Phylogenetic Relationships and the Evolution of BMP4 in Triggerfishes and Filefishes (Balistoidea)

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ABSTRACT

The triggerfishes (family Balistidae) and filefishes (family Monacanthidae) comprise a charismatic superfamily (Balistoidea) within the diverse order Tetraodontiformes. This group of largely marine fishes occupies an impressive ecological range across the world's oceans, and is well known for its locomotor and feeding diversity, unusual body shapes, small genome size, and ecological and economic importance. In order to investigate the evolutionary history of these important fish families, we used multiple phylogenetic methods to analyze molecular data from 86 species spanning the extant biodiversity of Balistidae and Monacanthidae. In addition to three gene regions that have been used extensively in phylogenetic analyses, we include sequence data for two mitochondrial regions, two nuclear markers, and the growth factorgene *bmp4,* which is involved with cranial development. Phylogenetic analyses strongly support the monophyly of the superfamily Balistoidea, the sister-family relationship of Balistidae and Monacanthidae, as well asthree triggerfish and four filefish clades that are well resolved. A new classification for the Balistidae is proposed based on phylogenetic groups. Bayesian topology, as well as the timing of major cladogenesis events, is largely congruent with previous hypotheses of balistid phylogeny. However, wepresent a novel topology for major clades in thefilefish family that illustrate the genera *Aluterus* and *Stephanolepis* are more closely related than previously posited. Molecular rates suggest a Miocene and Oligocene origin for the families Balistidae and Monacanthidae, respectively, and significant divergence of species in both families within the past 5 million years. A second key finding of this study is that, relative to the other protein-coding gene regions in our DNA supermatrix, *bmp4* shows a rapid accumulation of both synonymous and non-synonymous substitutions, especially within the family Monacanthidae. Overall substitution patterns in *bmp4* support the hypothesis of stabilizing selection during the evolutionary history of regulatory genes, with a small number of isolated examples of accelerated non-synonymous changes detected in our phylogeny.

Keywords: Balistoidea, taxonomy, phylogenetics, molecular evolution, *bmp4*

1. Introduction

Triggerfishes and filefishes are two important lineages of mostly coral reef- associated marine fishes. In addition to their charismatic color patterns and morphology which make them popular aquarium fishes, the diverse ecology and behavior of fishes in these two families have often attracted scientific inquiry. While their sister-family relationship has been supported by morphology and molecular data(Arcila et al., 2015; Holcroft, 2005; Matsuura, 2015; Santini et al., 2013a), biologists continue to add new species samples and new genes to the effort to understand the species-level phylogenetics of the Balistidae and Monacanthidae (Dornburg et al., 2008; 2011; Santini et al, 2013b; Yamanoue et al, 2009). Despite their close evolutionary relationship, there are fundamental differences between the triggerfishes and filefishes that raise intriguing evolutionary questions. There are 42 species of triggerfishes, nearly all of which are predatory carnivores distributed mostly on coral reefs and rocky shores in tropical and temperate waters. There are 107 species of filefishes with a more diverse range of ecological habitats and dietary patterns, as well as a wider range of body shapes. There are significant differences between balistids and monacanthids in nest building and parental care, the mechanics of fin-based locomotion and the cranial biomechanics associated with a biting mode of feeding (Nelson, 1994). The disparity between these two closely related families raises several interesting questions about their evolutionary histories, the timing of major ecological transitions, as well as the drivers of diversification within both phylogenetic groups.

Modern phylogenetic methods permit rapid statistical analyses of complex data matrices including dense and diverse character data for hundreds or thousands of taxa.Recent hypotheses of teleost interrelationships have tended toward densely sampled data matrices including a collection of nuclear genes (Near & Keck, 2013), ultraconserved elements (Faircloth

et al., 2013), or whole mitochondrial genome sequences (Yamanoue et al., 2009). Developmental regulatory genes represent one type of data that may have phylogenetic utility but as of yet, remain an underutilized source of data for reconstructing evolutionary relatedness (exceptions include Cook et al., 2001 and Smith et al., 2008). Here we investigate the utility and evolution of a developmental growth factor gene, *bmp4*, in combination with eight other gene regions from both the mitochondrial and nuclear genome.

Bone morphogenetic protein 4 (*bmp4*) has long been of interest to functional morphologists, developmental biologists, and geneticists due to its role in the development and diversification of cranial morphologies in a wide range of organisms. In particular, the clear correlation of *bmp4* expression levels to the incredible range of jaw morphology in African cichlids as well as beak biodiversity in Darwin's finches is well established (Albertson et al. 2005; Campas et al. 2010; Lee et al. 2004; Sinervo, 2005; Terai et al. 2002). Despite its clear contribution to the diversification of fish skulls, few researchers have explicitly tested the phylogenetic utility and evolution of *bmp4* and other regulatory genes known to have crucial roles in the development of the head, teeth, and jaws. We propose that the balistoidfishes provide an excellent experimental system within which to clarify the phylogenetic utility of the *bmp4* gene and the patterns of molecular evolution in this important developmental gene, due to their highly modified and robust skulls and teeth as compared to other teleost fishes.

Rapid progress in molecular phylogenetics has created opportunities to use phylogenetic inference to better understand complex evolutionary processes at many different levels of organization, from molecule to morphology (e.g. Agnarsson, 2004; Dornburg et al., 2011; Field et al., 1988; Near et al., 2013; Reid, 1989; Rokas et al. 2003). Indeed, comparative analyses based upon robust phylogenies of densely sampled taxa and diverse character data are central to the contemporary study of morphological evolution. In this study we explore balistoid phylogenetics with three primary objectives. First, we reconstruct the phylogenetic relationships of 86 balistoid fishes using Bayesian inference methods, and address long standing questions

regarding the taxonomic classification of several triggerfish species. How are the different species within the families Balistidae and Monacanthidae related to one another, and are phylogenetic studies in agreement so that a new taxonomy of the triggerfishes can be adopted? Second, we examine the timing of diversification events within the balistoid phylogeny. When did major cladogenesis events take place within the balistoid radiation and how different are triggerfishes and filefishes in the timing of major evolutionary events? Third, this research also aims to clarify the phylogenetic utility and evolutionary dynamics of the growth factor gene *bmp4* within the superfamily Balistoidea.Is there variability in the rate of accumulation of synonymous and non-synonymous substitutionsin the *bmp4* gene across the balistoid phylogeny, and if so, in what part of the tree?These three areas of inquiry will help to set up future analyses of morphological and functional evolution in the balistoid fishes.

2. Methods

2.1 Species sampling and genes sequenced

DNA sequence data for a total of 86 fish species were analyzed in this study, including 32 species of triggerfishes, 48 filefishes, and 6 outgroup taxa from the families Tetraodontidae, Diodontidae, and Ostraciidae (See Appendix A for a list of all taxa included in these analyses). With regard to taxonomic names, we follow the Catalog of Fishes database (Eschmeyer, 2015).We included at least one member of each balistoid genus, except the rare genera *Cantheschenia, Colurodontis, Enigmanthus, Lalmohania, Rudarius, Xenobalistes,* for which neither tissue samples nor sufficient GenBank sequences were available. All specimens were collected by the authors, purchased at local fish markets, or borrowed from outside institutions and most are associated with a voucher specimen (Appendix A). Portions of nine genes were sequenced and/or analyzed in the study, including four mitochondrial regions (12S, 16S, COI, and cytb) and 5 nuclear markers (rag1, rag2, rhodopsin, tmo4c4, and bmp4; Table 1).

Sequence data for recombination activating gene-1, cytochrome b, rhodopsin and cytochrome oxidase subunit I was downloaded from GenBank and included in the concatenated supermatrix (Dornburg et al., 2008; Santini et al., 2013b). Fragments of five genes, including three nuclear loci, were sequenced at the Pritzker Laboratory of the Field Museum of Natural History. Tmo4c4 is a single-copy nuclear DNA locus that has a high degree of similarity with part of the human titin protein. Titin is a large protein that contributes to the assembly and resting tension of muscle. Since its isolation by Streelman and Karl (1997), tmo4c4 has become popular for its utility in family-level fish phylogenetics (Dornburg et al., 2008; Farias et al., 2000; Lovejoy, 2000; Smith et al., 2008; Smith & Wheeler, 2004; Westneat & Alfaro, 2005). We generated 511 base pairs of tmo4c4.

Recombination activating gene-2 (rag2) is a single exon in vertebrates and works with rag1 to activate the process by which lymphocytes developmentally generate immune system diversity (Oettinger et al., 1990). 802 bp of rag2 were sequenced, all of which is located in the proposed functional core region (Cuomo & Oettinger, 1994).

Bone morphogenic protein-4 (bmp4) is a member of the transforming growth factor-β super-family (Kingsley, 1994). Bmp4 is the vertebrate homolog of *Drosophila* decapentaplegic, which is responsible for the establishment of dorsal-ventral polarity and segmentation (Irish & Gelbart, 1987). Similar patterning functions for bmp4 have been recognized in zebrafish (Chen et al., 2004; Nikaido et al., 1997). Bmp4 has also been implicated in tooth formation (Peters & Balling, 1999), bone and joint development and repair (Kingsley, 1994), and the evolution of skull diversity in cichlid fishes (Albertson et al., 2003, 2005; Cooper et al., 2011) and Darwin's finches (Abzhanov et al., 2004; Sinervo, 2005). We sequenced a total of 482 bp of bmp4 sequence data.

2.2 DNA extraction and amplification

DNA was extracted from muscle or gill tissue according to the PureGene animal tissue DNA isolation procedure (Gentra System). Briefly, cell lysis and Proteinase K solutions were added to roughly 2 mm cubed of tissue, mixed together, and then held at 55° C in a rotating incubator for 2 hours. Next, Protein Precipitate Solution was added to the lysate, and the resultant mixture was vortexedfor thirty seconds and centrifuged to form a tight protein pellet. The supernatant of this solution was added to a DNA carrier (glycogen) and isopropanol, inverted to mix and finally centrifuged until a pellet was formed. The resultant DNA pellet was then washed once with 70% ethanol. Finally, 50ul DNA Hydration Solution was added to the mixture, which was then incubated at 65° C for 1 hour, and then refrigerated at 4° C until PCR could be performed.

Double-stranded DNA products were amplified using polymerase chain reaction from aliquots of genomic DNA isolates of the 12S, 16S, tmo4c4, rag2, and bmp4 gene fragments. Each PCR had a total reaction volume of 25 ul and contained 1 ul of DNA, 2.5 ul of reaction buffer (100 mM Tris–HCl, 500 mM KCl, 15 mM MgCl2, pH 8.3; Roche, Mannheim, Germany), 1.5 ul of 2 mM each deoxynucleotide triphosphates, 0.1 ul of 5□ □ Taq polymerase (Roche, Mannheim, Germany), 0.5 ul of 10 mg/ml bovine serum albumin, and 1.0 ul of each oligonucleotide primer, each at 10 M concentration. Primers used for DNA amplification and sequencing are listed in Table 1.

Each 16S, 12S, tmp4c4, rag2, and bmp4 PCR included an initial denaturation step at 95° C for 5 minutes followed by 35 cycles of PCR. Each cycle included denaturation at 95° C for 30 s, annealing at 55° C for 60 s, and extension at 72° C for 90 s. After a final extension step at 72° C for 5 minutes, the PCR products were held at 4° C. PCR reactions were performed using an MJ Research PTC-200 Peltier Thermal Cycler (MJ Research Inc., Watertown, MA).

PCR products were electrophoresed on 1% low melt agarose gels stained with ethidium

bromide. Ultraviolet light allowed for the visualization of DNA bands, which were excised, melted at 70° C, and incubated with 1 ul GELase at 48° C for three hours. PCR products were then cycle-sequenced by creating a solution of 3.3 ul of buffer, 0.5 ul of primer, 0.7 ul of Big Dyereagent, 0.5 ul of PCR product template DNA, and 5.0 ul nuclease free water to bring the total reaction volume to 10 ul. The cycling protocol included one initial denaturation at 96° C followed by 25 cycles of denaturation at 96° C for 10 s, annealing at 50° C for 5 s, and extension at 60° C for 4 min. All products were purified and then loaded into a DNA sequencing machine. Sequences of both strands were generated on an ABI PRISM3730 Genetic Analyzer (Applied Biosystem, Foster City, CA). All raw sequence data have been deposited to GenBank (see Appendix A for accession numbers).

2.3 Sequence alignment

Sequences for 12S, 16S, tmo4c4, rag2, and bmp4 were manually aligned to homologous sequences for outgroup species (other Tetraodontiformes and Labridae) using Sequencher 4.2 (Smith et al., 2008; Gene Codes Corp., Ann Arbor, MI). Sequences obtained from GenBank for rhodopsin, rag1, COI, and cytB were automatically aligned using the Clustal module of Mesquite V2.75. Sequences from all genes sequenced, and those obtained from GenBank, were trimmed to the size of the smallest fragment to minimize the amount of missing data in the data matrix. Gaps and ambiguously alignable regions were also excluded from analysis. After excluding unalignable regions and trimming sequence ends, the character count for each gene was 12S (783), 16S (551), tmo4c4 (511), rag2 (802), bmp4 (482), rhodopsin (310), cytB (1072), rag1 (1337), and COI (607) for a total of 6455 nucleotide characters.

2.4 Phylogenetic analysis

Bayesian phylogenetic analyses of the concatenated DNA supermatrix were conducted using MrBayes (3.2.2; Ronquist et al., 2011) through the CIPRES Science Gateway V3.3, which provides access to the National Science Foundation's XSEDE computer network. *Acanthostraction polygonius, Diodon holocanthus, Takifugu rubripes, Tetraodon palembangensis, Arothron hispidus,* and *Arothron manilensis* were selected as outgroup taxa for all phylogenetic analyses. We performed four independent runs of a Markov Chain Monte Carlo analysis with six chains running for 10 million generations for Bayesian analyses. The nine gene partitions were assigned separate parameters with their own model of sequence evolution, determined using jModelTest 2 (Darriba et al., 2012; Guindon & Gascuel, 2003; Table 1). Trees were saved every 1000 generations for each run and used default priors for the transition/transversion rate ratio, branch length, alpha parameter of the gamma distribution for rate heterogeneity, proportion of invariant sites, base frequencies, and tree topology parameters. To help ensure that stationarity was reached, the first 2.5 million generations (2500 sampled trees) were discarded from each run as burn-in and used the remaining 7.5 million generations (7500 sampled trees for each of the four runs) used in all subsequent analysis. To determine the posterior probability of clades, a majority-rule consensus tree calculated from the post burn-in trees was constructed. We used the specialized MrBayes Max. A-Post. Tree Source in the Mesquite program to find the tree with maximum posterior probability. The majority-rule consensus tree and maximum posterior probability phylogenetic trees were then exported and viewed using FigTree version 1.4.0.

2.5 Divergence time estimation and rates of molecular evolution

To explore the timing of divergence events in the history of balistoid fishes, weconstrained five nodes in the balistoid tree. We calibrated the root age of the tree based upon

a stem tetraodontiform fossil, *Cretatriacanthus guidotti,* from the Nardo deposits in southern Italy that areknown to be at least 83.5 Myr old (Schlüter et al., 2008; Tyler & Sorbini, 1996). As such, we assigned an exponential distribution prior for the root with an offset of 83.5 Myrand a mean of 20. We assigned a secondary constraint to the age of the most recent common ancestor to the balistids based upon the estimate from Alfaro et al. (2007) and Dornburg et al. (2008). We chose a normally distributed prior to assign a mean age of 20 Myr to this split with a sigma of 10 Myr. The stem balistid fossils *Gornylistes prodigiosus, Balistomorphus orbiculatus, Balistomorphus spinosus,* and *Balistomorphus ovalis* were used to assign a mean age of 42 MYA with a sigma value of 20 to the most recent common ancestor of filefishes and triggerfishes using a normal prior. The fossil *Balistes procapriscus* was used to establish the age of crown *Balistes.* We assigned an exponential distribution with a sigma of 6.5 MYA and a mean of 2 for this calibration point. Lastly, the recently described fossil filefish, *Aluterus shigensis,*was used to establish the age of the *Aluterus* clade with an exponential distribution offset of 13 MYA and a mean of 3.

To estimate divergence times, we used our DNA supermatrix data under a relaxed clock of log normal distributed rates using BEAST 2.1.3 based upon the calibrations outlined above (Bouckaert et al. 2014). A standard birth death rate model estimated rates of cladogenesis. We partitioned the nine genes used in this analysis such that each region was modeled separately under its best substitution model. We then used Tracer v1.6 to assess convergence following two independent analyses for 10 million generations each. The first 25% of generations were used as burn-in, and the effective sample size (ESS) for all model parameters were checked for good mixing of the final Markov chain Monte Carlo (all ESS exceeded 200 in our analyses).

To examine the evolution of nuclear gene regions included in our dataset, the amounts and rates of synonymous and non-synonymous change were calculated for all nuclear gene sequences. To explore nucleic acid substitution patterns under a likelihood framework, we used

the Muse and Gaut (1994) codon model as implemented by the AnalyzeCodonData algorithm of the software package HyPhy v2.2.4 (Kosokovsky-Pond & Frost, 2005) in combination with the Bayesian maximum posterior probability tree that was pruned to only include taxa for which sequence data for the gene of interest was available. Using the HyPhy tree graphics utility, we also calculated the rates of synonymous and non-synonymous substitution per site and visualized these rates along each branch of the phylogeny. Several branches of the phylogeny within the filefish lineage appeared to have particularly high and/or variable rates of bmp4 dN/dS relative to other branches. The hypothesis was tested whether any branches within the balistoid lineage had significantly different dN/dS ratios using the TestBranchdNdS module in HyPhy. TestBranchdNdS runs a likelihood ratio test on selected branches of a phylogeny wherethe non-synonymous rate parameter is held constant over all branches under the null hypothesis, and the alternative hypothesis allows it to vary freely.This analysis was performed with a custom MG94xTrN93 nucleotide substitution model, a site-to-site rate variation model with dN and dS allowed to vary simultaneously, and the default amino acid class model. To test the hypothesis that dN/dS ratios in the filefish clade differs significantly from that of the triggerfish lineage, we conducted a second likelihood ratio test in TestBranchdNdS selecting only the branches and tips that are part of Monacanthidae. To identify which individual branches had significantly different dN/dS ratios compared to the rest of the phylogeny, we ran additional likelihood ratio tests in TestBranchdNdS on each branch of the balistoid phylogeny that had non-zero values for both dN and dS. Finally, we focused our analysis of substitution dynamics to each family independently in order to determine which, if any, clades within each family had significantly different dN/dS ratios using the model parameters described above.

3. Results

Bayesian-based phylogenetic analysis of a DNA supermatrix consisting of nine concatenated gene sequences produced well-resolved trees for all major clades of balistoid

fishes. Supermatrix-based species trees recovered two well-supported monophyletic families within the superfamily Balistoidea, offering solid support for the long-standing sister-family relationship of the Monacanthidae and Balistidae, as has been suggested in previous morphology and gene-based analyses (i.e. Holcroft, 2005; Santini et al., 2013a; 2013b; Winterbottom, 1974). Strong support for many groups was also found at finer levels in the tree, however the confidence levels for establishing interspecific relationships among several rare temperate filefish species remains generally low. The growth factor gene *bmp4* was found to be accumulating codon substitutions at significantly different rates among the various balistoid clades included in these analyses, with a high substitution rate in the filefishes.

3.1 Balistoid phylogenetics

Bayesian phylogenetic analyses of a DNA supermatrix consisting of nine gene regions for 32 species of triggerfishes, 48 filefish species and six outgroup species from within the order Tetraodontiformes produced a well-resolved phylogeny with good support for the balistoid fishes. We find that several genera are non-monophyletic and therefore in need of taxonomic revision.Our phylogenetic analyses recovered three major triggerfish clades (Figure 1). Clade 1 consists of all fishes in the genus *Balistes* as well as *Pseudobalistes naufragium* and *Pseudobalistes fuscus.* Clade 2 contains *Balistapus undulatus, Balistoides conspicillum*, *Odonus niger, Balistoides viridescens, Pseudobalistes flavimarginatus,* all fishes in the genera *Melichthys* and *Xanthicthys.* Clade 3 includes all fishes in the genera *Abalistes, Canthidermis, Rhinecanthus,* and *Sufflamen.*

Our analyses reveal fourwell-supported monacanthid clades. Fishes in the genera *Aluterus* and *Stephanolepis*, as well as *Oxymonacanthus longirostris*, *Monacanthus tuckeri,* and *Monacanthus ciliatus* comprise Clade A. Clade B contains *Acreichthys tomentosus, Monacanthus chinensis, Chaetodermis penicilligerus, Brachaluteres jacksonianus* and *Paraluteres prionurus*, as well as fishes in the genera *Pervagor and Paramonacanthus.* Clade C

includes *Amanses scopas* as well as all fishes in the genera *Pseudomonacanthus* and *Cantherhines*. Clade D includes fishes in the genera *Acanthaluteres, Meuschenia, Eubalichthys,* and *Thamnaconus*, as well as the two monotypic genera *Nelusetta* and *Scobinichthys.*

3.2Dates of origin and divergence times for balistoid fishes

The majority rule consensus BEAST topology revealed the same major clades with comparable support to the Bayesian maximum posterior probability tree that was calculated using MrBayes, with the exception of the relative placement of the monacanthid genera *Aluterus,Stephanolepis,* and *Pervagor* (Figure 2); compared to our MrBayes phylogeny, we observe a weakly supported sister genus relationship between *Stephanolepis* and *Pervagor* using five node calibrations (PP=61% and 55%, respectively) in BEAST 2.1.3. Based upon five node constraints, we recover a split between triggerfishes and filefishes at approximately 50 Myr. Our chronogram places the crown of the triggerfish lineage at 18.6 Myr, and dates the filefish crown to approximately 33 Myr. We find Balistidae Clades 1 and 3 are roughly the same age (14.5 Myr), whereas Clade 2 is slightly younger at around 13.1 Myr. Within the Monacanthidae, Clade A was found to be the oldest (28.6 Myr) and Clade D the youngest (14.1 Myr). Several relatively young groups within each family appear within the last 2 Myr (eg. *Melichthys vidua, Abalistes stellaris, Acanthaluteres vittiger, Thamnaconus modestus*).

3.3 Rates of molecular evolution

Rates of genetic evolution were found to be variable among gene regions and across the phylogeny for all data included in our phylogenetic analyses. Overall, the accumulation of changes was higher at the base of the balistoid radiation than the tips, and more accelerated in the filefish lineage than the triggerfishes. This is particularly true of bmp4, in which the substitution rate in filefishes is 10 times that of triggerfishes for synonymous and non-

synonymous substitutions alike (Figure 4). All coding genes showed considerably higher rates of synonymous than non-synonymous change, resulting in dN/dS ratios well below 0.5. We found two dN/dS greater than 0.5 within the balistoid radiation in which the rate ratio for *bmp4*at *Aluterus scriptus* was 0.5283 and for *Stephanolepis hispidus,* it was 0.6484. Whereas most mitochondrial and nuclear regions showed similar rates of substitution across the balistoid phylogeny (eg. Figure 3), we found that substitution patterns for *bmp4* showed accelerated dS and also many non-zero dN branch lengths, especially in the monacanthids (Figure 4). An initial likelihood ratio test of all branches in the phylogeny revealed that there were significant differences in dN/dS ratios within the balistoid lineage (p=0.0212).We also found that dN/dS ratios in the filefish lineage are significantly different than those in the triggerfishes (p=0.0394). While about 75% of thebranches in our analyses of *bmp4* molecular evolution had non-zero values for dN or dS, we identified only nine tip branches and one internal branch of the balistoid phylogeny that had non-zero dN and dS values for *bmp4*(Table 2). By testing each of these ten independently, we foundfive branches within the filefish lineage that had significantly different dN/dS rate ratios compared to all other branches in the balistoid phylogeny (Table 2). Our analysis of *bmp4* substitution dynamics within each family separately revealed no significant differences in dN/dS ratios among the three triggerfish clades (Clade 1, p=0.6342; Clade 2,p=0.9454; and Clade 3,p=0.8811). However, within the monacanthids,the branches of Clade A had a significantly different dN/dS ratio (p=0.0426) compared to the other two filefish clades for which we had *bmp4* sequence data (Clade B, p=0.7006; Clade C, p=0.7865; Table 2).

4. Discussion

Using a combination of mitochondrial and nuclear genes, we resolve the phylogenetic relationships of 86 tetraodontiform fishes. This combination of genetic data provides well-supported resolution at both deep and recent nodes for 32 balistid species and 48 monacanthid

species and confirms the long-held concept that the superfamily Balistoidea forms a natural group.The well resolved and strongly supported (PP=100%) sister-family relationship between triggerfishes and filefishes resolved here corroborates previous studies of tetraodontiform relationships based on both morphological and molecular data (eg. Arcila et al., 2015; Holcroft, 2005; Near et al., 2013; Santini & Tyler, 2004). With solid agreement among studies of triggerfish phylogenetics, we propose a new classification for the Balistidae involving three name changes at the genus level. The accumulation of synonymous and non-synonymous changes within the developmental growth factor gene *bmp4* was found to be markedly rapid compared to the other nuclear genes included in this study, with filefishes exhibiting a 10 fold increase in substitution rate relative to the triggerfishes. We focus our attention below on major patterns of species-level balistoid phylogeny, suggestions fortaxonomic reclassification based upon our phylogeny, the interpretation of emergent patterns of nucleotide substitutions, and the timing of origin and diversification we estimate for major balistoid clades.

4.1Triggerfish phylogenetics

These results confirm the monophyly of Balistidae (PP=100%) and suggest that the triggerfishes can be grouped into three main clades that are generally well supported using Bayesian inference phylogenetics. With the exception of the moderately supported (PP=81) close association of *Balistapus undulatus* and *Balistoides conspicillum*, and the questionable placement (PP=81) of this sister pairing as the nearest relative of fishes in the monophyletic genus *Melichthys,* all balistid clades are strongly supported (PP>97%) in our phylogenetic analyses. Clade 1 includes only six triggerfish species, whereas Clades 2 and 3 are roughly equal in size, containing 11 or 15, respectively. We find strong ecological and biogeographic distribution signals within the phylogenetic framework of triggerfishes. Clade 1 contains fishes that are large, durophagous, and mostly pelagic. Clade 2 consists of fishes with more varied

distribution and feeding ecology, however most members of this clade are benthopelagic. Clade 3 is a mostly Indo-Pacific radiation of generalist fishes. The morphology of the skull and biomechanics of the jaws are unique and diverse among the triggerfishes, with increases in muscle subdivisions in the jaw adductors that have significant implications for the evolution of feeding within the group (McCord, 2014). Future work will explorepatterns of phylomorphospace using the present phylogeny and examine the diversification of anatomical and functional traits in balistoid fishes.

Our topology of triggerfish relationships corroborates the close evolutionary relationships of *Canthidermis, Abalistes,Rhinecanthus,*and *Sufflamen* as well as *Melichthys, Odonus niger, Balistapus undulatus,* and *Balistoides conspicillum* that several others have recovered based upon various molecular datasets (eg. Dornburg et al., 2011; Holcroft, 2005; Yamanoue et al., 2009; Santini et al., 2013b, Arcila et al., 2015). However, our topology identifies *Balistes* (Clade 1) as sister to the two other triggerfish clades (Clades 2 and 3). This topology is in agreement with some previous molecular studies (eg. Dornburg et al., 2008; Yamanoue et al., 2009), but differs from more recent work (Dornburg et al., 2011; Santini et al., 2013b) that found*Balistes* to be nested deeply within the triggerfish radiation.A second disparity between this and previous balistid phylogenies is the relationship between *Balistapus undulatus, Balistoides conspicillum, Odonus niger,* and fishes in the genus *Melichthys.* Here,we report the novel finding of a, albeit weakly supported, sister-group relationship between *Balistapus undulatus* and *Balistoides conspicillum.* All previous studies have shown *Balistoides conspicillum* as sister to fishes in *Melichthys*, with *Balistapus undulatus* and *Odonus niger* often reconstructed as sister taxa (eg. Dornburg et al., 2008; 2011).

Our data illustrate that the genera *Balistes, Pseudobalistes,*and *Balistoides* are not monophyletic and are, therefore, in need of revision. Phylogenetic relationships as reconstructed here, andin all previous phylogenies involving these genera, strongly support readopting theoriginal or new senior synonymsfor *Pseudobalistes fuscus, Balistoides viridescens,* and

Pseudobalistes naufragium that were proposed by Bloch and Schneider (1801) and Jordan (1895). By rolling back the taxonomy for*Pseudobalistes fuscus* to *Balistes fuscus*, *Pseudobalistes naufragium* to *Balistes naufragium,* and *Balistoides viridescens* to *Pseudobalistes viridescens, the* monophyly of *Balistes* and *Pseudobalistes* will be retained, respectively. These revisions restore Clade 1 to include exclusively fishes in the genus *Balistes*, and also render the *Pseudobalistes* clade monophyletic, and sister to the strongly supported *Xanthichthys* clade. In addition to *Xenobalistes, Balistapus,*and *Odonus,* this suggested reclassification would make *Balistoides* the fourth monotypic genus in the family Balistidae.

4.2Filefish phylogenetics

The Monacanthidae is a large and diverse family with several enigmatic temperate species that has yet to be the sole focus of a rigorous investigation of molecular phylogenetics. With the exception of the low support attributed to the sister pairs *Pervagor aspricaudus* + *Pervagor nigrolineatus* (PP=55%) and *Stephanolepis cirrhifer* + *Stephanolepis setifer* (PP=73%), sister group pairs generally receive high support in our analyses of a broad taxonomic range of filefishes (PP>87%). Deeper nodes are typically more uncertain than terminal branching patterns. In particular, the close association of *Pervagor spilosoma* and the *Pervagor janthinosoma* + *Pervagor melanocephalus* clade (PP=62%), the position of *Cantherhines verecundus* as sister to the clade formed by all other fishes in the genus *Cantherines* + *Amanses scopas* (PP=73%), as well as the placement of *Acreicithys tomentosus* as sister to fishes of the genus*Paramonacanthus* + *Monacanthus chinensis* clade (PP=77%) were only weakly supported by our molecular dataset.

In contrast to the rich history and diversity of species-level phylogenies that are available for Balistidae, a single species-level molecular phylogeny has been reconstructed for Monacanthidaeprior to the analyses presented here (Santini et al., 2013b). Our data

corroboratemany of the major clades reconstructed in that manuscript.For example, we find that *Monacanthus chinensis* is not closely related to other species currently named to the genus *Monacanthus* and is instead nested within a clade containing the genus *Paramonacanthus* and *Acreicththys tomensus.*Our data also support the same close association among the ornamental, colorful, dewlapped filefishes in the genera *Brachaluteres, Acreichthys, Paraluteres, Paramonacanthus, Pervagor,* and *Chaetodermis* that Santini et al. (2013b) reported (Figure 1).

Our molecular phylogenetic analyses also support numerous novel findings for filefish phylogenetics. In particular, the relationships of filefishes we classify as belonging toClade A differ significantly from Santini et al. (2013b). Santini and colleagues found *Oxymonacanthus longirostris* to be sister to all other filefishes with weak support (BS=72). We find strong support (PP=100%) that *Oxymonacanthus longirostris* is sister to the *Stephanolepis +Monacanthus tuckeri* and *Monacanthus ciliatus* clade (Figure 1). Santini also reported that *Monacanthus tuckeri* and *Monacanthus ciliatus* are nested within the genus *Aluterus,* and that this clade is sister to *Amanses* + *Cantherhines* + *Pseudomonacanthus* and the subtropical and temperate Indian Ocean filefishes of the genera *Thamnaconus, Nelusetta, Scobinichthys, Eubalichthys, Meuschenia* and *Acanthaluteres*. We find these temperate and subtropical monacanthids form their own clade (Clade D) that is not closely related to *Aluterus* + *Monacanthus ciliatus* + *Monacanthis tuckeri*(Figure 1).

Here, we propose a novel filefish phylogeny, and also highlight a need for increased research attention for the monacanthid lineage. Additional taxonomic sampling is clearly necessary to solidify phylogenetic relationships in various parts of the filefish tree, and there are additional filefish fossils that might be used in future efforts (Sorbini and Tyler, 2004). Given that our phylogeny is based on 48 of the 106 total species of filefishes, we do not deem it appropriate to formally propose taxonomic revisions. However, our findings convincingly show

that the genera *Scobinichthys*, *Thamnaconus, Acanthaluteres, Meuschenia, Nelusetta*, *Eubalichthys,* and *Monacanthus*likely do not form natural groupings, and that these classifications will need to be revised when sufficient taxonomic and molecular sampling have been compiled.

4.3Dates of origin and divergence times for balistoid fishes

There is a rich diversity of fossil representatives from the order Tetraodontiformes (Santini & Tyler, 2003). However, with the exception of the recently described *Aluterusshigensis*, fossil filefishes are rare. The divergence timing analyses presented here are the first to incorporate a specific fossil calibration within the filefish lineage. Our findings for the timing of major cladogenesis events using a set of five node constraints in the balistoid lineage all fall within the 95% confidence intervals of previously proposed dates (Arcila et al, 2015; Dornburg et al., 2008; Santini et al., 2013b; see Table 4).However, the timing of Balistoidean diversification we observe is much younger than dates proposed by Arcila and colleagues (2015) and also Santini and colleagues (2013b), but older than the dates proposed by Dornburg et al. (2008). We observe a lower Miocene origin for crown Balistidae with the three major triggerfish clades having originated in the mid Miocene. Within Balistidae, we find multiple young clades, with *Rhinecanthus*, *Sufflamen*, and *Melichthys* rising in the Pliocene and upper Miocene. The youngest genera in the triggerfish lineage are *Abalistes*, and *Canthidermis*, both of which originated within the last two million years during the Pleistocene. We report Monacanthidae as a more ancient lineage compared to Balistidae, having originated during the lower Oligocene. Within the monacanthid lineage, the oldest major genera appear to have originated during the mid Miocene (eg. *Aluterus*and *Cantherhines*).Substantial diversification also took place during the upper Miocene when *Pervagor*, *Thamnaconus*, and several other genera originated. We also report multiple young filefish clades that originated during the Pliocene, such as

Monacanthus and *Stephanolepis*, as well as the Pleistocene when *Pseudomonacanthus* and*Acanthaluteres* appear to have evolved.

4.4 Evolution of bmp4 in balistoid fishes

An elevated level of variation in developmental regulatory genes has been shown to be associated with morphological diversity in several groups of organisms (Aagaard et al, 2006; Abzhanov et al. 2004; Barrier et al., 2001; Smith et al. 2008; Terai et al. 2002). The cranial diversity and rapidly evolving jaw mechanisms of fishes may make them a particularly interesting part of the vertebrate tree of life in which to search for associations of structural or functional diversity with elevated variation in the protein coding genes that play a role in development. It has long been known that balistoid fishes represent a morphologically diverse and unique radiation of marine fishes (Friel & Wainwright, 1997; Winterbottom, 1973). In addition to an overall reduction in skull bones and teeth, which produces a notably robust cranium with premaxillae that are incapable of protrusion, the jaw-closing musculature of Balistoidea has undergone several rounds of physical subdivision with important consequences for jaw function through evolution (McCord, 2014). Quantitative genetic analyses demonstrate that the relative expression of *bmp4* directly affects the biodiversity of dentition and lower jaw biomechanics in cichlids (Albertson et al., 2005). Therefore, we included a detailed analysis of the regulatory gene *bmp4* due to its known importance in the development of cranial elements in fishes.

By analyzing the substitution patterns in protein-coding genes, one can reveal the phylogenies and the genes in which an increase in synonymous or non-synonymous changes serve as a signal for selection (Guindon et al., 2004; Seo et al., 2004; Smith et al., 2008). All of the genes we examined showed higher rates of synonymous than non-synonymous change, which resulted in dN/dS ratios smaller than 0.5, suggesting that stabilizing selection has acted

on all these gene regions. However, we do note that dN and dS were highly variable on the phylogeny, and among the genes we investigated. In particular, we found that *bmp4* sequencescontain more non-zero dN scores than any other region we examined, predominantly in the filefishes where dN/dS ratios for this gene were elevated overall. Furthermore, our molecular analyses of *bmp4* revealed that Monacanthidaeexhibitsthe only two examples of dN/dS ratio greater than 0.5, both of which were found to be significantly different from all other branches of the balistoid phylogeny. Compared to the members of Balistidae, monacanthid fishes, especially taxa in Clades A, had notably elevated dN which may indicatea phylogenetic region of positive selection for protein evolution.Considered together, the discovery of elevated dN/dS ratios at major nodes in the balistoid fishes for *bmp4* suggests that these portions of the phylogeny may be undergoing changes to significant developmental patterns in the skull, a result that has been reported for the parrotfishes as well (Smith et al., 2008). Broader taxonomic and molecular sampling that include additional filefish species as well as other developmentally relevant regulatory genes (such as *otx1* and *dlx2*) are needed to discover whether higher rates of substitution to developmental regulatory genes are correlated with cranial functional or morphological evolution in diverse fish lineages.

4.5 Conclusions

There is a rich literature focused upon reconstructing the phylogenetic relationships of balistoid fishes and their relatives. Here, we build upon this strong history by adding new species and novel molecular data to reconstruct the phylogenetic relationships of Balistoidea. Our findings corroborate much of the earlier work that has gone into resolving the general topographic and divergence timing of this fascinating group of fishes. However, we also report a novel topology for major clades in the family Monacanthidae, and also present a fresh understanding of how the relative rates of synonymous and non-synonymous substitutions contribute to phylogenetic signal in balistoid fishes. From our findings, it is clear that broader

taxonomic and molecular sampling are required to finely assess the Balistoidea at both deep and recent levels of phylogenetic relatedness. In particular, the captivating biodiversity of temperate and sub-tropical monacanthids is currently poorly understood, and few viable tissue samples are available for molecular analyses. Broader genomic sampling techniques are also now available (eg. exon capture, ultra-conserved elements, and genotype-by-sequencing approaches) and hold great promise for resolving the questionable nodes found in this and other studies of balistoid phylogeny. By developing a comprehensive species-level tree, rich layers of data that describe the staggering biodiversity of Balistoidea may be mapped onto a robust phylogeny to better understand the unique morphological, behavioral, functional, and ecological evolutionary history of balistoid fishes that has captivated researchers for more than a century. Given the extreme diversity of the balistoid cranium, and the known importance of multiple regulatory genes to the development of these trophic structures, we also suggest that Balistoidea is an ideal system for examining the relationship of genotype and phenotype.

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Appendix A.

Family	Species	12S	16S	4c4	rag2	bmp4	rhodopsin	rag1	cytB	COI
Balistidae	*Abalistes stellaris*		KT600988	KT600805	KT600890	KT600846		KF025871.1	KF025733.1	HQ149782.1
	Abalistes stellatus	KT600929	AY679632.1	EU108823.1			EU108845.1	AY700318.1		JF492756.1
	Balistapus undulatus	KT600937	KT600995	KT600808	KT600894	KT600852	EU108849.1	EU108869.1	JQ861146.1	FJ582893.1
	Balistes capriscus	KT600938	KT600996	KT600809	KT600895	KT600853	DQ197830.1	AY700308.1	DQ197928.1	JQ623916.1
	Balistes polylepis	KT600939	KT600997	KT600810	KT600896	KT600854	KF025912.1	AY700309.1	KF025738.1	GU440245.1
	Balistes punctatus	KT600940	KT600998	KT600811	KT600897	KT600855	EU108848.1	EU108868.1	KF025739.1	KF025674.1
	Balistes vetula	KT600941	KT600999	KT600812	KT600898	KT600856	EU108850.1	AY700310.1	KF027544.1	JQ839714.1
	Balistoides conspicillum	KT600942	KT601000	KT600813	KT600899	KT600857	EU108847.1	AY700311.1	KF025737.1	FJ582895.1
	Balistoides viridescens	KT600943	KT601001	KT600814	KT600900	KT600858	KF025913.1	AY700320.1	KF025740.1	JQ431476.1
	Canthidermis maculata	KT600950	KT601007	KT600818	KT600904	KT600862	EU108851.1	AY700312.1	KF025741.1	GU225160.1
	Canthidermis sufflamen	KT600951	KT601008	KT600819	KT600905	KT600863				JQ841088.1
	Melichthys niger	KT600953	KT601010	KT600821	KT600907	KT600865	EU108852.1	AY700313.1	KF025743.1	FJ583654.1
	Melichthys vidua	KT600954	KT601011	KT600822	KT600908	KT600866	EU108853.1	EU108870.1	KF025744.1	JQ431904.1
	Odonus niger	KT600958	KT601014	KT600823	KT600909	KT600867	EU108854.1	EU108871.1		FJ583744.1
	Pseudobalistes flavimarginatus	KT600964	KT601020	KT600827	KT600915	KT600872	EU108855.1	EU108872.1		FJ583961.1
	Pseudobalistes fuscus	KT600965	KT601021	KT600828	KT600916	KT600873		AY700314.1	KF025745.1	FJ583962.1
	Pseudobalistes naufragium	KT600966	KT601022	KT600829	KT600917	KT600874	GU014454.1	GU014460.1	KF025746.1	KF025682.1
	Rhinecanthus abyssus	KT600967	KT601023	KT600830	KT600918	KT600875				
	Rhinecanthus aculeatus	KT600968	KT601024	KT600831	KT600919	KT600876	KF027976.1	AY308790.1	KF027545.1	FJ584054.1
	Rhinecanthus assasi	KT600969	KT601025	KT600832	KT600920	KT600877	EU108858.1	AY700315.1	KF025748.1	KF025684.1
	Rhinecanthus lunula	KT600970		KT600833			EU108859.1	EU108873.1	KF025749.1	JQ432086.1
	Rhinecanthus rectangulus	KT600971	KT601026	KT600834	KT600921	KT600878	EU108860.1	EU108874.1	KF025750.1	FJ584060.1
	Rhinecanthus verrucosus	KT600972	KT601027	KT600835		KT600879	EU108861.1	EU108875.1	KF025751.1	FJ584061.1
	Sufflamen albicaudatum	KT600976	EU108820.1	KT600838			KF025944.1	KF025904.1	KF025798.1	
	Sufflamen bursa	KT600977	KT601031	KT600839	KT600924	KT600882	EU108863.1	AY700319.1	KF025752.1	FJ584128.1
	Sufflamen chrysopterum	KT600978	KT601032	KT600840	KT600925	KT600883	EU108864.1	AY700321.1	KF025753.1	FJ584131.1
	Sufflamen fraenatum	KT600979	KT601033	KT600841	KT600926	KT600884	KF025916.1	KF025874.1	KF025754.1	JF494610.1
	Sufflamen verres	KT600980	KT601034	GU014452.1			GU014455.1	GU014459.1	KF025755.1	KF025688.1
	Xanthichthys auromarginatus	KT600985	KT601035	KT600843	KT600927	KT600887	EU108865.1	AY700316.1		FJ584243.1
	Xanthichthys mento	KT600986	KT601036	KT600844		KT600888	EU108866.1	EU108877.1	KF025757.1	HM032024.1
	Xanthichthys ringens	KT600987	KT601037	KT600845	KT600928	KT600889	EU108867.1	EU108878.1	KF025758.1	GU225064.1
Ostraciidae	*Acanthostracion polygonius*	KT600930	KT600989	KT600806		KT600847				
Tetraodontidae	*Arothron hispidus*	KT600935	KT600993		KT600892	KT600850	JQ682374.1	AY700367.1	FJ434546.1	EU148578.1
	Arothron manilensis	KT600936	KT600994		KT600893	KT600851	JQ682376.1	JQ682296.1	JQ681859.1	KC409387.1
	Pao palembangensis	KT600982				KT600886	JQ682446.1	JQ682358.1	AB741989.1	JQ681839.1
	Takifugu rubripes	KT600981	AB199321.1	KT600842		KT600885				
Diodontidae	*Diodon holocanthus*	KT600952	KT601009	KT600820	KT600906	KT600864	JQ682369.1	AY700325.1	JQ681853.1	FJ583356.1

Appendix A con't

Family	Species	12S	16S	4c4	rag2	bmp4	rhodopsin	rag1	cytB	COI
Monacanthidae	*Acanthaluteres spilomelanurus*							KF025877.1	KF025759.1	KF025693.1
	Acanthaluteres vittiger		EU848434.1					KF025878.1	KF025760.1	KF025694.1
	Acreichthys tomentosus	KT600931					KF025919.1	KF025879.1	KF025761.1	KF025695.1
	Aluterus heudelotii									
	Aluterus monoceros		JX974406.1				JX996162.1	KF025881.1	EU216740.1	EU216739.1
	Aluterus schoepfi	KT600932	KT600990			KT600848	KF025922.1	KF025882.1	KF025764.1	JQ841844.1
	Aluterus scriptus	KT600933	KT600991	KT600807		KT600849	EF427450.1		EF392567.1	EU216741.1
	Amanses scopas	KT600934	KT600992		KT600891			AY308793.1	KF027550.1	JQ431401.1
	Brachaluteres jacksonianus	KT600944	AY679651.1				KF025924.1	AY700337.1	KF025767.1	KF025701.1
	Cantherhines dumerilii	KT600945	KT601002			KT600859		AY700332.1	EU791284.1	JQ349816.1
	Cantherhines fronticinctus	KT600946	KT601003	KT600815	KT600901					
	Cantherhines macrocerus	KT600947	KT601004			KT600860				JQ842801.1
	Cantherhines pardalis	KT600948	KT601005	KT600816	KT600902	KT600861	KF027983.1	KF027949.1	KF027551.1	JF493013.1
	Cantherhines pullus	KT600949	KT601006	KT600817	KT600903		KF025926.1	AY700333.1	KF025770.1	FJ582914.1
	Cantherhines sandwichiensis							KF025884.1	KF025771.1	JQ431523.1
	Cantherhines verecundus									DQ521021.1
	Chaetodermis penicilligerus						KF025927.1	KF025885.1	KF025772.1	KF025704.1
	Meuschenia freycineti								KF025775.1	KF025707.1
	Meuschenia hippocrepis								KF025776.1	KF025708.1
	Meuschenia trachylepis	KT600955					KF025928.1	AY700338.1	KF025777.1	KF025709.1
	Monacanthus chinensis		KT601012				KF027984.1	KF027950.1	EU216742.1	EF607586.1
	Monacanthus ciliatus						GU014458.1		KF025779.1	KF025711.1
	Monacanthus tuckeri	KT600956	KT601013					AY700331.1	KF025780.1	JQ840165.1
	Nelusetta ayraudi	KT600957	DQ533261.1					AY700340.1	KF025781.1	KF025712.1
	Oxymonacanthus longirostris	KT600959	KT601015	KT600824	KT600910	KT600868	KF027985.1	AY700339.1	KF025782.1	FJ583796.1
	Paraluteres prionurus	KT600960	KT601016		KT600911	KT600869	GU014457.1	AY700336.1	KF027554.1	JF494082.1
	Paramonacanthus choirocephalus						KF025933.1	KF025890.1		KF025715.1
	Paramonacanthus filcauda						KF025934.1	KF025891.1	KF025786.1	KF025717.1
	Paramonacanthus oblongus								KF025785.1	KF025717.1
	Paramonacanthus sulcatus									EF607471.1
	Pervagor aspricaudus									JQ431993.1
	Pervagor janthinosoma	KT600961	KT601017		KT600912	KT600870	GU014456.1	KF025893.1	KF025787.1	JQ350210.1
	Pervagor melanocephalus	KT600962	KT601018	KT600825	KT600913	KT600871	KF025936.1	KF025894.1	KF025788.1	FJ583846.1
	Pervagor nigrolineatus	KT600963	KT601019	KT600826	KT600914		KF025937.1	KF025895.1	KF025789.1	FJ583850.1
	Pervagor spilosoma									DQ521020.1
	Pseudomonacanthus macrurus						KF025938.1	KF025896.1	KF025791.1	AB853874.1
	Pseudomonacanthus peroni						KF025939.1	KF025897.1	KF025792.1	KF025724.1
	Scobinichthys granulatus							KF025899.1	KF025793.1	KF025726.1
	Stephanolepis auratus						KF025941.1	KF025900.1		KF025727.1
	Stephanolepis cirrhifer	KT600973	KT601028				KF027987.1	KF025901.1	KF027555.1	JF952869.1
	Stephanolepis hispidus	KT600974	KT601029	KT600836	KT600922	KT600880	DQ197910.1	AY700335.1	DQ198008.1	JQ841973.1
	Stephanolepis setifer	KT600975	KT601030	KT600837	KT600923	KT600881		AY700334.1	KF025796.1	GU225483.1
	Thamnaconus arenaceus									KF489781.1
	Thamnaconus fajardoi							KF025905.1	KF025799.1	KF489782.1
	Thamnaconus modestoides	KT600983							JN212083.1	EU145722.1
	Thamnaconus modestus	KT600984					KF025943.1	KF025903.1	KF025797.1	JQ738546.1
	Thamnaconus tessellatus									JQ681351.1

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FIGURE CAPTIONS*

*Please use color version of illustrations online only

Figure 1. Bayesian majority-rule consensus cladogram for the Balistidae, Monacanthidae, and six outgroup taxa. Posterior probabilities are written at each node. Clades represent those identified in the text above. Fish images modified under Creative Commons license from original photographs retrieved from http://www.fishbase.org.

Figure 2. Chronogram based upon five node constraints (listed in the text) illustrating the ages of origin and diversification of balistoid cladesimplemented in BEAST 2.1.3.

Figure 3. A) Synonymous and B) non-synonymous substitution rate trees for *cytB* calculated using the Bayesian maximum posterior probability topology for all taxa included in this study, pruned to only include those taxa for which we had *cytB* sequence data, and the Muse-Gaut (1994) substitution model implemented in HyPhy.

Figure 4. A) Synonymous and B) non-synonymous substitution rate trees for *bmp4*calculated using the Bayesian maximum posterior probability topology for all taxa included in this study, pruned to only include those taxa for which we had *bmp4* sequence data, and the Muse-Gaut (1994) substitution model implemented in HyPhy.

TABLES

Gene	Primer name	Primer sequence	Best fit model of nucleotide substitution*
12S rDNA	12S53F 12S489F 12S613R 12S991R	CAC AAA GGC TTG GTC CTG ACT TT CTG GGA TTA GAT ACC CCA CTA TGC TCG GTT CTA GAA CAG GCT CCT CTA G GGT ACA CTT ACC ATG TTA CGA CT	GTR + G
16S rDNA	16AR 16BR	CGC CTG TTT ATC AAA AAC AT CCG GTC TGA ACT CAG ATC ACG T	GTR + I + G
tmo4c4	Tmo-f1-5 Tmo-f1-6 Tmo-r1-3	CCT CCG GCC TTC CTA AAA CCT CTC GAA AAG AGT GTT TGA AAA TGA CAT CGT GCT CCT GGG TGA CAA AGT	GTR + G
rag2	RAG2-f1 RAG2-r2	GAG GGC CAT CTC CTT CTC CAA GTC TGT AGA GTC TCA CAG GAG AGC A	TrN + I + G
bmp4	Bmp4-2Fa Bmp4-2Fb Bmp4-2R	TCT YAT YTC AGA GCA CAT GGA GAG G AAC CTC ACC AGC ATT CCA GA ATC GCT GAA GTC CAC GTA CA	TrN + I
*rhodopsin***			HYK + I + G
*COI***			GTR + I + G
*cytB***			GTR + I + G
*rag1***			SYM + G

Table 1 Primers and primer sequences used for amplification and cycle sequencing in this study. *Best fit nucleotide substitution model, based on Akaike information criteria score, for each generegion was determined using jModelTest 2. **Gene regions retrieved from GenBank.

Branch	dN	dS	dN/dS	*p*-value
Balistes capriscus	0.0039	0.0125	0.3116	0.3938
Aluterus scriptus	0.0058	0.0110	0.5283	0.0191*
Aluterus schoepfii	0.0101	0.0516	0.1957	0.0266*
Oxymonacanthus longirostris	0.0212	0.2283	0.0930	0.6674
Stephanolepis hispidus	0.0079	0.0122	0.6484	0.0409*
Pervagor melanocephalus	0.0492	0.4978	0.0989	0.0414*
Cantherhines dumerilii	0.0079	0.0619	0.1270	0.5233
Node 63 (*Pervagor* + *Cantherhines* + *Paraluteres*)	0.0511	0.9055	0.0564	0.0318*
Balistapus undulatus	0.0078	0.0382	0.2040	0.3785
Abalistes stellaris	0.0039	0.0891	0.0438	0.4489

Table 2. Analysis of synonymous and non-synonymous changes in *bmp4* calculated using the Muse-Gaut codon model. Branches with non-zero values for both dN and dS are reported above. *P*-values are from likelihood ratio tests of the statistical significance of differences in dN/dS compared to all other branches in the balistoid phylogeny.

Current classification, species and authors	Revised classification, if applicable
Clade 1:	
Balistes capriscus Gmelin 1789	
Balistes vetula Linnaeus 1758	
Balistes polylepis Steindachner 1876	
Pseudobalistes naufragium (Jordan & Starks 1895)	*Balistes naufragium* Jordan and Starks 1895
Pseudobalistes fuscus (Bloch and Schneider 1801)	*Balistes fuscus* Bloch and Schneider 1801
Balistes punctatus Gmelin 1789	
Clade 2:	
*Balistapus undulatus (*Park 1797)	
Balistoides conspicillum (Bloch and Schneider 1801)	
Melichthys indicus Randall and Klausewitz 1973	
Melichthys vidua (Richardson 1845)	
Melichthys niger (Bloch 1786)	
Odonus niger (Rüppell 1836)	
Balistoides viridescens (Bloch and Schneider 1801)	*Pseudobalistes viridescens* (Bloch & Schneider 1801)
Pseudobalistes flavimarginatus (Rüppell 1829)	
Xanthichthys auromarginatus (Bennett 1832)	
Xanthichthys mento (Jordan and Gilbert 1882)	
Xanthichthys ringens (Linnaeus 1758)	

Table 3 Revised taxonomy for species in Clades 1 and 2 of the family Balistidae.

Taxon/clade	Alfaro et al. (2007)	Dornburg et al. (2008)	Santini et al. (2013)	Arcila et al. (2015)[1]	This study[2]
MRCA Balistidae & Monacanthidae	40 (44-35)	36.6 (39.7-35.2)	52 (60-42)	64	49.7 (69.7-22)
Balistidae	22.9 (30-16)	11.3 (15.9-8.2)	20 (16-25)	34	18.6 (26-12.4)
Monacanthidae	24.6 (31-18)	-	38 (44-31)	47.5	33.2 (46-15.5)
Crown *Balistes*	-	7.9 (10.8-5.8)	15	9	14.5 (20.5-8.9)
Crown *Rhinecanthus*	-	3.3 (5.3-2.0)	7	5	3.8 (6-0.5)
Crown *Sufflamen*	-	6.2 (9.1-4.0)	8	4	6.2 (9.8-0.3)
Crown *Melichthys*	-	2.5 (4.1-1.3)	7	6	3.9 (6.9-0.1)
Crown *Aluterus*	-	-	17	36	14.8 (18.3-13)
Crown *Cantherhines*	-	-	13	16	15 (21.5-7)
Crown *Pervagor*	-	-	7	12.5	9.1 (14.3-0.2)
Crown *Stephanolepis*	-	-	8		4.8 (8.3-0.3)

Table 4. Comparisons of divergence times for major balistoid clades inferred from this and previous studies (in Myr; mean and range of 95% HPD when available). [1]Implemented in BEAST with exponential root prior. [2]Clade ages for an analysis using five node constraints, as outlined in the text.

Figure 1

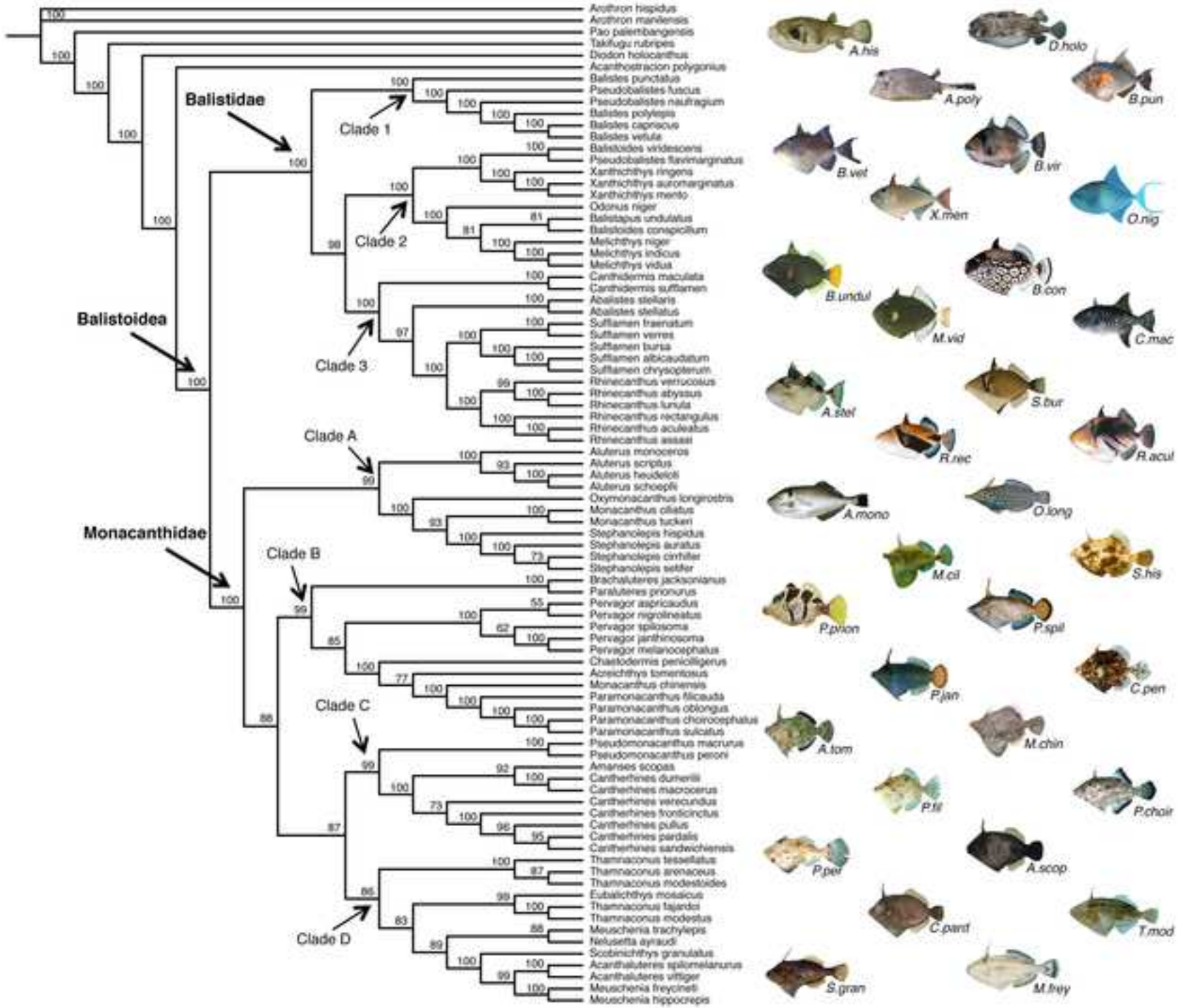

Balistidae
Balistoidea
Monacanthidae
Clade 1
Clade 2
Clade 3
Clade A
Clade B
Clade C
Clade D
Arothron hispidus
Arothron manilensis
Pao palembangensis
Takifugu rubripes
Diodon holocanthus
Acanthostracion polygonius
Balistes punctatus
Pseudobalistes fuscus
Pseudobalistes naufragium
Balistes polylepis
Balistes capriscus
Balistes vetula
Balistoides viridescens
Pseudobalistes flavimarginatus
Xanthichthys ringens
Xanthichthys auromarginatus
Xanthichthys mento
Odonus niger
Balistapus undulatus
Balistoides conspicillum
Melichthys niger
Melichthys indicus
Melichthys vidua
Canthidermis maculata
Canthidermis sufflamen
Abalistes stellaris
Abalistes stellatus
Sufflamen fraenatum
Sufflamen verres
Sufflamen bursa
Sufflamen albicaudatum
Sufflamen chrysopterum
Rhinecanthus verrucosus
Rhinecanthus abyssus
Rhinecanthus lunula
Rhinecanthus rectangulus
Rhinecanthus aculeatus
Rhinecanthus assasi
Aluterus monoceros
Aluterus scriptus
Aluterus heudelotii
Aluterus schoepfii
Oxymonacanthus longirostris
Monacanthus ciliatus
Monacanthus tuckeri
Stephanolepis hispidus
Stephanolepis auratus
Stephanolepis cirrhifer
Stephanolepis setifer
Brachaluteres jacksonianus
Paraluteres prionurus
Pervagor aspricaudus
Pervagor nigrolineatus
Pervagor spilosoma
Pervagor janthinosoma
Pervagor melanocephalus
Chaetodermis penicilligerus
Acreichthys tomentosus
Monacanthus chinensis
Paramonacanthus filicauda
Paramonacanthus oblongus
Paramonacanthus choirocephalus
Paramonacanthus sulcatus
Pseudomonacanthus macrurus
Pseudomonacanthus peroni
Amanses scopas
Cantherhines dumerilii
Cantherhines macrocerus
Cantherhines verecundus
Cantherhines fronticinctus
Cantherhines pullus
Cantherhines pardalis
Cantherhines sandwichiensis
Thamnaconus tessellatus
Thamnaconus arenaceus
Thamnaconus modestoides
Eubalichthys mosaicus
Thamnaconus tajandoi
Thamnaconus modestus
Meuschenia trachylepis
Nelusetta ayraudi
Scobinichthys granulatus
Acanthaluteres spilomelanurus
Acanthaluteres vittiger
Meuschenia freycineti
Meuschenia hippocrepis
A.his
D.holo
A.poly
B.pun
B.vet
B.vir
X.men
O.nig
B.undul
B.con
M.vid
C.mac
A.stel
S.bur
R.rec
R.acul
A.mono
O.long
M.cil
S.his
P.pyron
P.spil
P.jan
C.pen
A.tom
M.chin
P.til
P.choir
P.pel
A.scop
C.pant
T.mod
S.gran
M.frey

Figure 2

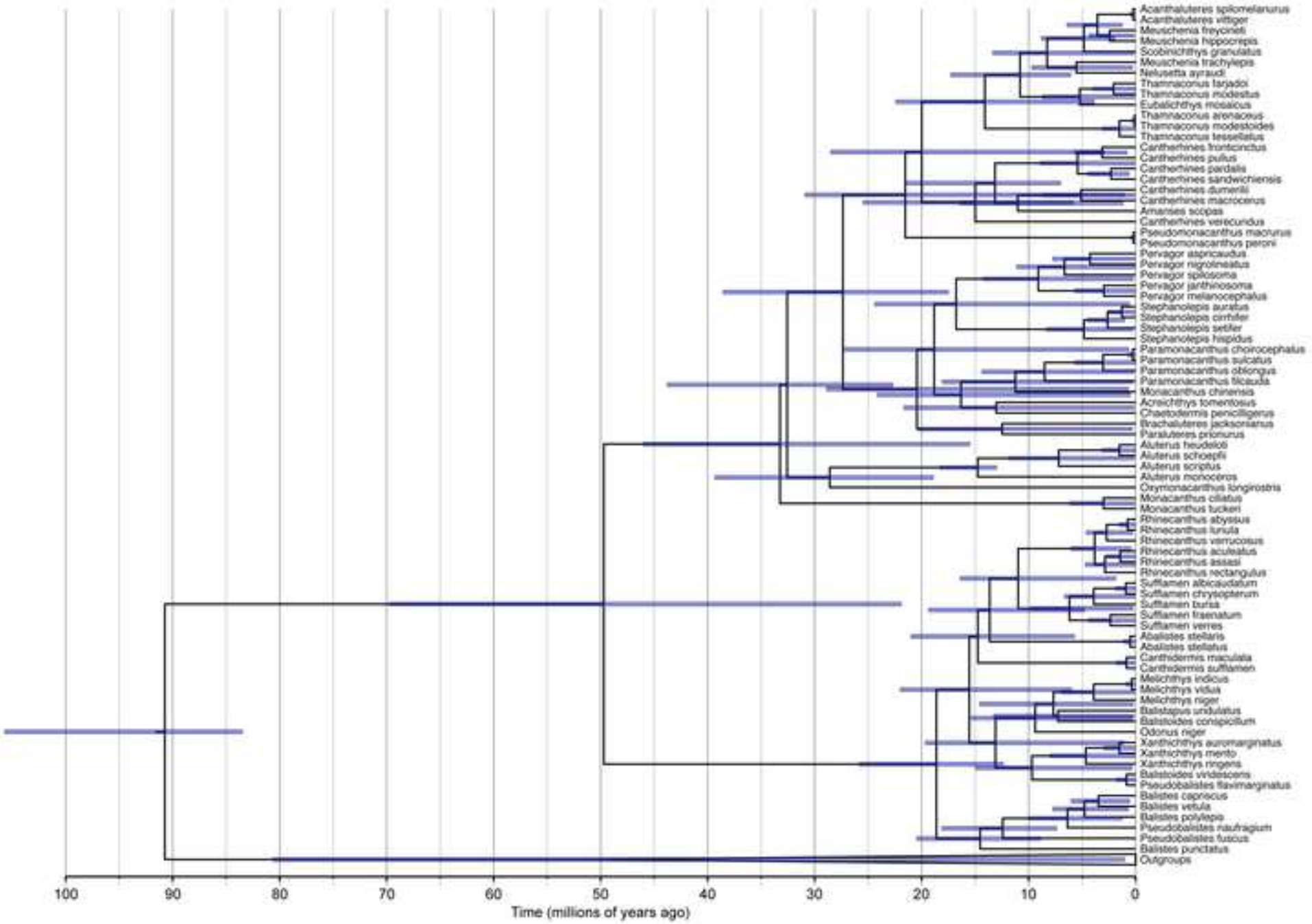
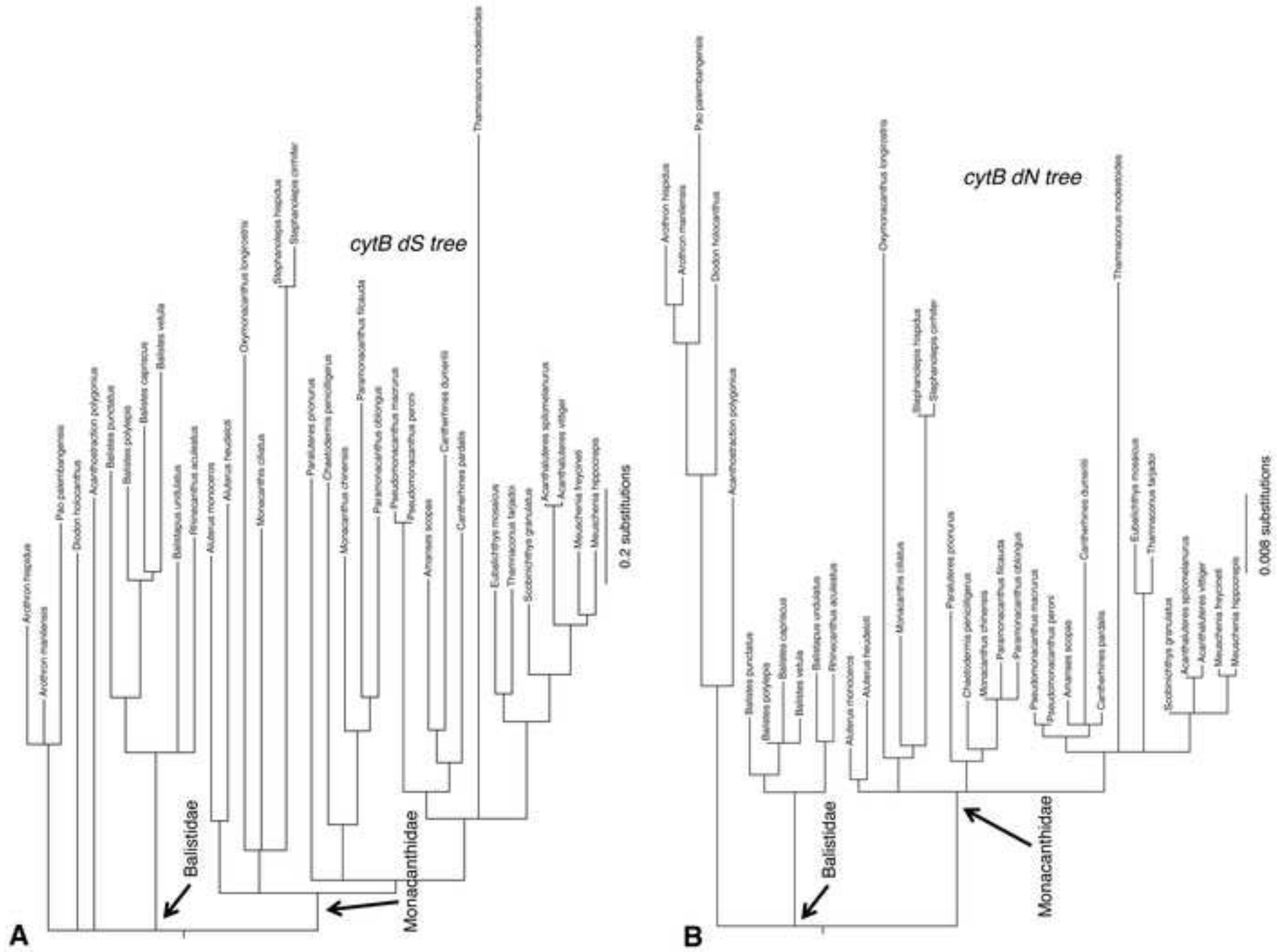

Acanthaluteres spilomelanurus
Acanthaluteres vittiger
Meuschenia freycineti
Meuschenia hippocrepis
Scobinichthys granulatus
Meuschenia trachylepis
Nelusetta ayraudi
Thamnaconus tarjadoi
Thamnaconus modestus
Eubalichthys mosaicus
Thamnaconus arenaceus
Thamnaconus modestoides
Thamnaconus tessellatus
Cantherhines fronticinctus
Cantherhines pullus
Cantherhines pardalis
Cantherhines sandwichiensis
Cantherhines dumerilii
Cantherhines macrocerus
Amanses scopas
Cantherhines verecundus
Pseudomonacanthus macrurus
Pseudomonacanthus peroni
Pervagor aspricaudus
Pervagor nigrolineatus
Pervagor spilosoma
Pervagor janthinosoma
Pervagor melanocephalus
Stephanolepis auratus
Stephanolepis cirrhifer
Stephanolepis setifer
Stephanolepis hispidus
Paramonacanthus choirocephalus
Paramonacanthus sulcatus
Paramonacanthus oblongus
Paramonacanthus filicauda
Monacanthus chinensis
Acreichthys tomentosus
Chaetodermis penicilligerus
Brachaluteres jacksonianus
Paraluteres prionurus
Aluterus heudelotii
Aluterus schoepfii
Aluterus scriptus
Aluterus monoceros
Oxymonacanthus longirostris
Monacanthus ciliatus
Monacanthus tuckeri
Rhinecanthus abyssus
Rhinecanthus lunula
Rhinecanthus verrucosus
Rhinecanthus aculeatus
Rhinecanthus assasi
Rhinecanthus rectangulus
Sufflamen albicaudatum
Sufflamen chrysopterum
Sufflamen bursa
Sufflamen fraenatum
Sufflamen verres
Abalistes stellaris
Abalistes stellatus
Canthidermis maculata
Canthidermis sufflamen
Melichthys indicus
Melichthys vidua
Melichthys niger
Balistapus undulatus
Balistoides conspicillum
Odonus niger
Xanthichthys auromarginatus
Xanthichthys mento
Xanthichthys ringens
Balistoides viridescens
Pseudobalistes flavimarginatus
Balistes capriscus
Balistes vetula
Balistes polylepis
Pseudobalistes naufragium
Pseudobalistes fuscus
Balistes punctatus
Outgroups
100
90
80
70
60
50
40
30
20
10
0
Time (millions of years ago)

Figure 3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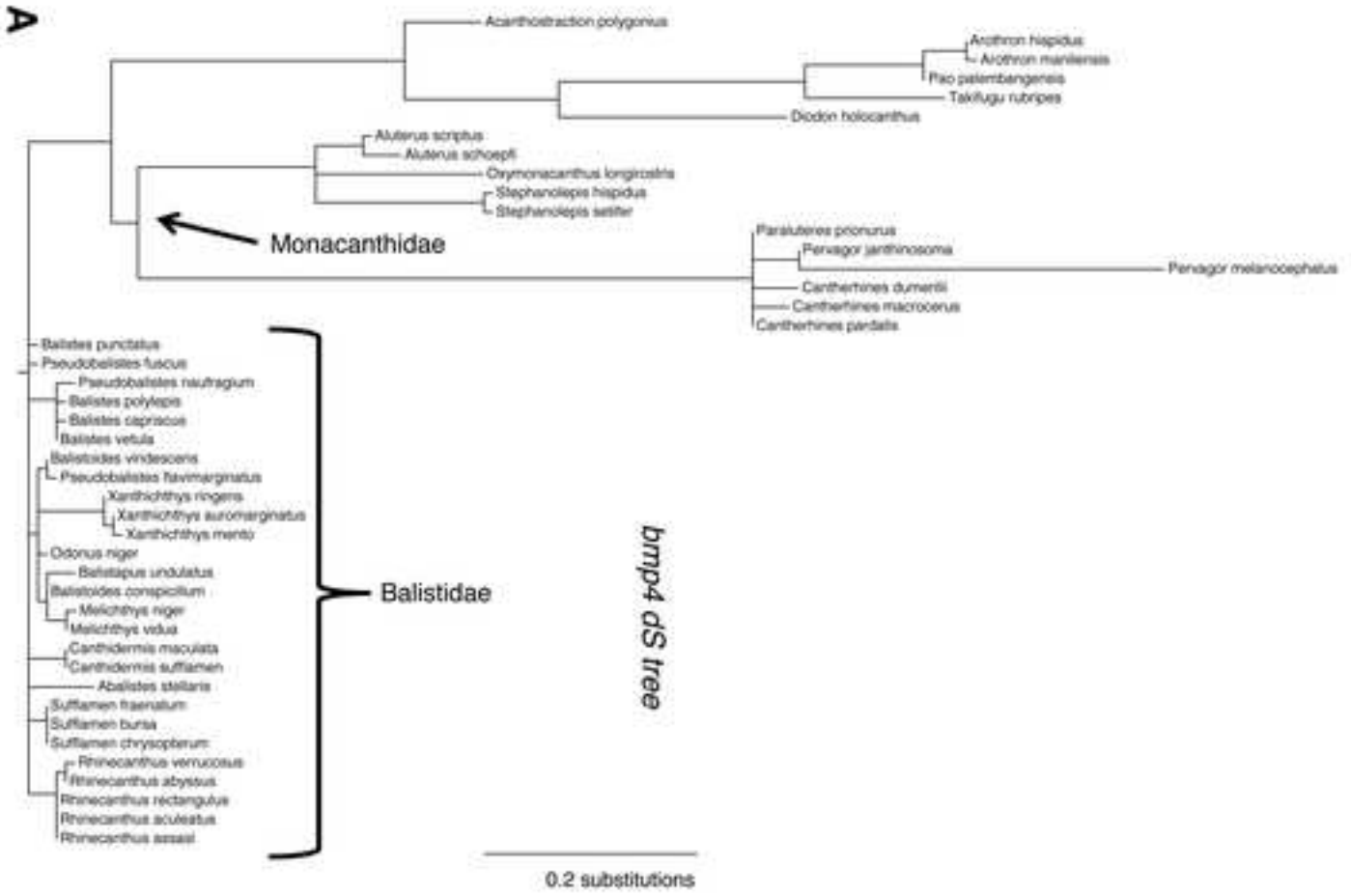
Balistidae
Monacanthidae
0.2 substitutions
B
cytB dN tree
Balistidae
Monacanthidae
0.008 substitutions

Figure 4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Pao palembangensis
Takifugu rubripes
Diodon holocanthus
Aluterus scriptus
Aluterus schoepfi
Oxymonacanthus longirostris
Stephanolepis hispidus
Stephanolepis setifer
Monacanthidae
Paraluteres prionurus
Pervagor janthinosoma
Pervagor melanocephalus
Cantherhines dumerilii
Cantherhines macrocerus
Cantherhines pardalis
Balistes punctatus
Pseudobalistes fuscus
Pseudobalistes naufragium
Balistes polylepis
Balistes capriscus
Balistes vetula
Balistoides viridescens
Pseudobalistes flavimarginatus
Xanthichthys ringens
Xanthichthys auromarginatus
Xanthichthys mento
Odonus niger
Balistapus undulatus
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Melichthys vidua
Canthidermis maculata
Canthidermis sufflamen
Abalistes stellaris
Sufflamen fraenatum
Sufflamen bursa
Sufflamen chrysopterum
Rhinecanthus verrucosus
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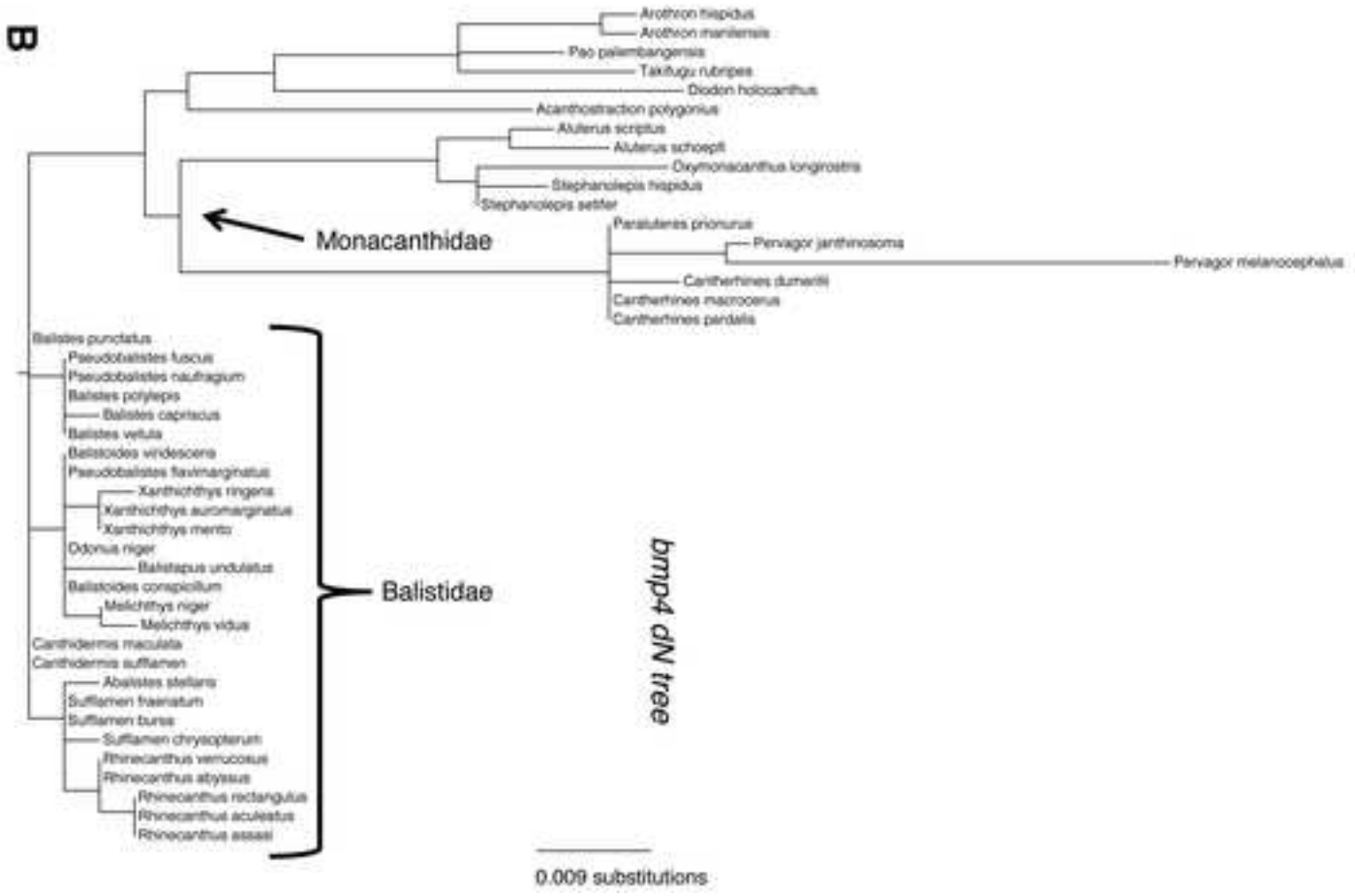
Rhinecanthus assasi
Balistidae
bmp4 dS tree
0.2 substitutions


B
Arothron hispidus
Arothron manilensis
Pao palembangensis
Takifugu rubripes
Diodon holocanthus
Acanthostracion polygonius
Aluterus scriptus
Aluterus schoepfi
Oxymonacanthus longirostris
Stephanolepis hispidus
Stephanolepis setifer
Monacanthidae
Paraluteres prionurus
Pervagor janthinosoma
Pervagor melanocephalus
Cantherhines dumerilii
Cantherhines macrocerus
Cantherhines pardalis
Balistes punctatus
Pseudobalistes fuscus
Pseudobalistes naufragium
Balistes polylepis
Balistes capriscus
Balistes vetula
Balistoides viridescens
Pseudobalistes flavimarginatus
Xanthichthys ringens
Xanthichthys auromarginatus
Xanthichthys mento
Odonus niger
Balistapus undulatus
Balistoides conspicillum
Melichthys niger
Melichthys vidua
Canthidermis maculata
Canthidermis sufflamen
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Sufflamen bursa
Sufflamen chrysopterum
Rhinecanthus verrucosus
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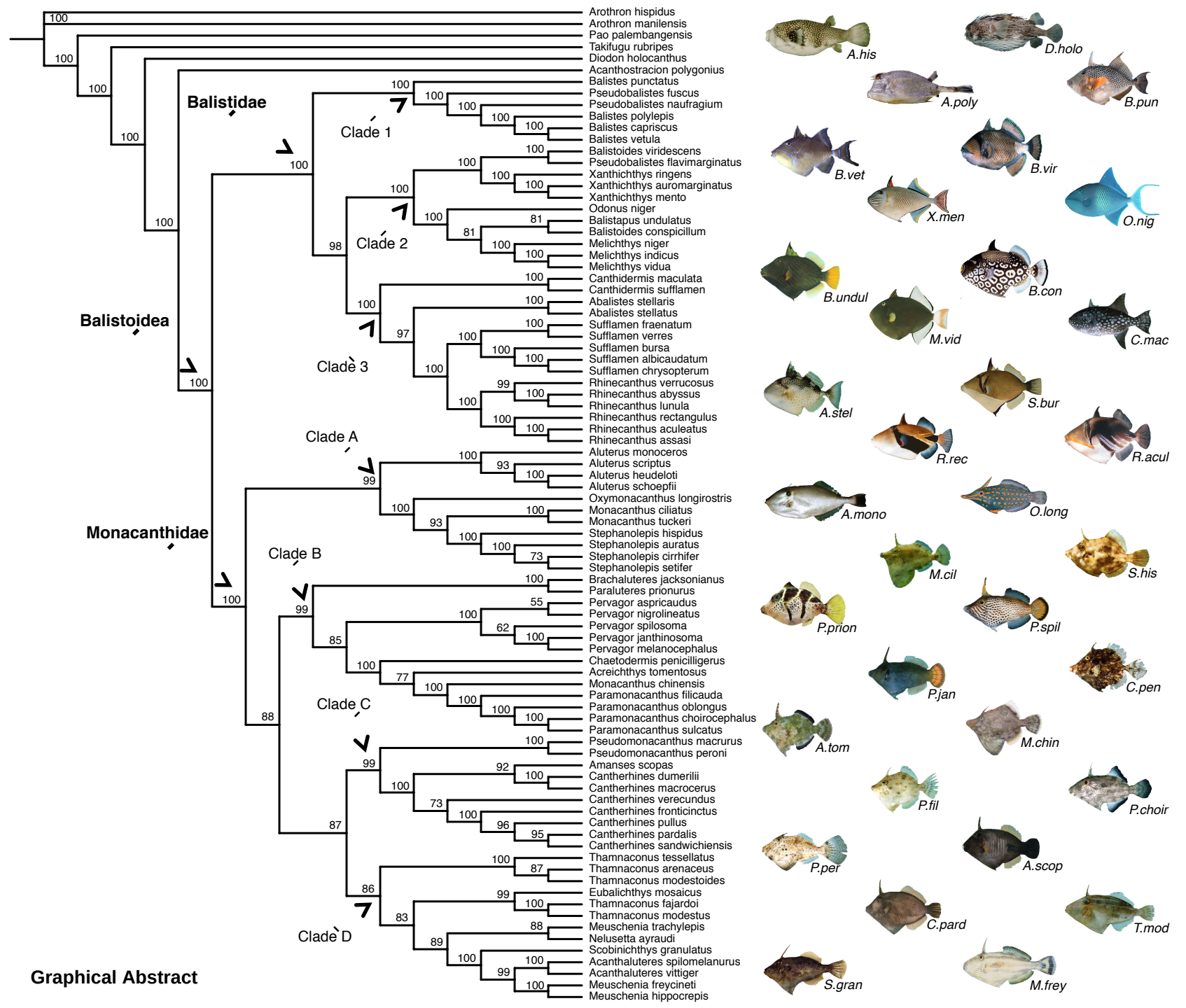
Balistidae
bmp4 dN tree
0.009 substitutions



Graphical Abstract