaceous families are highly diverse morphologically, but they are no more different from one another than are many of the other familes of tetraodontiforms (e.g., compare the skeletons of a triacanthodid and a monacanthid, or of a triodontid and a molid, etc.). If only a triacanthodid and a molid were available for comparison, and all other intermediary families of tetraodontiforms were unknown, their common intraordinal ancestry would not be so apparent; the absent intermediaries would have the same effect of enhancing differences as they have in the incomplete fossil record. This great morphological diversity also is evident, albeit to a lesser extent, within some tetraodontiform families. For example, compare *Triacanthodes* with the long tubular-snouted *Halimochirurgus* among the Triacanthodidae, or compare *Monacanthus* with the elongate *Anacanthus* among the Monacanthidae (for documentation of tetraodontiform morphological diversity see osteological and scale illustrations, descriptions, and comparative diagnoses of higher taxa in Tyler (1980)). Furthermore, many families of acanthomorph fishes, including those of the fossil and extant tetraodontiforms, are characterized by a unique combination of a large number of characters, some of which are also present in different unique combinations in other groups of fishes.

The main point to the above commentary is that these suggestions to exclude †*Plectocretacicus* from consideration within the plectocretacicoids are not supported by a single significant morphological feature (nor a single synapomorphy), whereas many morphological synapomorphies supporting its placement within the plectocretacicoids as early-branching tetraodontiforms have been presented by Tyler and Sorbini (1996) and Santini and Tyler (2003). To help clarify future discussions concerning the plectocretacicoids, we also find it necessary to correct a statement in Santini et al. (2013b) that the phylogenetic conclusions of Santini and Tyler (2003) regarding plectocretacicoids are suspect because lophiiforms are more likely their sister group (as opposed to zeiforms, at times considered close relatives of tetraodontiforms). But Santini and Tyler (2003) used the caproid *Antigonia capros* as the main outgroup (in addition to the zeiform *Cyttus novaezelandiae*), which is indisputably a close tetraodontiform relative

according to molecular and morphological evidence. Santini and Tyler (2003) also showed that use of either *Cyttus* or *Antigonia* as the outgroup had no influence on the topology. In summary concerning the plectocretacicoids, we welcome any new morphological data and analyses that clarify the phylogenetic relationships of †*Plectocretacicus* and any of the other members of the superfamily, and we hope that new specimens will become available, but, in the interim, all morphological evidence presented to date strongly supports the basal tetraodontiform position of the three families of this Upper Cretaceous clade.

A significant finding of this study is the implication of a Late Cretaceous origin of tetraodontiforms in relation to the divergence times of major lineages of acanthomorphs (spiny-finned fishes; subsection Acanthomorphata) and percomorphs (a large clade of acanthomorphs; subdivision Percomorphaceae). The acanthomorphs first appear in the fossil record of the Cenomanian (Late Cretaceous), with some of the most significant fossil-bearing formations found in Morocco and Lebanon (Forey et al., 2003; Patterson, 1993). The Cenomanian acanthomorph fauna consists of more than 20 fossil genera representing at least four major orders of non-percomorph (Polymixiiformes, Zeiformes, Beryciformes) and percomorph (tetraodontiform plectocretacicoids) lineages, as currently recognized (Betancur-R. et al., 2013a; Near et al., 2013). Because acanthomorphs are absent from the fossil record of the Early Cretaceous and throughout the early-mid Mesozoic, the remarkable Cenomanian diversity of spiny-finned forms has led to the suggestion that the group diversified rapidly following its first appearance (Patterson, 1993) and that the absence of acanthomorph fossils in earlier deposits is authentic rather than an artifact in the fossil record of teleost fishes (Friedman, 2010).

The pattern of a rapid acanthomorph radiation in the Cenomanian predicted by Patterson (1993) is rejected by the nested position of plectocretacicoids among percomorphs as well as by other lines of evidence. For instance, based on multiple fossil calibrations and multi-locus datasets, recent molecular phylogenies do not show the signature of a burst of acanthomorph diversification near the group's origin (see Figs. 1 and 2 in Betancur-R. et al., 2013a; Near et al., 2013). In fact, there is a pattern of constant lineage accumulation from their origin, followed by a global decline in the Cenozoic (Near et al., 2013). These molecular phylogenies also place the divergence of spiny-finned fishes in the Jurassic, although their absolute time estimates differ among these studies (185–146 Ma in Betancur-R. et al., 2013b; 152–133 Ma in Near et al., 2013). It is likely that the younger acanthomorph age estimate in the Near et al. (2013) study is due to the omission of a calibration based on the plectocretacicoids (or at least on †*Cretatriacanthus*). Regardless of the absolute molecular dates, the absence of pre-Cretaceous acanthomorph fossils is nonetheless surprising given that this period in time includes some of the best-known marine Lagerstätten (e.g., Cerin, Monte San Giorgio, Oxford Clay, Solnhofen; Friedman, 2010).

4.4. Phylogenetic relationships among tetraodontiforms

This study of tetraodontiform fishes combining molecular data with morphological characters from extant and fossil taxa provides new insights into their evolutionary history. Although discussion of phylogenetic relationships among tetraodontiforms is not the main goal of this study, analyses presented herein provide the most comprehensive hypothesis of tetraodontiform evolution to date, including molecular data from 16 loci and morphological data for recent and fossil taxa (131 species examined). Analyses incorporating molecular and morphological data suggest a different placement of fossils relative to those based on morphological data alone (online Supplementary File 1), which is also consistent with findings from previous studies (Cobbett et al., 2007; Jenner et al., 2009; Ronquist et al., 2012; Wiens et al., 2010).

The early branching pattern among tetraodontiform lineages could not be resolved with confidence. Low clade support in the combined analyses can be explained by the inclusion of taxa with unstable phylogenetic position, which can reduce the support for otherwise well-supported relationships (Cobbett et al., 2007; Geisler et al., 2011), or conflicting signals in the morphological and molecular partitions (Sites et al., 1996; Wiens and Hollingsworth, 2000). Because fossils are more prone to be unstable in position (likely due to a lower number of informative characters scored in many cases), they are also more likely to reduce support in the rest of the tree (Cobbett et al., 2007; Geisler et al., 2011). For instance, depending on the analysis, taxa such as †*Eoplectus* and †*Moclaybalistes* are found in up to four different positions across the tetraodontiform tree (Figs. S2–S3). Although there are specific fossil nodes for which support improves considerably when molecular data are added (e.g., †Plectocretacicoidea; see Table 1), this is far from being the norm (online Supplementary File 1; Table S4).

Despite the weak signal in the combined dataset, missing data in fossil taxa does not prevent tetraodontiform extinct lineages from being placed (in most cases) in their expected familial and subordinal clades. Ronquist et al. (2012) also found that missing data in fossil terminals had overall little impact in combined analyses of their Hymenoptera dataset. Exceptionally incomplete fossils such as †*Eomola* (only the upper jaw is known) with >90% missing data, however, are more susceptible to being misplaced outside the family Molidae (suborder Moloidei) in some analyses (e.g., Bayesian TD and parsimony trees). This result differs from the parsimony analysis reported by Santini and Tyler (2004), which places †*Eomola* branching off the molid stem. Although the correct placement of tetraodontiform fossils cannot be known with certainty, simulation analyses have shown that phylogenetic accuracy for fossils can be greatly improved with the addition of molecular sequences from extant species (Wiens, 2009).

4.5. Conclusions

Tip dating is a promising method that allows direct incorporation of fossil taxa and their temporal metadata for simultaneous estimation of phylogeny and divergence times in Bayesian analyses. This approach provides a powerful tool for integrating extinct and extant taxa into a single time tree, with the potential to greatly improve inferences about tempo and mode of diversification using phylogenetic comparative methods (Slater, 2013; Slater et al., 2013). Implementation of tip dating can also help to overcome the pitfalls of using fossils as *ad hoc* age constraints in traditional node-dating calibration-density analyses. This study shows that whereas precision of tip-dating estimates depends upon the number and intercalation of fossil taxa in the tree, it seems that the current implementations lead to unexpectedly old ages of tetraodontiform divergences, which are largely incongruent with the results obtained with node-dating analyses (using both calibration densities and the FBD model) and with consensus interpretations of the fossil record. In most cases, clade ages inferred with tip dating imply the existence of unlikely and prolonged ghost lineages. The TD analyses of tetraodontiforms are also considerably more sensitive to the choice of root prior distribution (exponential or lognormal) relative to ND. Future methodological updates implementing ghost lineage penalization may help to improve the accuracy of tip dating.

Studies of molecular clocks rely heavily on paleontological information. Even though the addition of molecular data can change the position of fossil taxa, the support for their placement will ultimately be contingent upon morphological evidence.

Without new explorations of fossiliferous sites and without new phylogenetic studies coding morphological characters for both extant and fossil taxa, molecular studies concerned with age calibrations remain challenging and based on a background that is clearly incomplete. Integration of paleontological and neontological information is key to improving our understanding of the tree of life and macroevolution.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev. 2014. 10.011.

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