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DO REEFS DRIVE DIVERSIFICATION IN MARINE TELEOSTS? EVIDENCE FROM THE PUFFERFISH AND THEIR ALLIES (ORDER TETRAODONTIFORMES)

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A major challenge in evolutionary biology lies in explaining patterns of high species numbers found in biodiversity hot spots. Tropical coral reefs underlie most marine hot spots and reef-associated fish faunas represent some of the most diverse assemblages of vertebrates on the planet. Although the standing diversity of modern reef fish clades is usually attributed to their ecological association with corals, untangling temporal patterns of codiversification has traditionally proved difficult. In addition, owing to uncertainty in higher-level relationships among acanthomorph fish, there have been few opportunities to test the assumption that reef-association itself leads to higher rates of diversification compared to other habitats. Here we use relaxed-clock methods in conjunction with statistical measures of species accumulation and phylogenetic comparative methods to clarify the temporal pattern of diversification in reef and nonreef-associated lineages of tetraodontiforms, a morphologically diverse order of teleost fish. We incorporate 11 fossil calibrations distributed across the tetraodontiform tree to infer divergence times and compare results from models of autocorrelated and uncorrelated evolutionary rates. All major tetraodontiform reef crown groups have significantly higher rates of diversification than the order as a whole. None of the nonreef-associated families show this pattern with the exception of the aracanid boxfish. Independent contrasts analysis also reveals a significantly positive relationship between diversification rate and proportion of reef-associated species within each family when aracanids are excluded. Reef association appears to have increased diversification rate within tetraodontiforms. We suggest that both intrinsic factors of reef habitat and extrinsic factors relating to the provincialization and regionalization of the marine biota during the Miocene (about 23–5 MY) played a role in shaping these patterns of diversity

KEY WORDS: **Coral reefs, divergence time, diversification, relaxed molecular clock, Tetraodontiformes.**

A fundamental challenge in evolutionary biology lies in explaining the high species numbers found in biodiversity "hot spots" (Gaston and Blackburn 1996; Bellwood and Hughes 2001; Ricklefs 2004; Wiens and Donoghue 2004; Jablonski et al. 2006; Marshall 2006; Wiens et al. 2006). In the marine realm, tropical coral reef ecosystems underlie several critical hot spots that cover less than 1% of the surface of the ocean but contain more than 50% of the known aquatic restricted-range species (Roberts et al. 2002). The reef-associated fish fauna is one of the most conspicuous elements of reef ecosystems and marine evolutionary biologists have long been interested in explaining how and when this staggering diversity of fish evolved (Bellwood and Hughes 2001; Mora et al. 2003; Bellwood et al. 2006).

Reefs might promote diversification in a number of ways. Intrinsic factors of the reef habitat such as high productivity (Fraser and Currie 1996), high spatial complexity (e.g., Gratwicke and

Speight 2005; Lingo and Szedlmayer 2006), and high ecological complexity (meaning that reef species generally interact with a large number of other members of the community) have all been implicated in explaining the high standing levels of species diversity (see Bellwood and Wainwright 2002 for a review). It seems reasonable to infer that these same factors could provide an ideal "substrate" for driving elevated rates of cladogenesis in the fish lineages that inhabit them. In addition to these intrinsic factors of reef habitats, external paleogeographic vicariant events acting on reefs might have also driven speciation of resident fish species. Reefs show a dynamic pattern of diversification of the Late Eocene and early Miocene (40–23 million years [MY]) and only come to dominate shallow-water marine tropical ecosystems over this time (Wood 1999). It seems plausible that the factors driving coral diversification could also be responsible for promoting diversification in some reef fish clades. Indeed, Oligocene and Miocene processes are implicated in the diversification of at least some marine fish genera (Bernardi et al. 2002; McCafferty et al. 2002; Streelman et al. 2002; Klanten et al. 2004; Read et al. 2006). Sea-level changes and associated climatic events during the Pliocene/Pleistocene have also been implicated in the diversification of several fish groups, particularly in the Indo-Pacific (Palumbi 1997; McMillan et al. 1999; Carpenter and Springer 2005) and it is during this time that many Recent acanthomorph fish genera appear in the fossil record (Bannikov and Parin 1997; Tyler et al. 2002; Baciu et al. 2005; Carnevale 2006). Broadly speaking, to assess the biological significance of any of the disparate paleogeographic events after the end Cretaceous that might have shaped patterns of reef fish diversity, such as cooling at high latitudes, the closing of the Tethys, Gondwanan fragmentation, and the establishment of the east Pacific barrier (reviewed in Bellwood and Wainwright 2002) one needs information about the pattern and timing of diversification events.

Both the intrinsic and extrinsic hypotheses described above predict that lineages on reefs should show higher diversification rates than lineages in other habitats. Although many studies of reef fish implicitly assume that the reef habitat itself has played a fundamental role in shaping patterns of diversity, there have been no explicit phylogenetic comparative studies that examine patterns of diversification in nonreef sister lineages. One factor hindering comparative study is that most reef fish belong to the crown group Acanthomorpha, a clade that comprises approximately 60% of the total diversity of teleosts (Froese and Pauly 2006). Although many reef-associated acanthomorph clades have been identified (reviewed in Bellwood and Wainwright 2002), it has proved challenging to resolve higher-order relationships within the group (Johnson and Patterson 1993; Chen et al. 2003; Miya et al. 2003; Dettai and Lecointre 2005; Tyler and Santini 2005). As a result, there are few instances in which the sister lineage to reef clades can be identified with confidence.

Fishes in the Order Tetraodontiformes offer a happy exception to this general pattern. The order comprises approximately 430 species divided into 10 families (Santini and Tyler 2003; Froese and Pauly 2006). Five of them, the triggerfish (Balistidae, 43 species), filefish (Monacanthidae, 107 species), trunkfish (Ostraciidae, 25 species), pufferfish (Tetraodontidae, 185 species), and porcupinefish (Diodontidae, 22 species), contain a large fraction of species normally found on coral reefs in shallow tropical or warm-temperate waters (Table 4). The triplespines (Triacanthidae, seven species) inhabit tropical coastal environments but are often found at depths too deep for scleractinian coral reefs (see collecting data in Tyler 1968). Aracanid boxfish (Aracanidae, 13 species) are found in deep-waters throughout the Western Pacific Ocean and in coastal temperate waters in southern Australia. Two families, the morphologically primitive spikefish (Triacanthodidae, 22 species), and the monotypic three-tooth puffer (Triodontidae) also inhabit deep waters. The worldwide oceanic and pelagic sunfish (Molidae, four species) complete the order. The presence of both predominately reef and predominately nonreef-associated families makes tetraodontiforms an ideal group for examining the influence of one of the planet's most distinctive ecosystems on historical patterns of diversification in marine teleosts.

Several aspects of tetraodontiform evolutionary biology are also notable. Diversity in adult size spans six orders of magnitude, from a few grams (the Malabar pufferfish *Carinotetraodon travancoricus*), to over 2000 kg (the ocean sunfish, *Mola mola).* A major theme of tetraodontiform evolution is mechanical defense (Brainerd 1984; Wainwright et al. 1995; Brainerd and Patek 1998) and various lineages possess elaborate inflation mechanisms, heavily armored scale plates, and/or spiny dermal processes and dorsal fins. Skeletal evolution reflects strong trends toward reduction, simplification, and/or loss of skeletal elements (Tyler 1980; Santini and Tyler 2003, 2004), although many muscles, especially in the cranial region, have undergone extensive duplication (Winterbottom 1974; Friel and Wainwright 1997; Nakae and Sasaki 2004). Members are also notable for possessing compact genomes. The smallest known genomes in vertebrates occur in pufferfish (Hinegardner 1968) and several other lineages in the group show reduced genome size (Hinegardner 1968; Brainerd et al. 2001).

Tetraodontiforms possess what is arguably one of the best-studied fossil records of teleost fish (recently reviewed by Tyler and Santini 2002). Fossils exist for all 10 extant as well as nine other extinct families (Santini and Tyler 2003, 2004). Many of these are exquisitely preserved and in some cases are assignable to extant genera (e.g., Tyler and Patterson 1991; Tyler et al. 1992). The fossil record of tetraodontiforms spans back to the late Cretaceous (95 MY) (Tyler and Sorbini 1996), where three families form a stem lineage, sister to the remaining tetraodontiforms (Santini and Tyler 2003, 2004). Tetraodontiforms assignable to the crown group first appear in the Paleocene (58–59 MY) in both

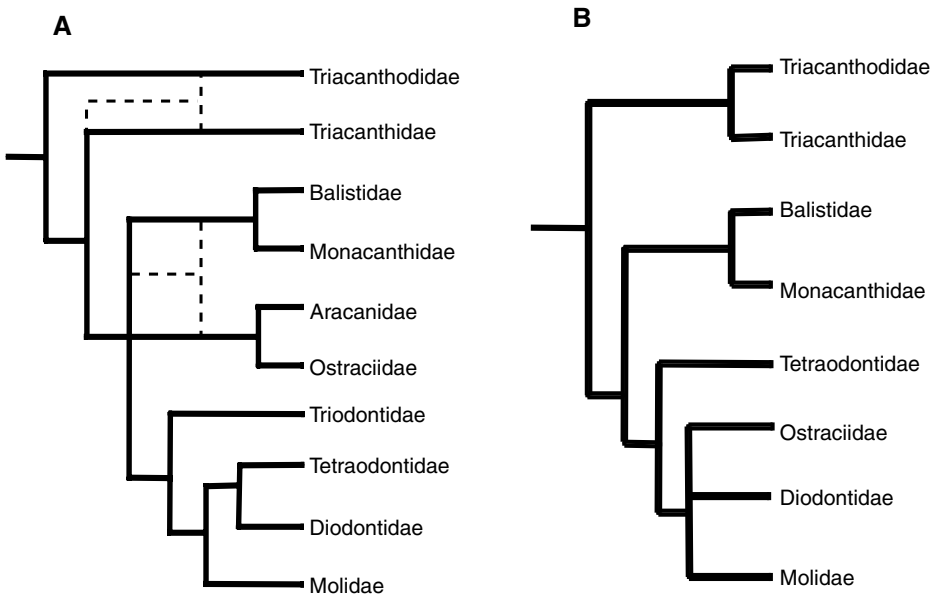

Figure 1. Morphological hypotheses of extant tetraodontiform families. (A) Topology based on analysis of adult skeletal (solid lines, Santini and Tyler [2003]) and musculature characters (dashed lines, Winterbottom [1974]). (B) Topology based on analysis of larval characters (Leis 1984).

northern Europe (Moclay deposits in Denmark) and in the Caucasus (southwestern Turkmenistan). The best-preserved tetraodontiforms date from the middle Eocene of Monte Bolca (northern Italy, approximately 50 MY). This locality contains at least six extant families as well as several extinct lineages. Except for the monacanthids, all extant families appear in the fossil record by the end of the Oligocene (23 MY). Almost all of the fossil taxa recovered from the Paleocene to the beginning of the Oligocene (35 MY) that have been included in phylogenetic analyses have been shown to be stem lineages to the extant crown families. The only exception is the Tetraodontidae, where *Archaeotetraodon winterbottomi*, from the early Oligocene of the Maikop deposits of the Caucasus in southwestern Russia, (about 35 MY), appears to be nested within the crown pufferfish. All fossils younger than the early Oligocene (Tyler et al. 1993; Tyler and Santini 2002; Sorbini and Tyler 2004) appear to be crown taxa, suggesting that the radiation of the crown families had started by then.

Previous phylogenetic analyses. Due to their highly modified morphological appearance, and their presence in European waters, tetraodontiforms have long attracted the attention of natural historians (e.g., Cuvier and Valenciennes 1828), and many taxonomic and phylogenetic hypotheses have been proposed for the group (see extensive reviews in Tyler 1980; Santini and Tyler 2003). The most influential of these include Winterbottom (1974), based on musculature characters of extant species; Tyler (1980) and Santini and Tyler (2003, 2004), based on skeleton features of extant and fossil species; Winterbottom and Tyler (1983), based on myological and skeletal data for extant species; Leis (1984), based on larval characters, and most recently, Holcroft (2004, 2005), based on molecular data. The morphological hypotheses largely agree on the placement of extant families (Fig. 1A). All assign subordinal status to three main lineages of extant tetraodontiforms: the Triacanthodoidei, sister group to all other extant tetraodontiforms, which includes the family Triacanthodidae and Triacanthidae; the Balistoidei, divided into two subgroups, with Balistidae + Monacanthidae sister to Aracanidae + Ostraciidae; the Tetraodontoidei, or gymnodonts, with Triodontidae being the sister taxon of (Tetraodontidae + Diodontidae) + Molidae. Santini and Tyler's (2003, 2004) topology conflicts with previous studies over the position of the Triacanthidae and the boxfish (Aracanidae + Ostraciidae). Leis' (1984) analysis of larval characters produced a conflicting topology in suggesting a sister group relationship between ostraciid boxfish and the tetraodontoids + Molidae (Fig. 1B). The molecular tree is strongly incongruent with previous morphological hypotheses (Holcroft 2004, 2005). Among the many novel relationships suggested by the molecular data is that the Molidae + boxfish form the sister group to the rest of the tetraodontiforms. Notably, *Triodon macropterus*, the sole member of the family Triodontidae, was not sampled in the molecular analysis.

The source(s) of incongruence between the morphological and molecular hypotheses is currently not known and will require additional data collection of both types of characters to resolve. Due to substantial differences in the taxonomic sampling of the molecular and morphological studies it is currently not possible to perform a satisfactory analysis of the combined data. In addition, neither of the common relaxed-clock methods available for divergence time estimation currently allow for the

incorporation of morphological data partitions when estimating branch lengths. For these reasons we rely on molecular characters only in this study although we acknowledge that uncertainty exists in higher-level tetraodontiform relationships.

Analysis of Tetraodontiform divergence and diversification. A recent molecular study of fish model organisms using calibrations distributed across vertebrate history suggests that the tetraodontiform crown group may have originated in the Jurassic (Yamanoue et al. 2006), and that the largest reef-associated families date back to the late Jurassic or early Cretaceous. If true (a Jurassic age of crown tetraodontiforms predates the appearance of the first stem tetraodontiform fossils by at least 64 MY), this suggests that the appearance of scleractinian coral reefs in the Tertiary was not a factor in the origin and early evolution of extant reef-associated families. However, if patterns of increased diversification within these families coincide with either the timing of reef association or with the diversification of the reef organisms themselves, this would still suggest that coral reefs have played a significant role in shaping patterns of standing tetraodontiform diversity.

Recent methodological developments in the area of statistical phylogenetics facilitate the kinds of temporally explicit analyses necessary to understand reef fish evolution and test for the role of reefs in driving diversification. Relaxed-clock methods in conjunction with fossil information improve divergence time estimation by allowing rates to vary across the tree (Thorne and Kishino 2002; Sanderson 2003; Drummond et al. 2006). In turn, chronograms provide the ages of stem and crown groups necessary to quantify patterns of diversification and test for unexpectedly rich or poor levels of standing species diversity (Magallon and Sanderson 2001). Here we demonstrate the utility of this approach by adopting a statistical phylogenetic framework to test whether (1) tetraodontiform evolutionary history extends back to the Jurassic, (2) diversification within reef-associated families coincides with the first appearance of scleractinian reefs or with later episodes of reef diversification, and (3) whether reef-associated families have diversified at a higher rate than nonreef-associated families.

To achieve these goals, we add new DNA sequence data from *T. macropterus* and *Pseudotriacanthus strigilifer* to the recently published molecular dataset of Holcroft (2005) and analyze it using two Bayesian divergence time estimation methods: the autocorrelated rates model implemented in MULTIDIVTIME (Thorne et al. 1998; Thorne and Kishino 2002) and a new uncorrelated lognormal rates model in BEAST (Drummond et al. 2006). We test for consistency among fossil calibrations using a cross-validation procedure (Near et al. 2005b) and explore the effects of inconsistent fossils and data-partitioning schemes on the divergence time estimation for 18 focal nodes in the Tetraodontiform tree. We also explore the effect of data partitioning on divergence time estimation. To quantify the temporal pattern of the tetraodontiform radiation, we plot the number of lineages through time and test for deviations from a model of constant rates of diversification. We test the hypothesis that reef-associated lineages have speciated more quickly than nonreef-associated families by two methods. First, by comparing standing levels of species diversity to those predicted under null models of cladogenesis. Second, by using phylogenetic comparative analysis to test for a positive relationship between diversification rate and proportion of reef-associated species within each family.

Materials and Methods

TAXON SAMPLING

We downloaded sequences from Genbank for two mitochondrial gene fragments (12S and 16S) and one nuclear gene fragment (RAG1) for 64 species of tetraodontiform fish species as well as seven outgroups (Appendix). Sixty-three of these species and all of the outgroups were from a recent molecular phylogenetic study of the group (Holcroft 2005). We downloaded sequence data from the molid *Ranzania laevia* from another recent study (Yamanoue et al. 2004) to increase the number of fossil-calibrated nodes available for divergence time estimation. In addition, we obtained a loan of tissue for the rare *T. macropterus* from the Australian Museum (sole member of the family Triodontidae). Finally, we obtained a specimen of the triacanthodid *Pseudotriacanthus strigilifer* from a commercial wholesaler. This specimen has been accessioned as a voucher to the Connor Museum of Natural History CRCM 06–240. Our taxonomic sampling includes representatives all 10 tetraodontiform families; the extent of sampling within each family is indicated in Table 4.

DNA EXTRACTION, GENE AMPLIFICATION, AND SEQUENCING

We used the PureGene Animal Tissue DNA Isolation Protocol (Gentra Systems, Inc., Minneapolis, MN) to obtain genomic DNA from *Triodon* and *Pseudotriacanthus*. We amplified mitochondrial gene fragments encoding approximately 425 bp of 12S, and 650 bp of 16S ribosomal RNA by PCR (94°C 45 sec, 50–55°C 30 sec, 72°C 1 min), using universal primers (Kocher et al. 1989; Palumbi 1996). A fragment encoding part of the recombination activating gene 1 (RAG1) was amplified using primers and protocols from Holcroft (2005). PCR products were sequenced in both directions using Big Dye version 3 chemistry (Perkin Elmer, Waltham, MA) and ethanol precipitated. We assembled and edited contigs in Sequencer 4.1 (Genecodes, Ann Arbor, MI).

SEQUENCE ALIGNMENT

We used Sequencher 4.1 (Genecodes) to produce initial alignments for all gene sequences. We manually aligned 16S and 12S sequence to previously published secondary structure models for the large and small ribosomal subunits in fish (Orti et al. 1996;

Wang and Lee 2002) using BBEdit (BareBones Software). Ambiguously alignable sites were identified by eye using conserved regions in the secondary structure models and were excluded from further analysis. RAG1 alignment was trivial. We used Se-Al (A. Rambaut) to visually inspect all alignments and to concatenate sequences into a single matrix. In total the concatenated dataset consisted 833 bp of 12S, 535 bp of 16S, and 1400 bp of RAG1.

PHYLOGENETIC ANALYSES

Maximum likelihood. We assessed the fit of common phylogenetic models to the concatenated dataset using ModelTest 3.6 (Posada and Crandall 1998; Posada 2003) in conjunction with PAUP* 4.0 b 10.0 (Swofford 2003). The GTR + G + I model received a decisive amount of the Akaike weight (about 1.0) (Burnham and Anderson 2003), and we used this model for all subsequent analyses of the concatenated dataset. We performed a heuristic search using this model in PAUP* with TBR branch swapping and 10 random addition sequence replicates. Strength of statistical support for phylogenetic results was assessed using nonparametric bootstrapping (Felsenstein 1985a) with 300 pseudoreplicates and two random addition sequences per pseudoreplicate.

Bayesian. We defined stem pairings for each ribosomal gene sequence and assigned separate doublet models to 12S and 16S stems using the unlink command in MrBayes 3.1 (Huelsenbeck and Ronquist 2001). We also assigned separate GTR + I + G models to each loop partition and to the RAG1 partition. Default priors were used for all model parameters (topology: uniform, revmat: Dirichlet(1.0,1.0,1.0,1.0,1.0,1.0), pinvar: Uniform(0.0,1.0), brlengths: Exp(10.0)). We set the Markov chain to run for a maximum of 10 million generations but also implemented a stopping rule so that the analysis would halt when the average deviation of the split frequencies was less than 0.1%. We ran four independent analyses each with one cold and four heated chains with the default heating parameter (temp = 0.2). We sampled every 100 generations and discarded the first 25% of MCMC samples as burnin. As a secondary check of convergence, we examined potential scale reduction factors (Gelman and Rubin 1992) for all parameters in the model.

DIVERGENCE TIME ESTIMATION

Fossil calibrations. We assigned fossil calibrations to 11 nodes on the Bayesian consensus tree (Table 1, Fig. 1). These calibrations provided hard lower bounds in all analyses. Upper age constraints represent the 95% cumulative distribution of the lognormal prior (i.e., a soft bound) in BEAST analyses and hard bounds in MULTIDIVTIME. In several calibrations below we assume an upper bound of 120 MY. The earliest acanthomorph fossils are reliably dated to 90–100 MY (Patterson 1993). Even taking into consideration the uncertain dating of some fossiliferous strata, we estimate that acanthomorphs originated sometime before 120 MY. Thus, the 120 MY upper bound that we use to constrain many nodes below reflects our prior belief that no living lineages of tetraodontiforms are older than this.

Age of the Root Between Tetraodontiforms and Outgroups

The sister group of the Tetraodontiformes is not currently known (Chen et al. 2003; Miya et al. 2003; Dettai and Lecointre 2004, 2005; Tyler and Santini 2005), and most acanthomorph groups that are thought to be closely related to the tetraodontiforms have a fossil record that is much younger (Patterson 1993; Baciu et al. 2005; Tyler and Santini 2005). The minimum age of the root must be at least 95 MY as this is the date of the earliest stem tetraodontiform (Tyler and Sorbini 1996; Santini and Tyler 2003, 2004). Placing an upper limit on the root is problematic. Recent molecular studies suggest that many lineages of fish (e.g., cichlids, ostariophyisans) are much older than their earliest fossil appearances (Kumazawa et al. 1999; Inoue et al. 2005;

Table 1. Bounds on fossil calibrations. Min/Mean/95% refer to the bound on the minimum age, the mean, and the value at the 95% lognormal prior distribution. m and SD specify the shape and scale parameters of the lognormal distribution used to specify the prior.

Calibration	Description	Min/Mean/95%	Ln(m, SD)
1	*Mola* versus *Masturus*	13/41/70	(3.20, 0.51)
2	MRCA of Triodontidae, Molidae, and Ostracioidea	53/70/120	(1.78, 1.47)
3	Aracanidae versus Ostraciidae	50/70/120	(2.30, 1.19)
4	*Diodon* versus *Chilomycterus*	5/18/50	(2.00, 1.10)
5	Tetraodontidae versus Diodontidae	50/70/120	(2.30, 1.19)
6	MRCA of tetraodontids	35/70/120	(3.30, 0.70)
7	*Sphoeroides* versus sister tetraodontids	5/18/50	(3.32, 0.30)
8	MRCA of Triacanthidae, Triacanthodidae, and Balistoidea	59/80/120	(0.09, 2.44)
9	MRCA of monacanthids	5/35/70	(3.21, 0.59)
10	Balistidae versus Monacanthidae	35/50/70	(2.50, 0.65)
11	*Balistes* versus *Pseudobalistes*	5/19/50	(2.00, 1.10)
	root	95/120/250	(1.97, 1.57)

Lavoue et al. 2005; Peng et al. 2006; Yamanoue et al. 2006). Our prior assigned a minimum age of 95 MY to the root and an upper bound of 250 MY, which is more than twice the currently accepted time for the origin of acanthomorphs (see above), and far exceeds the presently available date for the origin of Teleostei (Cloutier and Arratia 2004; Benton and Donoghue 2007).

Mola versus Masturus

The earliest fossils that can be assigned to the genus *Mola* are 13 MY (Fig. 2, node 1; Weems 1985). The oldest fossil that can be assigned to the Family Molidae is *Eomola* (41 MY) (Tyler and Bannikov 1992). Molas are open water dwellers and possess a very spongy skeleton, both features that make fossilization unlikely, so the split between *Mola* and *Masturus* might be considerably older than this fossil. Our prior assumed a minimum age of 13 MY, a mean time of 41 MY to reflect the known occurrence of other molid genera, and placed 95% of the weight on divergence times within the timespan marked by *Plectocretacicus clarae*, the first stem tetraodontiform (95 MY) (Tyler and Sorbini 1996; Santini and Tyler 2003, 2004).

MRCA of Triodontidae, Molidae, and Ostracioidea

The oldest fossil known to belong to this clade is *Triodon antiquus*, from the early Eocene of the London Clay (about 53 MY; Fig. 2, node 2; Tyler and Patterson 1991). In all phylogenetic analyses performed by Santini and Tyler (2004), *T. antiquus* appears as sister taxon to the only extant species of Triodontidae, *T. macropterus*. In our preliminary molecular analyses, however, the relationships between *T. macropterus*, the ostracioids, and the molids are not stable. For this reason the entire clade was constrained and assigned a lower bound of 53 MY and an upper bound of 120 MY (maximum likely age of tetraodontiform lineages, see above).

Aracanidae versus Ostraciidae

Eolactoria sorbinii (Tyler 1973) is a stem ostraciid, and *Proaracana dubia* is a stem aracanid (Fig. 2, node 3; Tyler and Santini 2002). Both are from the middle Eocene of Monte Bolca. We assigned a lower bound of 50 MY and an upper bound of 120 MY (maximum likely age of tetraodontiform lineages, see above) to this calibration.

Diodon versus Chilomycterus

The fossil *Diodon acanthodes* places a minimum age estimate on the split between *Diodon* and *Chilomycterus* of 5 MY (Fig. 2, node 4; Santini and Tyler 2003). We used this date as a lower bound and a date of 50 MY (first appearance of stem diodontids, below) as the upper bound.

Tetraodontidae versus Diodontidae

Several stem diodontids, *Prodiodon erinaceus, Prodiodon tenuispiis*, *Heptadion echinus*, and *Zignodon fornasieroae* (Fig. 2, node 5; Tyler and Santini 2002), and the stem tetraodontid, *Eotetraodon pygmaeus* (Tyler and Santini 2002; Tyler et al. 2006) are known from the middle Eocene of Monte Bolca (50 MY). We used this date as a lower bound and assigned an upper bound of 120 MY (maximum likely age of tetraodontiform lineages, see above) to the calibration.

MRCA of tetraodontids

The fossil *A. winterbottomi* (Tyler and Bannikov 1994) has been assigned to the crown Tetraodontidae, and provides a minimum age estimate of 35 MY for the most recent common ancestors (MRCAs) of the family (Fig. 2, node 6; Santini and Tyler 2004). We assigned an upper bound of 120 MY (maximum likely age of tetraodontiform lineages, see above) to this calibration.

Sphoeroides versus sister tetraodontids

Preliminary analysis and previous studies (Holcroft 2005) suggest that *Sphoeroides* forms a sister group to a clade consisting of *Canthigaster*, *Arothron*, *Monotreta*, and *Tetraodon* (Fig. 2, node 7). The fossil *Sphoeroides hyperosteus* from the Pliocene of North Carolina (5 MY) (Tyler et al. 1992) provides a minimum age of 5 MY for the split. We assigned an upper bound of 50 MY (corresponding to the first appearance of stem tetraodontids) to this calibration.

MRCA of Triacanthidae, Triacanthodidae, and Balistoidea

Preliminary data analysis revealed strong support for a group containing Triacanthidae, Triacanthodidae, and Balistoidea (Fig. 2, node 8). The relationships within this clade, however, were uncertain. The triacanthids generally appear as the sister taxon of triacanthodids + balistoids, but different relationships are occasionally recovered. For this reason we constrained the entire clade and assigned it a lower bound of 59 MY. This corresponds to the age of the stem balistoid *Moclaybalistes danekrus* (Tyler and Santini 2002). We assigned an upper bound of 120 MY (maximum likely age of tetraodontiform lineages, see above) to this calibration.

MRCA of monacanthids

The fossil genus *Frigocathus*, with the two species *F. stroppanobili* and *F. margaritatus*, dates from the late Pliocene and early Pleistocene of Italy, and is closely related to the extant *Aluterus* (Fig. 2, node 9; Sorbini and Tyler 2004). Preliminary molecular analysis revealed that the position of the only *Aluterus* in our sample is uncertain within a monophyletic Monacanthidae. We used this fossil to assign a lower bound of 5 MY to the age of the family and assigned an upper bound of 70 MY (corresponding to the age of the youngest stem tetraodontiforms) to this calibration.

Balistidae versus Monacanthidae

The stem balistids *Balistomorphus*, which includes the species *B. orbiculatus*, *B. ovalis*, and *B. spinosus*, and *Oligobalistes robustus* are all from the early Oligocene of, respectively, Switzerland and Caucasus (35 MY) (Fig. 2, node 10; Tyler and Santini 2002). They provide a minimum age estimate for the split between balistids and monacanthids. Our prior assigns a minimum age of 35 MY to this calibration, a mean age of 50 MY (reflecting the appearance of several other tetraodontiform families in Monte Bolca), and an upper bound of 70 MY to this calibration (reflecting the age of the youngest stem tetraodontiforms).

Balistes versus Pseudobalistes

The fossil *Balistes procapriscus* from the late Miocene can be assigned to the Recent genus *Balistes* and thus provides a minimum age estimate for the split between *Balistes* and *Pseudobalistes* of 5 MY (Fig. 2, node 11; Santini and Tyler 2003). We used an upper bound of 50 MY corresponding to the age of the Monte Bolca tetraodontiform families.

Autocorrelated rates model. One of the most commonly used parametric procedures for relaxing the assumptions of a strict molecular clock assumes that substitution rates are autocorrelated across the tree (Thorne et al. 1998; Kishino et al. 2001; Thorne and Kishino 2002). We used the program MULTIDIVTIME (J. Thorne), which implements this model in a Bayesian framework, to perform several analyses of our data. For each, we used the BASEML program (part of the PAML package, Yang 1997) to estimate branch lengths of the maximum-likelihood topology (Fig. 2) under the F84 model (Felsenstein 1984) with gamma-distributed rate heterogeneity (Yang 1994). After pruning outgroups, we used MULTIDIVTIME to estimate posterior distributions of divergence times. We specified a prior age of 70 MY (SD 35) for the expected age of the root, 0.1735 (SD 0.087) for the mean rate of substitution at the root, 0.4 (SD 0.8) for the Brownian motion parameter, and 250 MY for the upper limit of the analysis. We used 20,000 burnin cycles followed by 30,000 generations of the MCMC chain with sampling every 10 generations. Fossil first occurrences provided minimum age estimates for nodes and the 95% upper bounds (Table 1) were treated as fixed maximum ages. We repeated all analyses at least three times as a check that the MCMC sampler had converged on the target distribution.

Uncorrelated rates model. We used BEAST version 1.3 (Drummond and Rambaut 2003) to estimate divergence times under a model of uncorrelated but lognormally distributed rates (Drummond et al. 2006). We assigned soft upper bounds to the prior distributions of all 11 fossil calibrations using lognormal distributions as described above (Table 1). We also specified a Yule prior on rates of cladogenesis. The concatenated dataset was assumed to have evolved under a GTR model (Rodriguez et al. 1990) with invariant sites and gamma-distributed rate heterogeneity (Yang 1994). Five independent analyses of 10,000,000 generations each were run; output from each run was analyzed using TRACER 1.2 (A. Rambaut and A. J. Drummond).

Partitioning strategy. Both autocorrelated and uncorrelated rates models depend ultimately on inferred branch lengths to estimate divergence time and are thus potentially sensitive to the choice of phylogenetic model employed. To investigate the effects of partitioning scheme on divergence time estimation within and between methods, we performed three parallel sets of the above analyses. In the first, we combined the data into a single partition. In the second, we estimated branches for each gene partition (12S, 16S, and RAG1) independently and treated these partitions as having independent autocorrelation parameters. In the third, we assigned separate partitions and autocorrelation parameters to ribosomal stem and loop positions for each gene and to codon positions in RAG1, for a total of seven partitions. AIC scores calculated using Modeltest and PAUP revealed that a five or six parameter substitution model was the best-fitting model for every partition we examined. Furthermore, we found that the GTR + I + G model always fell within the 95% credible interval. For these reasons we assigned independent GTR + I + G models to each partition in our BEAST analyses.

Cross validation procedure. We used the procedure developed by Near et al. (2005a, b) to identify inconsistent fossil calibrations and explored the effects of including these fossils on our divergence time estimates. Briefly, this process involves three steps. In the first, each calibration is used in a separate analysis to estimate the ages of the other calibrations. Second, fossils are ranked by their error in estimating the other calibrations and F-tests are used to determine if the sequential removal of these calibrations produces a significant decrease in the pooled error of the remaining fossils. A significant F-test is taken as evidence that a calibration or set of calibrations provides estimates that are inconsistent with the others. Finally, a jackknife procedure is used to identify the single calibration point that is estimated with the least error by the others. The age of this fossil is then treated as fixed in subsequent analysis. We performed the cross-validation procedure using MULTIDIVTIME by constraining each node in turn with a minimum age as given in Table 1 and a maximum age equal to the minimum age + 1 MY and then estimating the ages of the other 10 fossil calibration points. After identifying and removing inconsistent fossils we reran MULTIDIVTIME. We assigned minimum age estimates to the consistent fossil-calibrated nodes as described in Table 1 with the exception of the node best-estimated by fossil jackknifing which also received maximum age estimate of minimum age + 1 MY. We also explored the influence of inconsistent fossils on divergence time estimation using the uncorrelated rates model by running BEAST analyses with these calibrations excluded.

Diversification Statistics. All diversification statistics were performed in R (R Development Core Team 2006) using routines written by L.J. Harmon, W. Challenger, and J. Weir. Rates of cladogenesis through time were investigated using the CR test of Pybus and Harvey (2000), which estimates the γ statistic of a given chronogram. Under a Yule (pure birth) process, γ values of completely sampled phylogenies have been shown to fit a standard normal distribution with mean $= 0$ (Cox and Lewis 1966; Pybus and Harvey 2000). Significantly negative γ values ($\gamma < -1.645$ for a one-tailed test) are indicative of decreasing rates of cladogenesis through time (i.e., internal nodes are distributed more toward the root than expected under a pure birth process). However, incomplete taxon sampling has been shown to inflate the Type I error rate of the CR test (Pybus and Harvey 2000). To correct for the undersampling in our analysis (65 of about 430 extant species), we used the Markov chain constant rates (MCCR) test (Pybus and Harvey 2000) in which full topologies are simulated under the Yule process and then randomly subsampled to generate a corrected null distribution. We compared our observed γ to a null distribution based on 10,000 simulated topologies.

The CR and MCCR tests both assume that diversification rates do not differ significantly among lineages over time. To test for violation of this assumption we estimated the relative cladogenesis statistic (Nee et al. 1992) for the seven-partition UC BEAST chronogram (Fig. 5). This statistic identifies lineages with significantly faster or slower rates of cladogenesis than their sister lineage by calculating the probability that the n total taxa descending from an internal node are partitioned into subclades of size r and s using a broken-stick distribution as the null expectation (Nee et al. 1992).

The diversification rate, r, was estimated for the tetraodontiforms using the method of Magallon and Sanderson (2001). Because this estimate is conditioned on the rate of extinction, which is unknown, we estimated r across extinction rates in increments of 0.1 from 0 to 0.9.

We used a corrected version of equation 11a from Magallon and Sanderson (2001) to calculate the probability of observing the standing levels of species diversity in each tetraodontiform family given global estimates of diversification (including extinction) across the order (r_G). For the Triacanthodidae and Triodontidae only one species was included in our study (in the latter only one extant species is known) and thus crown group ages were unavailable. For these families, equation (10a) from Magallon and Sanderson (2001) was used to calculate the above probability for a stem group age. All probabilities were calculated using source code written in R language by CDB.

We note here that the method of Magallon and Sanderson (2001) is less sensitive than others (e.g., Nee et al. 1994) to the effects of taxon sampling. However, inference of elevated diversification rates is still conditioned on underlying estimates of the age of the crown group (i.e., t in the equations of Magallon and Sanderson 2001). In our study, two sources of error could lead to misspecification of the crown group ages: errors in divergence time estimation and insufficient taxonomic sampling. Despite incomplete taxonomic sampling (Table 3), we believe that our crown group estimates are reliable for most nodes (meaning that the sampled taxa span the root of the focal clade). For some families (Molidae, Triodontidae, and Balistidae), our sample includes all or nearly all of the extant genera (Froese and Pauly 2006). Sampling of other focal groups generally includes morphologically distinct taxa thought to be the sister group to most remaining members of the clade (for example, *Lagocephalus* in the Tetraodontidae [Tyler 1980]). One exception to this is the aracanid boxfish. A previous morphological analysis (Winterbottom and Tyler, 1983) suggests that the crown group spanned by our taxonomic sampling excludes two genera (*Polycapros* and *Kentocapros*) and five species. To explore the robustness of our statistical results to crown group age misspecification, we performed a sensitivity analysis by progressively raising the crown age for focal nodes (assuming no extinction) until the difference in standing species diversity for the clade rate was no longer significantly different from that expected given the global rate. We also calculated aracanid diversification rates assuming that the crown group contained alternatively eight and 13 species to explore the possibility that our taxonomic sampling captured only a subclade of the family.

To test for elevated rates of lineage accumulation over specific time intervals, a lineage through time (LTT; Nee et al. 1992) plot for the Tetraodontiformes was compared with that of the expectation for a pure birth model (see Harmon et al. 2003 for a description of the construction of the pure birth plot). To test whether tetraodontiforms show greater than expected lineage accumulation early in their history, the area between the two plots was calculated for the first ~30% of the group's history. To assess the significance of this area, we simulated 20,000 trees equal in length and number of the included tetraodontiform taxa of our tree (65) under a Yule process with a diversification rate parameter equal to that estimated above with extinction $= 0$. For each simulated tree, the area between the LTT and the pure birth expectation plot was calculated for the given interval, producing a null distribution of the test statistic. P-values were calculated as the proportion of simulated trees that showed an area between curves greater than that calculated for the tetraodontiform tree. Our area was considered significantly greater than expected by chance for a time interval when this proportion was < 0.05 (one-tailed test). The above test was run with source code written in R language (R Development Core Team 2006) by C.D.B.

Influence of reef association on diversification rate. To test whether reefs lead to increased rates of diversification, we calculated the fraction of reef-associated species within each tetraodontiform family using habitat data taken from Fishbase (Froese

and Pauly 2006). We calculated the absolute diversification rate for each family under a pure birth model using the maximum-likelihood estimators and the BEAST-generated chronogram. Family-level diversification rates were calculated using the crown group estimator described by Magallon and Sanderson (2001) except in two cases (Triodontidae and Triacanthodidae). As both of these families were represented in our study by a single lineage, we used the stem group calculation of Magallon and Sanderson (2001) to estimate diversification rates. To test for an evolutionary association of diversification rate and reef habitat, we performed an independent contrasts analysis (Felsenstein 1985b) with branch lengths based on the BEAST chronogram as well as with all branch lengths set to 1 following a similar approach taken in a recent study of amphibian diversification (Wiens et al. 2006). The percent of reef-associated species within each family was arcsine transformed to meet the assumptions of the *F*-test.

Results

Likelihood and Bayesian Phylogenetics. Our heuristic search strategy produced a single most likely tree ($-\ln L = 32181.15$) (Fig. 2). Our topology as well as the general pattern of Bayesian posterior probability and bootstrap support is largely congruent with the Bayesian consensus tree presented in Holcroft (2005) and here we focus on the position of the three taxa newly added to the dataset. *Ranzania* is strongly supported as the sister group to *Mola* + *Masturus*, consistent with morphological (Santini and Tyler 2002b) and recent molecular hypotheses (Yamanoue et al. 2004; Bass et al. 2005). *Pseudotriacanthus strigilifer* receives strong support as the sister group to the other triacanthid, *Trixiphichthys,* in agreement with the phylogenetic studies based on morphological characters (Santini and Tyler 2002a). However, the addition of this second triacanthid did not affect the unexpected position of triacanthids as the sister to *Triacanthodes* + Balistoidea. Finally, *Triodon* appears in a nontraditional position as the sister group to the boxfish. This result did not receive strong support from either bootstrapping or Bayesian analysis. The Bayesian consensus topology (not shown) was congruent with the ML tree with the exception of *Triodon* that formed the sister group to the Molidae.

Autocorrelated Rates Model. MULTIDIVTIME estimates suggest a late Cretaceous origin of the crown group Tetraodontiformes (about 100 MY). Several splits among families occurred during the late Cretaceous the oldest of which are between Molidae and Aracanidae/Ostraciidae/Triodontidae and between Diodontidae/Tetraodontidae and Triacanthidae/Triacanthodidae/Balistidae/Monacanthidae. By the late Cretaceous all families except for the Monacanthidae and Balistidae had diverged from one another. The oldest crown family in our study was the Tetraodontidae with a MRCA of about 48 MY. The youngest were the Aracanidae (about 13.3 MY) and the Diodontidae (about 22.5 MY). Data partitioning had little effect on divergence time estimates (Table 2).

Uncorrelated Rates Model. The mean evolutionary rate was 0.0017 substitutions per site per million years (95% HPD: 0.0015–0.0019). The Yule process birth rate was 0.047 (HPD: 0.039–0.051). The data showed a great deal of unclock-like behavior with a coefficient of variation of 0.74 (95% HPD: 0.601–0.893). We found little evidence for autocorrelation of rates among parent and daughter branches (mean covariance: 0.0068, 95% HPD: −0.14–0.16). BEAST provided considerably younger estimates of divergence time across the tree compared to our MULTIDIVTIME analysis (Fig. 5). Crown tetraodontiforms appeared toward the end of the Cretaceous (about 70 MY). By the late Paleocene all familiar lineages except for the Monacanthidae and Balistidae had diverged; these last families split late in the Eocene (about 40 MY). Five families (Monacanthidae, Balistidae, Triacanthidae, Ostraciidae, and the Molidae) possess MRCAs with a late Oligocene/early Miocene age (about 20–25 MY). In addition, the clade containing the two most species-rich genera of pufferfish, *Sphoeroides* and *Canthigaster,* Node K, possesses a MRCA of similar age (about 18–19 MY). Branch lengths estimated under the three partitioning schemes did not vary substantially from one another (Table 2).

Cross validation. Removal of the two fossils with the highest squared error term (SS_x), calibrations 9 and 7 (Fig. 3) resulted in a significant decrease in the overall variance of age estimates ($F = 2.15$, df = 89, 71, $P < 0.001$). Jackknifing revealed the age of the split between Balistidae and Monacanthidae (calibration 10 in Table 1 and Fig. 2) as best estimated by the pooled consistent fossils. MULTIDIVTIME analysis with these calibrations excluded produced markedly younger mean divergence time estimates (age of crown tetraodontiforms = 76.8 vs. 99.9 for example) although most focal nodes still overlapped in their 95% credible intervals (Table 2). Reanalysis of the sequence data under the uncorrelated rates model using BEAST and the nine consistent calibrations produced similar estimates of the mean to those including all calibrations. The only exception to this was Node K that became significantly older (about 34.5 MY vs. about 19.4 MY) when the fossil constraining this node was removed.

Diversification statistics. We used age estimates from the uncorrelated rates (UC) seven-partition model (Table 2) because we judged these dates to be more reliable than those estimated under the uncorrelated model (Fig. 4) (see Discussion). The relative cladogenesis statistic was not able to reject the hypothesis of rate homogeneity across tetraodontiform lineages. The CR and MCCR tests showed no evidence for a slowdown in the rate of cladogenesis through time for the Tetraodontiformes ($\gamma = -0.024$, MCCR adjusted *P*-value = 1.0).

Estimates of r_G conditional on extinction decreased with extinction rate as expected (Table 3) (see Magallon and

Table 2. Divergence time estimates under autocorrelated and relaxed clock models. Nodes refer to focal nodes labeled in Figures 4 and 5. Bold rows indicate fossil-constrained nodes. UC = uncorrelated, lognormal distributed rates model, AC = autocorrelated rates model, P = number of gene partitions. The final two columns indicate divergence times estimated when inconsistent fossils as determined by cross-validation were excluded.

Node	Description	UC			AC			UC	AC
		1P	3P	7P	1P	3P	7P	1P	1P
A	crown tetraodontiforms	68.5 (61.9–76.2)	69.2 (61.7–77.6)	69.6 (61.6–78.6)	99.9 (83.2, 117.0)	102.4 (86.9, 119.4)	98.6 (84.4, 114.5)	72.1 (63.0–82.5)	76.8 (66.7, 91.3)
B	Molidae MRCA	25.9 (17.3–37.6)	24.2 (16.3–34.8)	25.5 (15.9–37.3)	36.4 (23.1, 51.9)	33.4 (20.6, 50.0)	31.1 (18.1, 46.8)	26.6 (17.7–37.8)	29.9 (20.1, 41.3)
C	*Mola* versus *Masturus*	15.7 (13.8–18.0)	15.6 (13.8–18.0)	15.6 (13.7–17.9)	16.9 (13.1, 26.0)	15.5 (13.1, 21.8)	15.1 (13.1, 20.3)	15.6 (13.8–17.9)	15.7 (13.1, 22.5)
D	***Triodon* + Molidae + "boxfish" MRCA**	**65.4** (57.2–74.5)	**65.7** (57.1–75.3)	**67.0** (58.6–76.5)	**97.7** (81.4, 114.5)	**97.2** (82.4, 113.6)	**93.3** (79.4, 108.8)	**68.8** (58.9–80.0)	**75.2** (65.5, 89.3)
E	Aracanidae MRCA	6.7 (1.5–13.9)	6.9 (1.5–14.3)	7.2 (1.9–14.4)	18.6 (8.0, 35.4)	14.5 (6.7, 25.1)	13.3 (5.7, 21.8)	7.6 (1.85–15.7)	17.6 (8.1, 30.6)
F	**Aracanidae versus Ostraciidae**	**52.2** (50.0–55.9)	**52.3** (50.0–55.9)	**52.1** (50.1–55.2)	**64.6** (51.4, 81.0)	**59.9** (50.6, 75.2)	**54.2** (50.1, 63.6)	**52.4** (50.1–56.4)	**54.1** (50.1, 63.6)
G	Ostraciidae MRCA	20.8 (9.1–36.2)	22.1 (8.9–38.6)	20.5 (8.1–39.1)	47.7 (32.1, 65.3)	41.7 (29.8, 56.8)	34.1 (24.6, 44.8)	22.5 (9.6–38.2)	41.0 (30.8, 51.7)
H	**Diodontidae MRCA**	**10.9** (6.4–16.1)	**11.8** (6.4–18.0)	**11.5** (6.7–16.4)	**25.5** (12.5, 41.8)	**24.6** (14.3, 37.6)	**22.5** (14.2, 32.2)	**11.9** (7.1–17.9)	**22.6** (11.6, 36.1)
I	**Diodontidae + Tetraodontidae**	**54.7** (50.1–60.6)	**54.8** (50.2–60.5)	**54.2** (50.2–59.9)	**84.5** (70.3, 99.0)	**86.9** (72.3, 102.8)	**84.8** (71.4, 99.0)	**58.0** (50.4–67.0)	**65.6** (56.9, 77.8)
J	**Tetraodontidae**	**38.3** (35.6–41.8)	**38.2** (35.6–41.6)	**38.7** (35.6–42.8)	**46.5** (36.5, 55.9)	**45.1** (36.3, 53.9)	**47.8** (38.5, 55.7)	**39.7** (35.7–45.1)	**39.5** (35.2, 48.2)
K	***Spheroides* versus sister tetraodontids**	**19.4** (13.5–26.2)	**19.2** (12.0–27.0)	**17.7** (11.6–25.5)	**41.4** (31.8, 49.4)	**41.5** (33.0, 49.3)	**43.9** (35.4, 49.7)	**34.5** (26.3–42.1)	**35.5** (29.9, 44.1)
L	"puffers" + balistoids MRCA	65.4 (57.7–73.0)	65.0 (59.0–71.1)	64.9 (56.8–73.0)	96.7 (80.8, 112.9)	100.1 (85.0, 116.6)	94.1 (80.8, 108.5)	68.8 (56.8–80.80	74.1 (64.5, 88.0)
M	Triacanthidae MRCA	21.0 (7.6–38.1)	21.8 (5.4–41.0)	23.4 (8.7–36.9)	33.7 (20.6, 49.6)	34.3 (18.6, 53.7)	34.0 (18.1, 51.9)	23.8 (9.1–41.9)	24.5 (14.5, 36.8)
N	"Balistoids"	60.2 (59.0–63.4)	60.3 (59.0–64.0)	60.7 (59.0–65.9)	93.0 (77.9, 108.2)	96.1 (81.5, 111.8)	89.7 (76.6, 103.7)	61.6 (59.0–69.2)	70.3 (61.1, 83.4)
O	Monacanthidae MRCA	24.6 (18.3–31.3	25.4 (18.3–31.8)	24.4 (17.7–29.8)	38.3 (35.1, 44.9)	38.2 (35.1, 44.6)	38.5 (35.1, 45.3)	28.1 (22.0–34.8)	15.9 (12.0, 20.2)
P	**Balistidae versus Monacanthidae**	**39.2** (35.6–44.2)	**39.3** (35.7–44.6)	**39.0** (35.5–43.6)	**65.0** (56.7, 69.8)	**64.4** (55.7, 69.7)	**62.6** (53.4, 69.5)	**41.6** (35.9–48.4)	**35.5** (35.0, 36.0)
Q	Balistidae MRCA	22.9 (16.0–29.9)	21.9 (14.9–29.7)	24.4 (16.7–32.0)	41.2 (31.2, 50.7)	36.0 (26.7, 45.6)	32.4 (23.8, 41.9)	24.8 (17.9–32.7)	18.5 (13.3, 24.0)
R	***Balistes* versus *Pseudobalistes***	**9.1** (5.3–13.5)	**12.0** (6.0–19.0)	**9.4** (5.5–13.4)	**19.9** (11.8, 29.7)	**20.3** (12.8, 29.2)	**17.4** (10.6, 25.3)	**10.0** (5.7–14.9)	**8.8** (5.5, 13.5)

Table 3. Departure of tetraodontiform families from global diversification rate estimates. Bold *P* values indicate significantly higher species diversity than expected under the global rate of cladogenesis. $\in$ is extinction rate, r_G is the estimated global tetraodontiform speciation rate conditional on a given rate of extinction.

Taxon	Age (MY)	Extant (No. Sampled)	$\in$=0.0 r_G=0.0750	$\in$=0.3 r_G=0.0736	$\in$=0.5 r_G=0.0708	$\in$=0.7 r_G=0.0685	$\in$=0.9 r_G=0.0512
predominately reef							
Balistidae	22.9	43 (14)	**0.002**	**0.009**	**0.022**	0.070	0.180
Diodontidae	10.9	22 (6)	**8.6×10^{-5}**	**0.0007**	**0.003**	**0.017**	0.094
Monacanthidae	24.6	108 (11)	**2.1×10^{-7}**	**1.0×10^{-5}**	**1.0×10^{-4}**	**0.002**	**0.020**
Ostraciidae	20.8	25 (5)	**0.025**	**0.050**	0.086	0.170	0.321
Tetraodontidae	38.3	185 (19)	**0.0002**	**0.0018**	**0.005**	**0.025**	0.059
pelagic, benthic, temperate							
Aracanidae	6.7	13 (2)	**2.7×10^{-4}**	**0.001**	**0.005**	**0.022**	0.106
Aracanini	*6.7*	*8 (2)*	***0.018***	***0.038***	*0.064*	*0.127*	*0.276*
Molidae	25.9	5 (4)	0.900	0.808	0.793	0.828	0.876
Triacanthidae	21.0	7 (2)	0.638	0.592	0.593	0.657	0.756
Triacanthodidae*	55.7	22 (1)	0.960	0.886	0.870	0.890	0.885
Triodontidae*	54.0	1 (1)	1.0	1.0	1.0	1.0	1.0

*Stem group ages for families with only one sampled species.

Sanderson 2001 for an explanation of this behavior). Notably, all of the reef-associated clades showed significantly higher than expected levels of diversity for all r_G with estimated for extinction rates between 0 and 0.3. With the exception of the aracanids, none of the nonreef lineages were more diverse than expected. Sensitivity analyses revealed that significance in all reef clades was robust to crown group ages 10 MY or greater than those reported in Table 3. Thus, we conclude that our results are moderately robust to misspecifications of the crown age due to dating error or incomplete taxonomic sampling.

Clade diversity over the first ~30% of crown tetraodontiform history is higher than expected under the pure birth model, however this result only approached significance ($P = 0.07$) in our parametric test (Fig. 6A, B). As the plot approaches the present, the area between the pure birth model and the observed becomes strongly negative, reflecting our reduced sample of the standing tetraodontiform diversity.

Influence of reef association on diversification rate. The proportion of reef-associated species within each tetraodontiform family ranged from 0% in four families to 84% in the triggerfish (Table 4). Preliminary regression using raw (not contrast values) diversification rate versus reef association were not significant ($r^2 = 0.17$; $F = 1.6$, df = 9, $P = 0.241$) but revealed the aracanids to be a distinct outlier with a high diversification rate and low fraction of reef species (Fig. 7A). Regressions of absolute contrast value on standard deviation were not significant for either reef association or diversification rate, indicating that evolution in both traits (assuming the chronogram branch lengths) did not violate the assumptions of the Brownian motion model underlying contrasts analysis (Garland et al. 1992; Diaz-Uriarte and Garland 1996; Diaz-Uriarte and Garland 1998). Initial regressions of diversification rate versus reef association approached significance ($r^2 = 0.25$; $F = 2.70$, df = 8, $P = 0.069$) when extinction rates were assumed to be 0 and were significant when extinction rates were assumed to be high ($\in = 0.9$: $r^2 = 0.33$; $F = 3.93$, df = 8, $P = 0.041$). The contrast between aracanids and ostraciids appeared as an outlier in these analyses due to the relatively high rate of diversification of the largely nonreef aracanids (Table 4). Exclusion of the aracanids from the analysis produced a highly significant result (Fig. 7B; $\in = 0.0$: $r^2 = 0.61$; $F = 11.08$, df = 7, $P = 0.006$). To further explore the sensitivity of the contrasts analysis to estimates of aracanid diversification rate we performed two additional analyses. In the first, we treated the split between the two aracanids in our study as an estimate of the age of a subclade of

Table 4. Summary of fraction of reef-associated species, and diversification rates (assuming no and high extinction rates) of tetraodontiform families. $\in$ is extinction rate, *r* is the estimated speciation rate conditional on a given rate of extinction.

Taxon	Reef species (%)	r ($\in = 0.0$)	r ($\in = 0.9$)
Aracanidae	8	0.29	0.12
Balistidae	84	0.13	0.07
Diodontidae	64	0.22	0.10
Molidae	0	0.03	0.01
Monacanthidae	53	0.17	0.10
Ostraciidae	76	0.10	0.05
Tetraodontidae	28	0.12	0.08
Triacanthidae	0	0.05	0.02
Triacanthodidae	0	0.02	0.01
Triodontidae	0	0.00	0.00

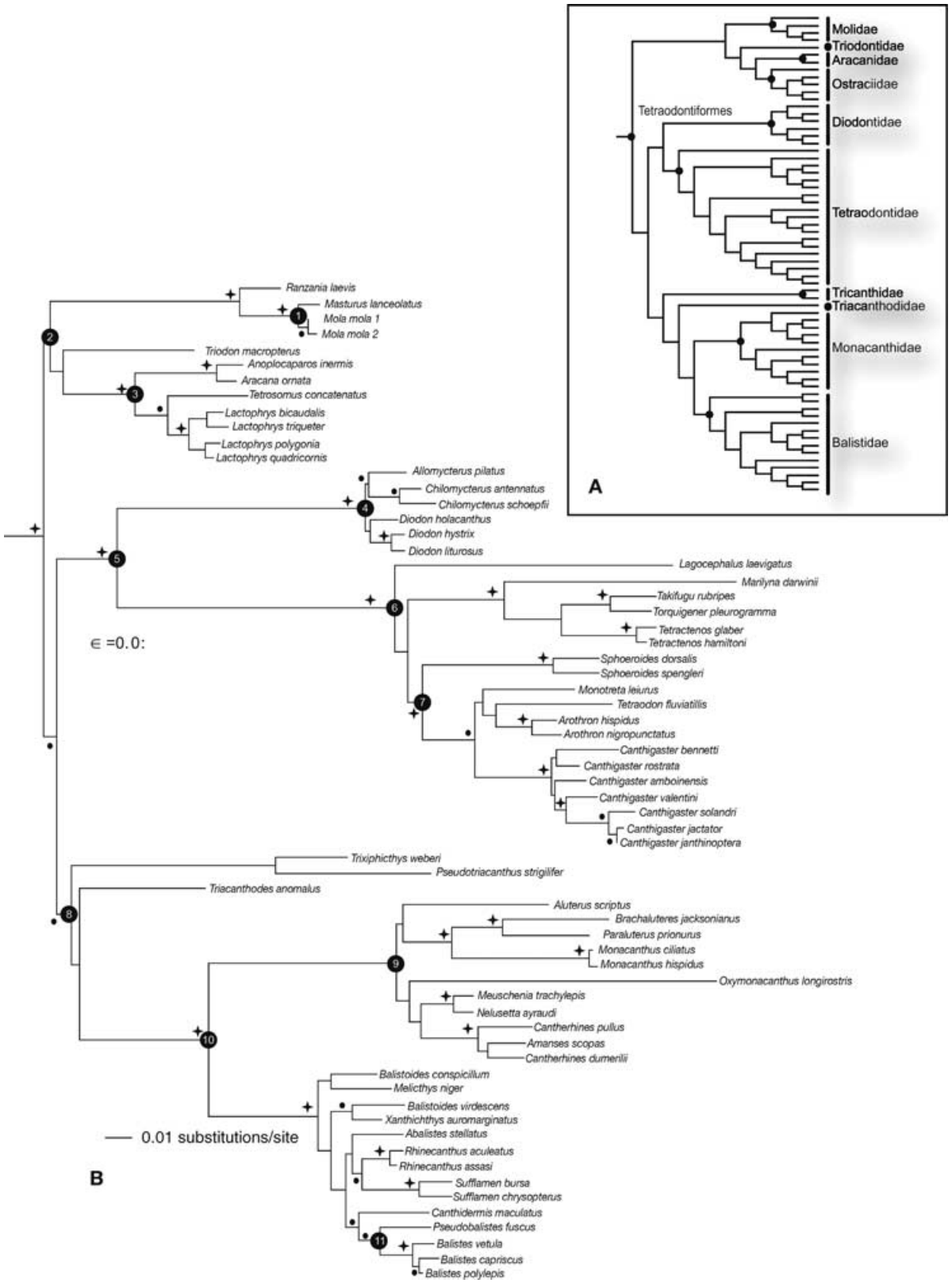

Figure 2. **Maximum likelihood tree based on concatenated gene sequences. Interfamilial relationships are highlighted in (A) with filled circles indicating the crown groups of each family. (B) Maximum-likelihood phylogram. Stars indicate branches nodes with bootstrap support $>$ 70% and posterior probability $>$ 90%; solid circles indicate nodes with posterior probability $>$ 90%. Numbered circles show the position of the 11 fossil-calibrated nodes used in this study.**

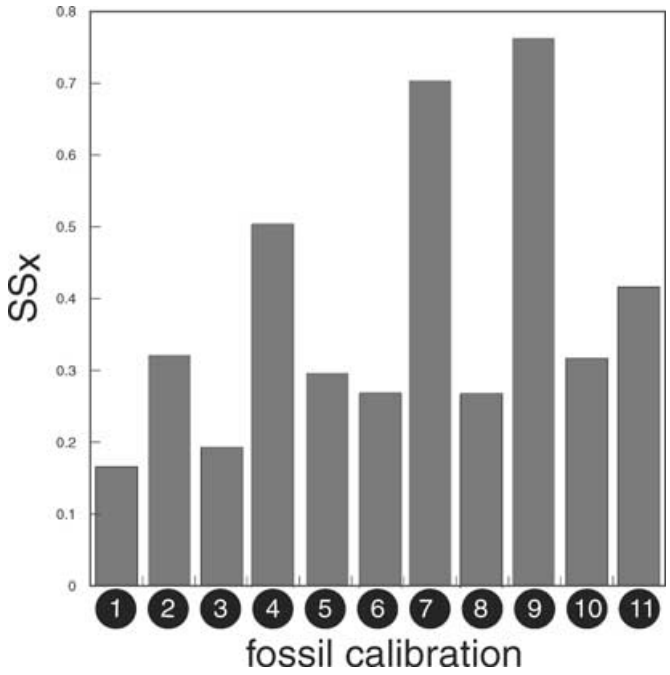

Figure 3. Assessment of fossils consistency. Histogram showing sum of squared error in age estimates of other calibrations when each calibration, in turn, is treated as known. Calibrations 7 and 9 have the highest error terms. Removal of these two calibrations results in a significant decrease in the variance of the pooled divergence time estimates (see Results).

the Aracanidae (Aracanini; containing only eight species) rather than as an estimate of the age of the entire family as suggested by the morphological phylogeny of Winterbottom and Tyler (1983) (see Methods). Regression analysis based on contrasts using this treatment of the data was significant ($\in = 0.0$: $r^2 = 0.39$; $F = 5.13$, df $= 8$, $P = 0.026$). In the second, we calculated aracanid diversification rate using the age of the stem group rather than the crown group. Contrasts analysis based on this estimate showed a highly significant relationship between reef association and diversification rate ($\in = 0.0$: $r^2 = 0.61$ $F = 12.56$, df $= 8$, $P = 0.004$). We obtained similar results for all analyses above assuming an extinction rate of 0.9 as well as when using a tree in which all branch lengths were set equal to 1.

Discussion

Our study confirms for the first time a widely held assumption by marine evolutionary biologists: that teleost lineages associated with reef habitats generally experience greater rates of diversification than nonreef-associated lineages. In addition, we find that the pattern of diversification is complex and does not suggest an ancient reef-fish association. Instead, diversification within tetraodontiform reef families coincides with periods of reef diversification and marine provincialization during the late Oligocene and Miocene. During this period, reef-associated crown groups show significantly elevated rates of diversification over the global Tetraodontiform rate; nonreef families, with the exception of the Aracanidae, do not (Table 3). We also find that tetraodontiforms are younger than previously suggested, with the crown group originating in the Late Cretaceous and all significant diversification unfolding over the Tertiary. Below we consider the methodological and evolutionary implications of our study in greater detail.

Data partitioning and divergence time estimation. We found divergence times of the focal nodes in this study to be generally robust to the branch lengths estimated under different models. The magnitude of these differences was generally small (Table 2). This is in general agreement with Yang and Rannala (2006) who found very similar divergence time estimates under a JC and HKY + G model despite the HKY + G model's significantly better fit to the data. The robustness of divergence time estimation is especially notable given the different ways that MULTIDIVTIME and BEAST estimate branches. MULTIDIVTIME currently uses a relatively simple model to obtain branch length estimates conditioned on a single topology. In contrast, our BEAST results were conditioned on far more complex models (GTR + I + G per partition) as well as on the posterior distribution of topologies. It is possible that poorly specified priors could prevent the data from yielding meaning posteriors no matter what partitioning scheme was used (Thorne and Kishino 2002; Yang and Rannala 2006). However, we feel that this is unlikely given the partition-insensitive results of the BEAST analyses where our use of soft bounded calibrations should mitigate against the effects of truncated priors (Yang and Rannala 2006).

Uncorrelated versus autocorrelated rate models. Like other recent studies comparing different divergence time methods, we found substantially different estimates between models (Bell and Donoghue 2005). The autocorrelated model suggests an early Upper Cretaceous origin of the tetraodontiforms with most diversification among families occurring before the Paleocene. In contrast, the uncorrelated model suggests an end Cretaceous origin of the order with family-level diversification during the Paleocene. In addition, BEAST suggests a late Oligocene/early Miocene origin for most of the crown families.

Hereafter, we focus on the uncorrelated divergence time estimates for a number of reasons. The covariance between parent and daughter branches was essentially zero, suggesting that the autocorrelated rates model may be inappropriate for the tetraodontiform sequence data. In addition, the UC-generated divergence times appear to be more robust to error potentially introduced by the fossil calibrations. The exclusion of inconsistent calibrations had little effect on divergence times estimated under the UC model in contrast to the AC estimated that dropped considerably. Finally, we prefer the UC procedure because the age estimates accommodate topological uncertainty.

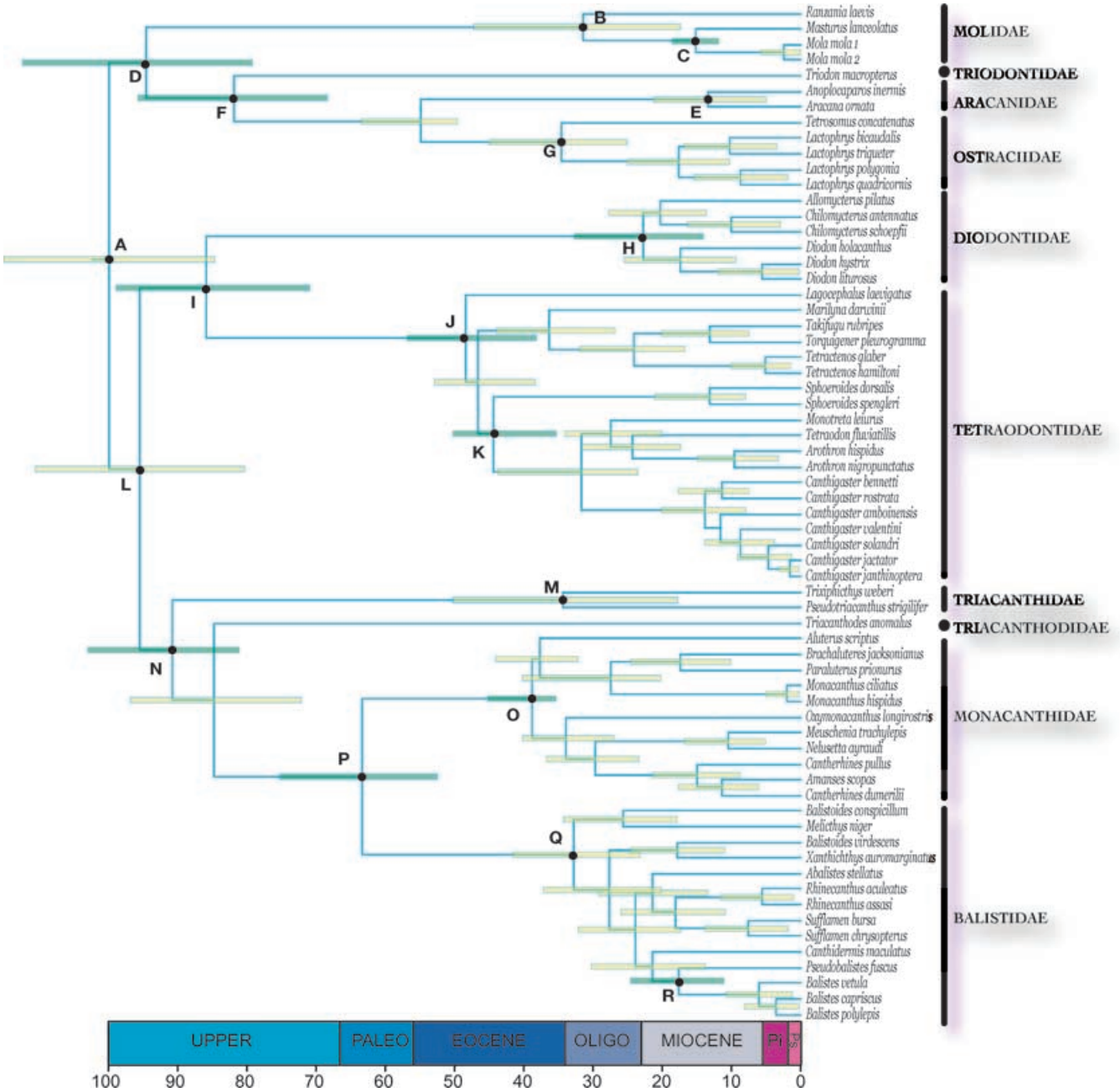

Figure 4. Chronogram estimated under the autocorrelated rates model with seven independent gene partitions. Branch lengths are proportional to the geologic time scale. Circles indicate the position of 18 focal nodes (ages given in Table 2). Credible intervals (95%) indicated by node bars and green bars indicate fossil-calibrated nodes.

Position of Triodon. The molecular data suggest a novel placement of the three-toothed puffer as the sister to either the Molidae or the Ostraciidae + Aracanidae. Both placements are incongruent with previous morphological trees that place *Triodon* as one of the sister groups to the Tetraodontidae + Diodontidae + Molidae. The statistical support for the position of the *Triodon*, Molidae, and Aracanidae + Ostraciidae is weak and so caution should be used in inferring patterns of character evolution from this topology (Fig. 2). However, we note that these trees suggest that the beak-like fusion of the jawbones might have occurred twice independently (once in the puffer + porcupinefish clade, and a second time in the molas + boxfish + three-tooth puffer clade). Interestingly, convergent evolution of beak-like jaws is known to have occurred in labrid fish as well (Westneat and Alfaro 2005).

Comparisons with earlier studies. Yamanoue and colleagues (2006) estimated much older divergence times for three tetraodontiform clades than we found in our study (Yamanoue et al. 2006). Their date for a split within the Tetraodontidae of 85 MY

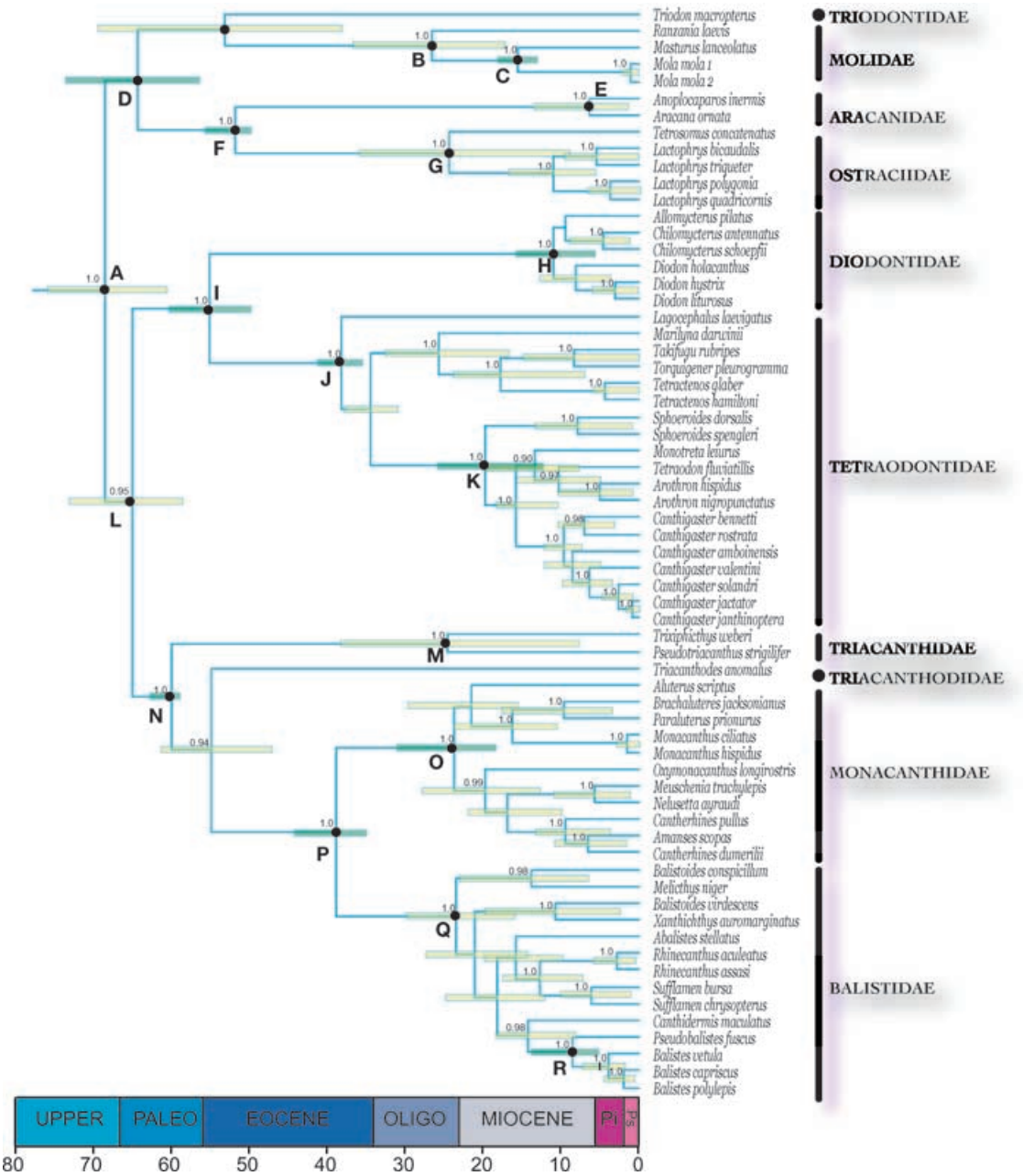

Figure 5. **Chronogram estimated under the uncorrelated lognormal-distributed rates model with seven independent gene partitions. Branch lengths are proportional to the geologic time scale. Circles indicate the position of 18 focal nodes (ages given in Table 2). Credible intervals (95%) indicated by node bars and green bars indicate fossil-calibrated nodes.**

exceeds our estimate of the MRCA for all extant Tetraodontiformes. Yamanoue et al's estimate of 130 MY for the split between filefish and triggerfish (Node P, Table 2) and 159 MY for the split between triggerfish and smooth puffers (Node L, Table 2) also vastly exceed our estimates.

We suspect that these conflicting date estimates arise from differences in the choice and treatment of calibrations between the studies. Yamanoue et al. do not take advantage of the younger fossils available to date splits within the Tetraodontidae or between the Balistidae and the Monacanthidae as we have done in this study. We also view some of their deeper calibrations as problematic. Although Yamanoue et al. use a putative gadiform fossil as a calibration with a listed age of 161 MY (Bathonian, Jurassic), the earliest fossils that can unquestionably be identified as gadiforms date from the Paleocene (about 100 MY later) (Benton 1993; Patterson 1993). Two other calibrations (the split between chondrichthyans and osteichthyans, 528 MY and between actinopterygians and sarcopterygians, 450 MY), derived

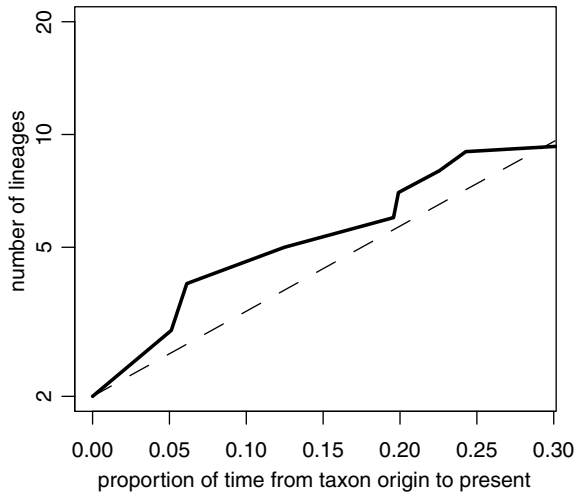

Figure 6. Test for accelerated cladogenesis in tetraodontiform early history. A lineage through time plot shows that there were more tetraodontiform species (solid line) over the first 30% of the groups history than expected under a pure birth model (dashed line). A parametric test for the significance of the area above curve based on 20,000 simulated evolutions under the pure birth process (r_G with $\in = 0.0$) approached significance ($P = 0.07$).

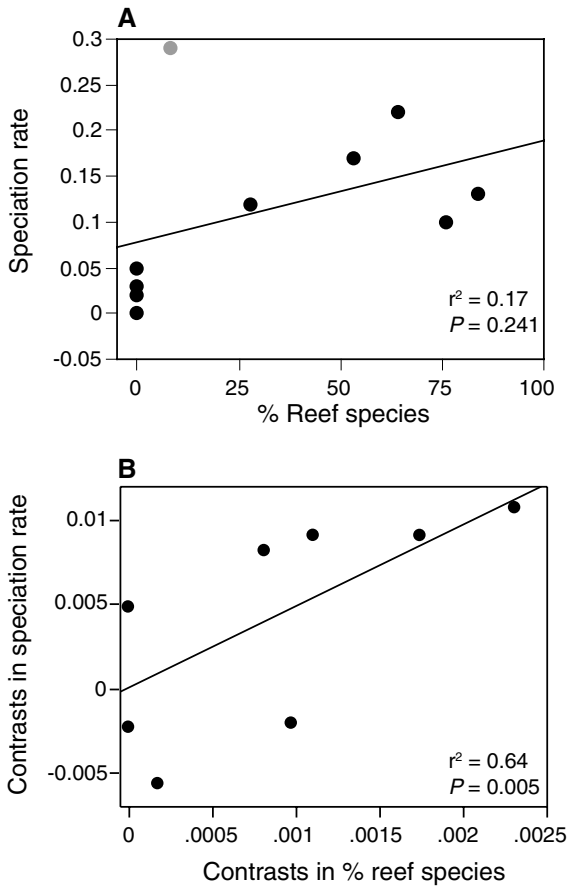

Figure 7. Influence of reef association on diversification rate: (A) Linear regression analysis of the tip values (not contrasts) shows a weak relationship between diversification rate and the proportion of reef species within each tetraodontiform family when the Aracanids (shaded point) are included in the analysis. (B) Contrasts among nonaracanid lineages reveal a significant positive correlation between reef association and diversification rate. Contrasts shown are based on branch lengths from the BEAST chronogram in Figure 5. Similar results obtain when all branch lengths are set to equal 1.

from previous molecular studies (Kumazawa et al. 1999; Hedges and Kumar 2003), are much older than the fossil evidence suggests. Ultimately, both are conditioned on the date of the mammal/bird split (Hedges and Kumar 2003), which itself has received recent criticism (Reisz and Muller 2004; Muller and Reisz 2005).

We argue that our results reflect the weight of the evidence of the tetraodontiform fossil record. When all 11 calibrations across the tree are considered, even the most unconstrained analyses (under the AC model) point to an Upper Cretaceous origin of crown Tetraodontiformes. Despite our accommodation of topological uncertainty, the use of soft upper bounds for all of our fossil calibrations (UC analysis) and a prior on the age of the root that could accommodate the placement of the tetraodontiform MRCA in the Jurassic in the AC and UC analysis, the 95% credible intervals indicate significantly younger ages than those found by Yamanoue et al. (2006). Thus, we reject the hypothesis that Tetraodontiformes originated and substantially radiated during the mid-Mesozoic.

Potential causes of elevated diversification in the Aracanidae. In our study, the Aracanidae have a very high diversification rate (Table 4) but are not associated with coral reefs and are thus an exception to the general pattern found in other tetraodontiforms. One possible explanation for this pattern is that we have underestimated the aracanid crown group age. The morphological phylogenetic study of Winterbottom and Tyler (1983) provides some evidence for this because it suggests that the genera *Polycapros* and *Kentocapros* fall outside of the clade spanned by our sampling of *Anoplocapros* and *Aracana*. With the five species in these genera excluded, the diversification rate for the remaining aracanid subclade (the "Aracanini" from Wintebottom and Tyler, 1983) becomes consistent with the pattern seen in other tetraodontiform families (see Results). Our relaxed-clock aracanid crown group age could also be an overestimate if the aracanids experienced an unexpectedly low rate of molecular evolution. Possible support for this comes from the observation that the difference in age between stem fossils and the age of the crown for aracanids is far larger than for all other tetraodontiform families (Fig. 5), even though morphological characters provide reasonably good support for the relationship between modern and fossil forms (Santini and Tyler 2003).

A second explanation is that the pattern is real and that historical factors acting on aracanids have resulted in high diversification rates in the absence of reef association. Most species of Aracanidae inhabit temperate waters around Australia, although some are found in deep waters throughout the western Pacific. Higher than expected diversification rate within this family could have been caused by the invasion of Australian waters, when the Australian plate collided and fused with the Asian plate during the Neogene (23–5 MY), after a long period of isolation following the Mesozoic breakup of Gondwana (Hallam 1994). It is also plausible that of the nonreef lineages aracanids are the most likely to have been affected by the climatic factors causing marine regionalization. The stem members of the family are known from the shallow water paleoenvironment of Monte Bolca (Tyler and Santini 2002) and the lineage might have experienced some diversification prior to or as a consequence of invading more temperate waters. The clade may also be more susceptible to vicariant events than some other nonreef tetraodontiforms due to a relatively widespread geographic distribution. However, these remain only speculations and future studies with more intensive sampling of these (and other) tetraodontiform clades will be necessary to determine whether the outlier status of the aracanids here is due to an underestimation of the crown group age or an accelerated rate of cladogenesis.

Diversification in tetraodontiforms and other fish: the role of coral reefs. Our results suggest that reef-associated tetraodontiform families have experienced greater rates of diversification than families not associated with coral reefs. But precisely what attributes of reefs are responsible for producing this macroevolutionary pattern? Given the historical nature of the data, the identification of causal factors underlying diversification of reef fish will likely remain a challenging problem. Here we argue that our phylogenetic comparative analysis is consistent with the hypothesis that diversification in tetraodontiform reef groups is driven in part by ecological opportunities provided by the unique and complex reef habitat itself. As clades independently become associated with reefs, there is a trend toward increased diversification rates within them (Fig. 7). However, we also suggest that the distribution of reef-family crown ages provides evidence for the role of extrinsic paleoclimatic events in shaping diversification patterns of reef-associated tetraodontiforms. If intrinsic factors were solely responsible for increasing diversification rates then we would expect the crown ages of extant groups to reflect the initial period of association between the tetraodontiform lineage and the reefs. However this appears implausible for at least some tetraodontiform families. For example, parsimonious reconstruction of ancestral habitat type would suggest that the common ancestor of filefish and triggerfish (Fig. 5, node P) was also reef associated. Yet a considerable gap in time (17 MY) separates the putative origin of reef-association and diversification within the crown families.

More generally, the age of first appearance of scleractinian reefs in the Eocene substantially predates both their widespread distribution (Wood 1999) as well as the crown ages of reef-associated tetraodontiforms, other reef fish (Bernardi et al. 2002; McCafferty et al. 2002; Streelman et al. 2002; Klanten et al. 2004; Read et al. 2006), and even the reef-building invertebrates themselves (Crame 2001; Wallace and Rosen 2006). This gap between the appearance of the first Cenozoic reefs and their widespread colonization of shallow-water ecosystems has been attributed to the appearance of more efficient herbivores and bioturbators, (e.g., parrotfish; Bellwood and Choat 1990; Streelman et al. 2002; Bellwood 2003) that allowed reefs to develop without the risk of being overgrown by algae (Horn 1989; Wood 1999). High ocean temperatures during the early part of the Cenozoic, preventing the establishment of successful symbiosis between corals and the photosynthetic Zooxantellae, or an efficient absorption of CO_2 (Wood 1999; Stanley 2003), might also have contributed to this gap between Cenozoic reef appearance and widespread colonization.

Although intrinsic opportunities for ecological diversification within reef-associated tetraodontiforms (and other reef fish as well) may have increased as reef habitats became more common over the Oligocene and Miocene, extrinsic factors in the form of major paleoclimatic events over this time period are also likely to have increased diversification rates in reef clades by fragmenting reef biotas. Starting at the end of the Eocene cooling ocean surface temperatures led to the transition from an Eocene "Greenhouse" world to an Oligocene "Icehouse" (Prothero et al. 2003; Bowen 2007). This trend continued into the Neogene (26–2.5 MY), over the course of which the oceanic temperatures may have dropped by as much as 10 degrees at high latitudes whereas increasing near the tropics (Jablonski et al. 1985; Crame 2001). Brief but intense glaciation events (e.g., Mi2 glaciation) may also have occurred during this period (Kiessling 2001; Zachos et al. 2001a, b). Sea levels during the early Miocene (23 MY) were also markedly low (Haq et al. 1987; Hallam and Wignall 1997; Miller et al. 2005). Finally, the effects of the closing of the Tethys sea and the fusion of the Indian and Asian plates during the late Oligocene and early Miocene on shallow-water marine biotas like reefs may have been pronounced (Hallam 1994; Briggs 1995). The formation of several mountain ranges, including the Himalayas, and the subsequent formation of major riverways would have increased siltation into the Indian ocean, disrupting the clear-water conditions required for the occurrence of scleractinian coral reefs and their associated biotas (Springer 1999). The uprising of the Himalayas also led to the origin of present-day monsoon cycles. Due to their effects on upwelling currents, these cycles partially determine significant biogeographic boundaries within the Indo-western Pacific (see Santini and Winterbottom 2002 and references therein). And the closing of the Tethys broke a corridor that had united what is now

the Mediterranean and northwestern Europe with the Indo-western Pacific, the most diverse marine region in the globe (Briggs 1995; Randall 1998; Carpenter and Springer 2005). Without the Tethys corridor, contact between the Atlantic and Indo-western Pacific biotas could only have occurred by passing through temperate waters around the southern tip of Africa, or by crossing the eastern Pacific barrier (Lessios et al. 1998; Lessios and Robertson 2006). Both routes would have presented formidable barriers to predominately coastal reef fish including many tetraodontiforms.

Conclusions

Fishes on coral reefs represent one of the most spectacularly diverse assemblages of vertebrates on the planet yet our understanding of the underlying causes of this diversity are limited. As higher-level percomorph relationships are resolved, more phylogenetically corrected comparisons of the relationship between reef association and diversification rate will become possible. In the absence of an overarching phylogenetic framework, we suggest that considerable insight into the macroevolutionary patterns of marine teleosts might still be gained by calculating diversification rates for major lineages following the example of Magallon and Sanderson for angiosperm clades (Magallon and Sanderson 2001).

More broadly, despite a growing interest in the investigation of rates and patterns of cladogenesis (e.g., Stanley 1997; see Ruber and Zardoya 2005 for a recent example in marine fish) especially in the context of adaptive radiations (e.g., horses, reviewed in MacFadden 1988,1994; after Hulbert 1993; Hawaiian silverswords, Baldwin 1998; Lake Malawi cichlids, Albertson 1999; Darwin's finches, Sato 1999; African ice plants, Klak 2004) the systematic study of rates of cladogenesis in a broad range of taxa, including teleost fish, is desperately needed. The identification of lineages with absolutely high (or low) rates of diversification is an integral step in the further elucidation of the macroevolutionary patterns and underlying processes responsible for the diversity of the tree of life.

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Appendix. Taxa used in this study.

Taxa	12S	16S	Rag1
Outgroup			
Antigonia caprosa	AY700233	AY679617	AY308786
Morone chrysops	AY700234	AY679618	AY308785
Drepane punctata	AF055589	AF055610	AY308767
Chaetodon striatusa	AF055595	AF055616	AY308772
Chaetodipterus faber	AF055592	AF055613	AY308775
Siganus doliatus	AF055596	AF055617	AY308773
Zebrasoma scopas	AY700235	AY679619	AY308777
Ingroup			
Abalistes stellatus	AY700248	AY679632	AY700318
Allomycterus pilatus	AY700254	AY679638	AY700324
Aluterus scriptus	AY700261	AY679645	AY700331
Amanses scopas	AY700271	AY679655	AY308793
Anoplocapros inermis	AY700276	AY679660	AY700346
Aracana ornata	AY700278	AY679662	AY700348
Arothron hispidus	AY700297	AY679681	AY700367
Arothron migropunctatus	AY700279	AY679663	AY700349
Balistes capriscus	AY700238	AY679622	AY700308
Balistes polylepis	AY700239	AY679623	AY700309
Balistes vetula	AY700240	AY679624	AY700310
Balistoides conspicillum	AY700241	AY679625	AY700311
Balistoides viridescens	AY700250	AY679634	AY700320
Brachaluteres jacksonianus	AY700267	AY679651	AY700337
Cantherhines dumerilii	AY700262	AY679646	AY700332
Cantherhines pullus	AY700263	AY679647	AY700333
Canthidermis maculatus	AY700242	AY679626	AY700312
Canthigaster amboinensis	AY700286	AY679670	AY700356
Canthigaster bennetti	AY700288	AY679672	AY700358
Canthigaster jactator	AY700280	AY679664	AY700350
Canthigaster janthinoptera	AY700296	AY679680	AY700366
Canthigaster rostrata	AY700281	AY679665	AY700351
Canthigaster solandri	AY700287	AY679671	AY700357
Canthigaster valentini	AY700282	AY679666	AY700352
Chilomycterus antennatus	AY700252	AY679636	AY700322
Chilomycterus schoepfii	AY700256	AY679640	AY700326
Diodon holacanthus	AY700255	AY679639	AY700325
Diodon hystrixc	AY700253	AY679637	AY308791
Diodon liturosus	AY700257	AY679641	AY700327
Lactophrys bicaudalis	AY700272	AY679656	AY700342
Lactophrys polygonia	AY700273	AY679657	AY700343
Lactophrys quadricornis	AY700275	AY679659	AY700345
Lactophrys triqueter	AY700274	AY679658	AY700344
Lagocephalus laevigatus	AY700295	AY679679	AY700365
Marilyna darwinii	AY700292	AY679676	AY700362
Masturus lanceolatus	AY700260	AY679644	AY308792
Melichthys niger	AY700243	AY679627	AY700313
Meuschenia trachylepis	AY700268	AY679652	AY700338
Mola mola	AY700258	AY679642	AY700328
Mola mola	AY700259	AY679643	AY700329
Monacanthus ciliatus	AY700264	AY679648	AY700334

Continued

Appendix. Continued

Taxa	12S	16S	Rag1
Monacanthus hispidus	AY700265	AY679649	AY700335
Monotreta leiurus	AY700294	AY679678	AY700364
Nelusetta ayraudi	AY700270	AY679654	AY700340
Oxymonacanthus longirostris	AY700269	AY679653	AY700339
Paraluterus prionurus	AY700266	AY679650	AY700336
Pseudobalistes fuscus	AY700244	AY679628	AY700314
Ranzania laevis	AP006047	AP006047	–
Rhinecanthus aculeatus	AY700247	AY679631	AY308790
Rhinecanthus assasi	AY700245	AY679629	AY700315
Sphoeroides dorsalis	AY700283	AY679667	AY308795
Sphoeroides spengleri	AY700284	AY679668	AY700354
Sufflamen bursa	AY700249	AY679633	AY700319
Sufflamen chrysopterus	AY700251	AY679635	AY700321
Takifugu rubripes	AY700293	AY679677	AY700363
Tetractenos glaber	AY700289	AY679673	AY700359
Tetractenos hamiltoni	AY700291	AY679675	AY700361
Tetraodon fluviatilis	AY700285	AY679669	AY700355
Tetrosomus concatenatus	AY700277	AY679661	AY308794
Torquigener pleurogramma	AY700290	AY679674	AY700360
Triacanthodes anomalus	AY700236	AY679620	AY308788
Trixiphichthys weberi	AY700237	AY679621	AY308789
Xanthichthys auromarginatus	AY700246	AY679630	AY700316
Newly sequenced species			
Pseudotriacanthus strigilifer	EF101312	EF101313	EF101314
Triodon macropterus	EF101309	EF101310	EF101311