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Mass extinction in tetraodontiform fishes linked to the Palaeocene–Eocene thermal maximum

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Integrative evolutionary analyses based upon fossil and extant species provide a powerful approach for understanding past diversification events and for assessing the tempo of evolution across the Tree of Life. Herein, we demonstrate the importance of integrating fossil and extant species for inferring patterns of lineage diversification that would otherwise be masked in analyses that examine only one source of evidence. We infer the phylogeny and macroevolutionary history of the Tetraodontiformes (triggerfishes, pufferfishes and allies), a group with one of the most extensive fossil records among fishes. Our analyses combine molecular and morphological data, based on an expanded matrix that adds newly coded fossil species and character states. Beyond confidently resolving the relationships and divergence times of tetraodontiforms, our diversification analyses detect a major mass-extinction event during the Palaeocene–Eocene Thermal Maximum (PETM), followed by a marked increase in speciation rates. This pattern is consistently obtained when fossil and extant species are integrated, whereas examination of the fossil occurrences alone failed to detect major diversification changes during the PETM. When taking into account non-homogeneous models, our analyses also detect a rapid lineage diversification increase in one of the groups (tetraodontoids) during the middle Miocene, which is considered a key period in the evolution of reef fishes associated with trophic changes and ecological opportunity. In summary, our analyses show distinct diversification dynamics estimated from phylogenies and the fossil record, suggesting that different episodes shaped the evolution of tetraodontiforms during the Cenozoic.

1. Introduction

Understanding the evolutionary history of life on earth requires comprehensive documentation of both extant and fossil species. Integrative approaches that combine evidence from palaeontological and neontological data sources are increasingly becoming standard practice in macroevolutionary research [1–3]. Despite the fragmentary nature of the fossil record, fossils provide an extraordinary window for understanding past evolutionary events because they can offer information about the direction of character evolution, the timing of divergence, diversification rates and biogeography. Fossils can also clarify phylogenetic relationships that would otherwise be problematic in their absence [1–4]. Although the use of fossils to elucidate diversification dynamics and the influence of ancient geological events (e.g. Cretaceous–Palaeogene (K-Pg)) in fishes has significantly increased during the last decade [5–7], a consensus regarding the phylogenetic placement of several fossil lineages is still lacking, which restricts the inference about their timing of divergence and their evolutionary history.

The Palaeocene–Eocene Thermal Maximum (PETM) event is of particular interest because it resulted in a rapid acidification of the oceans and an increase in the bottom-water temperature [8], providing a comparable model for climate change due to anthropogenic CO_2 emissions. The PETM is linked to the mass extinction of the deep sea benthic foraminifera fauna [9], changes in species composition of marine plankton [10], and extensive biogeographic and trophic shifts of terrestrial vertebrate faunas [11,12]; however, little is known about how this period shaped diversity in marine fishes.

Fishes of the order Tetraodontiformes provide a model clade to test macroevolutionary hypotheses regarding the influence of ancient geological events, given their extraordinary representation in the palaeontological record. The tetraodontiform fossil record dates back to the late Cretaceous and includes 10 extant (all with fossil representatives) and 12 exclusively extinct families (summarized in [13–15]). Tetraodontiforms also have the smallest genomes among vertebrates [13,16], and they encompass a broad array of ecological preferences, with a characteristic pattern of diversification associated with habitat transitions (e.g. from marine to freshwater habitats) [17,18].

Herein, we estimate a robust time-calibrated phylogeny of extant and fossil tetraodontiform species, which we use to infer diversification dynamics using integrative macroevolutionary approaches. These estimates are further compared with those based on the information extracted from the fossil record alone and from extant-only molecular phylogenies. Using novel methods that explicitly model speciation and extinction and account for missing species, we detected a mass-extinction event for tetraodontiform fishes that coincides with the PETM, followed by an increase in the rates of speciation. This signal is consistent with the mass extinction of the benthic foraminifera fauna during the PETM, suggesting that this period may have played a more important role than previously considered in shaping the evolutionary history of marine fishes.

2. Material and methods

Details on the expanded morphological matrix coded herein are provided in the electronic supplementary material. Morphological and molecular matrices are archived in Zenodo (http://doi.org/10.5281/zenodo.583772).

(a) Integration of fossil and extant species

We examined a total of 146 species in 10 extant and 12 extinct families of tetraodontiforms. These comprise 94 extant and 52 (out of 66) fossil tetraodontiform species; the remaining 14 fossil species were not coded because they are mostly represented by fragments or by specimens in poor preservation condition. Three outgroups were included: two non-tetraodontiform percomorphs (the lophiiform *Lophiodes monodi* and the caproid *Antigonia capros*) and a zeiform (*Cyttus novaezealandiae*). *Lophiodes monodi* was selected as the representative lophiiform given its early branching phylogenetic position and generalized morphology within the order (T Pietsch and J Caruso 2014, personal communications). In addition to *L. monodi*, all 16 tetraodontiform fossils described since the publication of the original morphological matrix by Santini & Tyler [13] were newly examined and coded. A list of the specimens examined is included in the electronic supplementary material, along with commentaries on particular coding decisions. Morphological character data for 132 taxa were based on Santini & Tyler [13] and Arcila *et al.* [19], with improvements to the coding for some of the

fossil taxa. For the molecular data, we analysed the combined dataset compiled by Arcila *et al.* [19] based on one mitochondrial and 15 nuclear sequences. Taking into account missing data and polymorphic characters, the molecular and morphological datasets are 70% and 49.3% complete, respectively.

To assess the robustness of the placement of the new fossil taxa examined, three phylogenetic analyses under different optimality criteria were implemented: Bayesian inference (see details below under 'Estimation of tempo of evolution'); maximum-likelihood (ML); and parsimony. The ML analyses were conducted in RAxML v. 8.2.4 using the GTRGAMMA and Mk models with five partitions: four for the molecular sequences (one for each codon position of the nuclear genes plus mtDNA 16S); and one for the morphological dataset. Taxa with polymorphic character states were recorded as missing (?) for the RAxML runs (RAxML cannot recognize polymorphic characters), a recording procedure that affected only 13 out of 14 858 cells (0.08%) in the matrix. Branch support was assessed using the rapid bootstraping algorithm with 1000 replicates under the GTRGAMMA model; the collection of bootstrapped trees was used to draw bipartition frequencies onto the optimal tree (estimated from 30 independent searches). Parsimony analyses were conducted in TNT v. 1.1 based on the sectorial ratchet and tree-fusing methodologies with default parameters. Under this setting, iterations were run until the minimum length tree was found in 500 separate replicates in order to hit as many tree islands as possible. Character states were treated with equal weights, and multistate characters were treated as non-additive. Bootstrap support was assessed using 1000 pseudoreplicates (performed via tree bisection–reconnection branch swapping) and summarized in a consensus tree.

(b) Estimation of tempo of evolution

By leveraging the extensive fossil record of tetraodontiform fishes, co-estimation of topology and divergence times was simultaneously performed using the fossilized birth–death (FBD) process [3] in MRBAYES v. 3.2.6 [20] under the total-evidence-dating (or tip-dating) approach [2]. Data for the 52 terminal fossils and a single calibration for the root (95 to 107.9 Ma) were used as priors for age parameterization. To account for the uncertainty in fossil ages, we applied uniform age priors based on the fossils' stratigraphical ranges (additional details provided in electronic supplementary material, table S4). The molecular clock was modelled under the independent gamma rates model. Eight independent chains were run for 50 million generations each, sampling every 1000 generations. The matrix was also run with empty data (all characters changed to '?') to assess the influence of the priors in the posterior distributions. The first 10% of trees sampled were discarded as relative burn-in. Convergence of the MCMC was verified using the ESS criterion for each parameter in TRACER v. 1.7. 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. We also used the most comprehensive time-calibrated molecular phylogeny of tetraodontiform fishes [19] based on extant species only and using node-dating approaches.

(c) Diversification analyses

We first estimated diversification rates based on the complete tree with fossil and extant species and the evidence from isolated sources of data (either fossil occurrences alone or extant-only molecular phylogenies; see below). To assess diversification patterns over time, LTT plots and γ-statistic analyses were conducted for molecular trees, and for trees combining extant and fossil taxa. The LTT plots were obtained using the R package phytools [21] by plotting log-lineages through time based on the species-level chronograms. Because incomplete taxon sampling may significantly affect

diversification rates [22], we augmented the taxonomic coverage of tetraodontiforms by grafting three densely sampled family-level phylogenies (Tetraodontidae [18], and Monacanthidae and Balistidae [23]) into the backbone time tree estimated with MrBayes. Tree grafting was performed using the R package APE; branch lengths leading to each of the grafted families were set to the age of the node where they attach. This resulted in a more complete chronogram with approximately 53% (223 species versus 94 species) of the extant tetraodontiforms diversity.

To assess whether diversification rates based on molecular-only trees varied through time, we used a stochastic birth–death branching process (BD), as implemented in the R package TESS [24] under a Bayesian framework. Four models were compared: BD process with constant rates; BD with continuously varying rates; BD with episodically varying rates; and BD with explicit mass-extinction events. Because interpreting Bayes factors can be highly subjective, we estimated model uncertainty by calculating the marginal likelihood values for each candidate model using the stepping-stone sampling function, and then the ratio of the marginal likelihoods was compared for the set of candidate models implemented in TESS. To identify whether the molecular phylogeny has been significantly shaped by mass-extinction events (an instantaneous loss of a substantial fraction of species diversity, approx. 90%), we used an independent compound Poisson process using the CoMET model [25] implemented in TESS.

In addition to the TESS analyses using the global tree, we also fitted four time-dependent models of diversification using the R package RPANDA v. 1.1 [26]: time constant BD (null) model; exponential speciation and constant extinction rate model; constant speciation and exponential extinction rate model; and exponential varying speciation and extinction rate model. Because RPANDA requires clades to be specified *a priori* to account for different rate shifts across clades, we selected the two largest suborders, Tetraodontoidei and Balistoidei, as the first two clades and the rest of the tetraodontiform suborders as the third clade.

To assess diversification rates directly from the fossil record, all fossil occurrences and extant species were selected to infer whether the patterns of diversification were best explained by changes in speciation or extinction using a birth–death process in PyRate [27] (Bayesian framework) with rates comparable with those estimated from molecular phylogenies. Because PyRate does not require a morphological matrix to conduct diversification analyses, we used data for all fossil occurrences assignable to a particular taxon based on stratigraphical ranges (66 fossil species, 128 occurrences) along with data from approximately 440 extant species to jointly estimate the following parameters: preservation rate; speciation and extinction times; and speciation and extinction rates and their variation through time. Fossil occurrences were assembled from the Paleobiology Database (http://paleodb.org), which was simultaneously compared and vetted against the literature [13,16,28,29]. All undetermined or dubious identification records were removed from the data. The dataset was randomly replicated 100 times and run for 10 million generations on each replicate dataset. The first 20% of these samples were discarded as relative burn-in, and the posterior estimates of the speciation and extinction rates across all replicates were used to generate rate-through-time plots. Convergence of the MCMC was verified using the log files in Tracer v. 1.7.

3. Results

(a) Integration of fossil and extant species
Evolutionary relationships among tetraodontiforms determined herein are largely congruent with previous studies that integrated extant and fossil species [19,30,31]. Our study strongly supports the monophyly of all tetraodontiform suborders (as delineated in [19,32]) and families, including extant and fossil taxa dating back to the late Cretaceous (stem †Plectocretacicoidea: figure 1). Phylogenetic placement of the 16 newly coded fossils species, most of which previously had uncertain phylogenetic positions, is largely resolved under the different phylogenetic criteria, with only a few exceptions (figure 1; see the electronic supplementary material for details). A unique result of our study is the assessment of the placement of the oldest stem tetraodontiform, the superfamily †Plectocretacicoidea, which is arguably one of the most informative fossil clades for the timing of the origin of acanthomorphs [19]. The available evidence confirms the traditional view of plectocretacicoids as stem tetraodontiforms (but see [33,34]). Additional details are provided in electronic supplementary material, figure S2.

(b) Tempo of evolution
The time-calibrated tree inferred herein for tetraodontiforms is, by far, the most comprehensive to date, including representatives for all extant and fossil families and the 16 fossil taxa described during the last two decades. Our results, using the FBD process for tetraodontiform divergence-time estimation, support a younger root age relative to that obtained in previous total-evidence-dating analyses [19]. We find a mean clade age origin during the late Cretaceous, from 110 Ma (total group; 95% HPD 120–102 Ma) to 101 Ma (crown group; 95% HPD 111–91 Ma). The new phylogeny shows all extant and fossil families originating prior to the end of the Palaeogene (figure 1), and the resulting ages are more congruent with the fossil record of tetraodontiforms (i.e. imply fewer ghost lineages) than those estimated previously [19].

(c) Diversification analyses
Net diversification rates estimated from the fossil record occurrences alone support a slight decrease in the rates of extinction after the K-Pg (66 Ma), whereas examination of extant-only molecular phylogenies shows the opposite, i.e. a slight increase in extinction rates (figures 2 and 3*a*). Diversification rates based on molecular phylogenies using a diversified sampling strategy in TESS support a birth–death process with an explicit mass-extinction event during the PETM (figure 3*b*; additional details provided in electronic supplementary material, table S1). This result was consistently obtained from the LTT plots that integrated extant and fossil species (figure 4). The LTT plots based on both combined and extant-only phylogenies suggest an increase in the number of lineages after the PETM and a moderate taxonomic turnover during the last approximately 20 million years (figures 1 and 3; electronic supplementary material, figure S1).

We further assessed patterns of tetraodontiform diversification in RPANDA, assuming a homogeneous rate of diversification, which supports a birth–death model with exponential variation in speciation and constant extinction rates for the entire tetraodontiform phylogeny (electronic supplementary material, figure S3 and table S2). As previously noted in cetaceans, a homogeneous assumption of diversification can be violated by the temporal distribution of the most-species-rich clades [35]. To account for non-homogeneous rates of diversification, we isolated the two most speciose suborders, Tetraodontoidei and Balistoidei (figure 1; electronic supplementary material, figure S3). The Tetraodontoidei exhibits a recent peak of diversity during the early Miocene followed
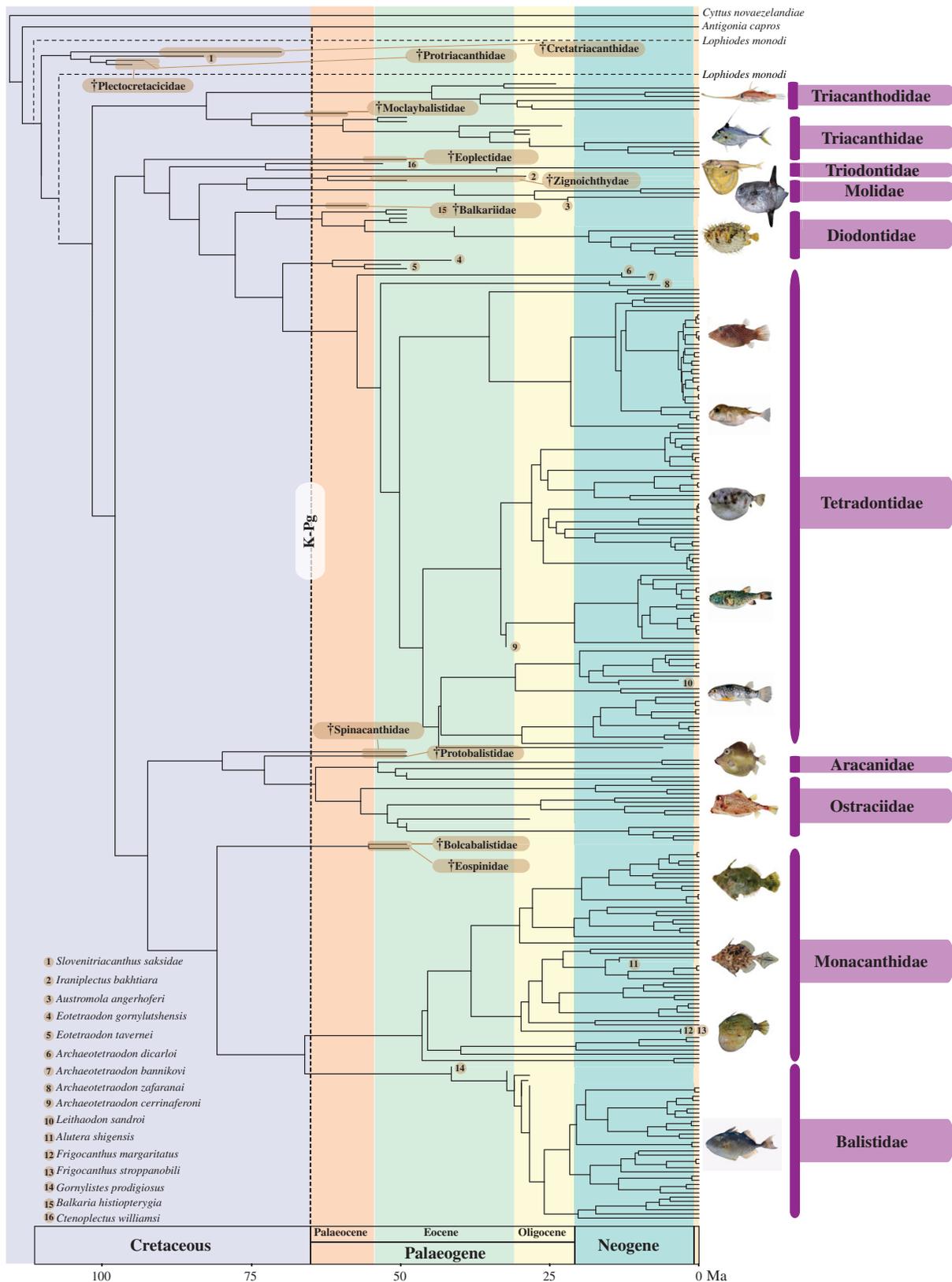
Figure 1. Chronogram of tetraodontiform fishes obtained by implementing the fossilized birth–death process using MrBayes. Names of the newly coded fossils are indicated by numbers (1–16). Alternative phylogenetic placements of *Lophiodes monodi* (Lophiiformes) are indicated by the two dash lines.

by a decline during the late Miocene (see electronic supplementary material, figure S3), a pattern obscured from the complete tetraodontiform phylogeny. The Balistoidei, on the other hand, exhibits exponential speciation and constant extinction rates,

suggesting that its diversity is presently expanding. The phylogeny of the remaining tetraodontiform suborders and families reveals constant speciation and extinction rates (see electronic supplementary material, figure S3).
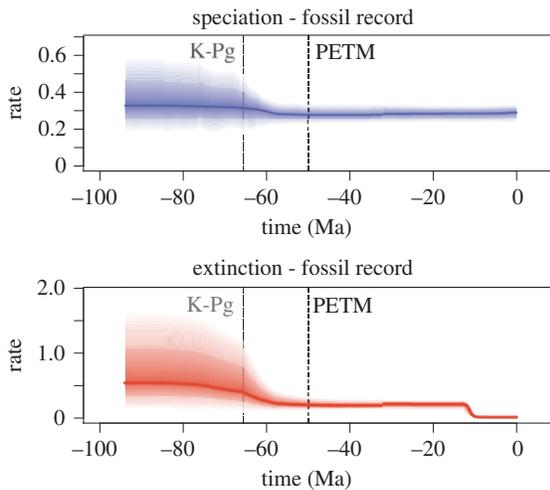
Figure 2. Rates of speciation and extinction through time estimated from the fossil record occurrences using PyRate.
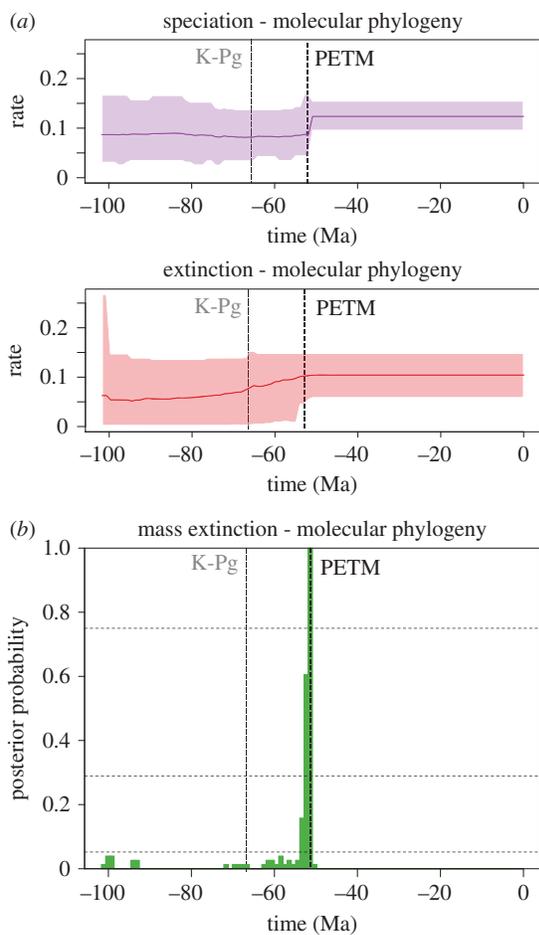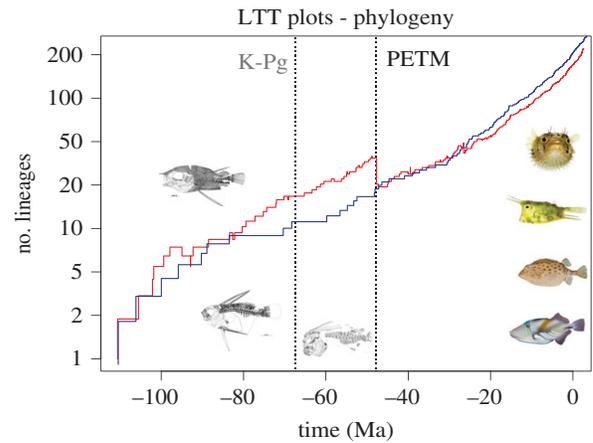

Figure 3. (a) Rates of lineage diversification estimated from the phylogeny for only extant species using TESS. (b) Mass-extinction events detected using the rjMCMC model, one significant mass-extinction event occurred 55 Ma.

Figure 4. Lineage-through-time (LTT) plots: red line represents the phylogeny including fossil and extant species; black line represents the same phylogeny after pruning all fossil species.

4. Discussion

(a) Diversification dynamics from fossils and extant-only phylogenies

The K-Pg mass extinction was a period of extensive changes in marine teleost faunas, which was followed by an explosive diversification as previously occupied niches became vacant (e.g. [36]). But, we show that tetraodontiform speciation rates from the fossil record and the phylogenetic analyses were constant during and after the K-Pg (figure 2), a result that is consistent with previous time-calibrated trees for acanthomorph fishes [5,37], we detected two opposite trends for the extinction rates: an increase revealed by the molecular phylogeny and a slight decrease indicated by the fossil record occurrences (figures 2 and 3). To confirm this, further studies are required comparing patterns of lineage diversification based on fossil occurrences and molecular phylogenies for other groups during the K-Pg. One possible explanation for this inconsistent result could be related to the incomplete nature of the fossil record. Despite tetraodontiforms having one of the best known palaeontological records among teleost fishes, there is a limited representation of fossils around the K-Pg, with only one extinct species described (†*Moclaybalistes danekrus* [13]). Given that PyRate uses fossil occurrences as input to estimate net diversification rates [27], a poor representation of tetraodontiforms in the fossil record during the K-Pg could be directly biasing the results towards a decrease in extinction rates. These results point out the importance of incorporating fossil-based and phylogenetic analyses in lineage diversification analyses.

An important outcome of this study was the detection of a major mass-extinction event that coincides with the PETM, followed by a significant increase in speciation rates (figure 3). Interestingly, this mass-extinction event coincides with a period of major global warming, ocean acidification, and high volcanic activity that has been linked to an abrupt drop in the diversity and turnover of benthic foraminiferal species, plankton and bottom-dwelling deep-sea organisms [10,38], and to a global decline in diversification rates of acanthomorphs [37]. Our results suggest that aspects of the PETM directly affected the diversification dynamics in marine fishes; however, it is uncertain which factors may have driven tetraodontiform fishes to undergo this mass-extinction event. Some likely explanations are low oxygen conditions due to an increase in deep-sea temperatures, an increase of $CaCO_3$ in the oceans as a result of CO_2 from the atmosphere, changes in trophic resources, or some combination of these [9,39]. Although the PETM may have driven many tetraodontiform lineages to extinction, it also may have promoted

tetraodontiform diversification by providing access to new habitats with abundant resources and few competitors, thus creating the conditions for increases in the rates of morphological and ecological diversification. Other studies have found similar diversification dynamics in other acanthomorph groups after the K-Pg [5], such as the radiation of major lineages within Labridae, Pomacentridae and Apogonidae [40]. These results are also consistent with patterns of diversity in placental mammals, neoavian birds and salamanders, for which diversity increased after the PETM extinction [41]. One question that warrants further exploration, however, is whether the extinction signal detected during the PETM for tetraodontiforms is also revealed by other acanthomorph groups. But, it is known that different groups may exhibit different responses to extinction [42] and that extinction can be selective, as noted in other groups during the K-Pg [5], it seems that there are also methodological artefacts that make the detection of mass extinctions a difficult endeavour from a phylogenetic perspective [43].

We similarly found support for a diversification shift of the suborder Tetraodontoidei (pufferfishes, porcupine fishes and allies) that coincides with the early Miocene (electronic supplementary material, figure S3). Changes in diversification dynamics during this period have also been documented in other groups of acanthomorpha fishes, such as Chaetodontidae, Labridae, Pomacentridae, Acanthuridae and Apogonidae, all of which are associated with coral reef habitats [40]. Explanations for the factors that may have contributed to the higher rates of diversification during the Oligocene/middle Miocene include extinctions through the late Eocene, tectonic events, changes in ocean circulation and upwelling, expansion of reefs in the Indo-West Pacific, and ecological or trophic opportunism [44]. Remarkably, a recent study on habitat shifts in pufferfishes showed an increase in the rates of cladogenesis associated with coral reefs occurring during the early middle Miocene [18], suggesting that coral reefs, ecological opportunity, or a combination of both may be responsible for this swing in Tetraodontoidei diversification.

(b) Reconciling palaeontological and molecular diversification rates

The estimation of diversification rates is crucial to understanding the causes that shaped patterns of species richness [45]. Although a limited number of studies have compared diversification rates inferred from the fossil record with those estimated from molecular phylogenies (e.g. [46]), the majority of these resulted in discrepancies when extinct species were included; for instance, trait evolution in fishes (e.g. [6]) and lineage diversification through time in plants (e.g. [47]). More recently, consistent diversification rates from the fossil record and from molecular phylogenies were obtained for cetaceans [35] and insects [48] when accounting for rates that varied over time and across clades. Further empirical comparisons are required, however, to evaluate the impact of fossils in deciphering diversification dynamics in marine fishes.

Our analyses reveal distinctive diversification dynamics estimated from the phylogeny and the fossil record (figures 2 and 3), suggesting that instead of a single event there had been several episodes that shaped the evolutionary history of tetraodontiform diversification since the late Cretaceous: a mass extinction during the PETM (figure 3); a rapid increase in speciation rates after the PETM (figure 3); and a shift in lineage diversification of the suborder Tetraodontoidei during

the Miocene detected under a non-homogeneous model of diversification (electronic supplementary material, figure S3).

(c) Timing of divergence based upon integrating fossils and molecules

Our analyses revealed robust estimates for the origin of tetraodontiforms, indicating a complex evolutionary history during the late Cretaceous and the Cenozoic, which was shaped by mass extinctions and changes in speciation and extinction rates. A large number of tetraodontiform fossil lineages have been described during the past few decade from deposits of the late Cretaceous to the early Pleistocene [28,30,31,49]. Moreover, a growing number of recent studies have addressed the timing of the origin of tetraodontiform fishes and of specific families within the order using either traditional node-dating or total-evidence-dating (tip-dating) approaches [19,23,31,49]. These studies, however, have largely ignored the most recent palaeontological information because they either used too few calibrations points for node-dating analyses or included only fossils that were previously coded [13] for tip-dating analysis [19].

We expanded the tetraodontiform dataset by coding more fossils, which we used in combination with molecular data to implement the FBD process that explicitly models diversification and fossilization as well as species sampling [20]. In agreement with the fossil record, our results find a late Cretaceous origin for crown tetraodontiforms (111–91 Ma; electronic supplementary material, table S3). These results, however, differ from previous studies that estimated either much older divergences (early Jurassic to early Cretaceous, 182–136 Ma) using tip-dating analysis, but without implementing the FBD process [19], or much younger dates (Palaeogene, 68–67 Ma) based on node-dating [17]. Similarly, other tip-dating studies using birth–death models without the FBD process have produced inconsistent results for Primates and Hymenoptera when compared with their corresponding fossil records [4,20]. Thus, these results confirm that previous implementations of total-evidence dating that failed to implement the FBD process can be sensitive to age overestimation (see electronic supplementary material, figure S4), a phenomenon recently referred to as 'deep-root attraction', and is attributable to either model misspecification or a failure to account for diversified sampling [50].

5. Conclusion

Previous studies estimating diversification dynamics in fishes, based upon the integration of fossil and extant species, have been restricted because of the absence of appropriate methodological approaches or by the scarce nature of the fossil record in many major groups. The relative completeness and exhaustive characterization of fossil and extant Tetraodontiformes makes this group an ideal model clade for conducting combined analyses that aim to infer macroevolutionary patters of lineage diversification. Our analyses show that the PETM, a period characterized by abrupt changes in climate and environmental conditions linked to the extinction of the benthic foraminiferal fauna and deep-sea biotas, is also responsible for shaping tetraodontiform diversification. Finally, our study provides a revised timescale of tetraodontiform evolution using the FBD process and a combination of molecular

and morphological datasets, including 16 newly coded fossil species. These results indicate that the age estimates under the FBD process are robust to the inclusion of new data and to different dataset assemblages, resulting in a late Cretaceous origin for Tetraodontiformes.

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