A multilocus molecular phylogeny of boxfishes (Aracanidae, Ostraciidae; Tetraodontiformes)

Francesco Santini, Laurie Sorenson, Tina Marcroft, Alex Dornburg, Michael E. Alfaro

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Abstract

Boxfishes (superfamily Ostracioida, order Tetraodontiformes) are comprised of 37 species within the families Aracanidae (13 sp.) and Ostraciidae (24 sp.). These species are characterized by several dramatic reductive trends in their axial and appendicular skeleton, and by the presence of a carapace formed by enlarged and thickened scale plates. While strong support exists for the monophyly of both families, interspecific relationships remain unclear as no species-level molecular phylogeny currently exists for either of these two clades, and the only hypotheses of relationships are based on morphological studies that were mostly restricted to generic-level relationships. Here we present the results of a new phylogenetic study of a dataset composed of 9 loci for 26 species of boxfishes using both likelihood and Bayesian methods. Our topology strongly supports the monophyly of both groups, and additionally provides strongly supported resolution for the vast majority of species-level interrelationships. Based on this new phylogeny, we suggest changing the taxonomic status of the species Lactoria fornasini to Tetrasomus fornasini, and Rhynchostracion nasus to Ostracion nasus. Using a Bayesian approach to divergence time estimation we inferred a Paleocene origin of the Ostracioida, with an estimated origin of the aracanids spanning the Eocene and Oligocene, and a Miocene/Pliocene origin of the aracanids.

1. Introduction

Ostracioids, from here on also referred to as boxfishes, represent one of the most distinct groups of teleost fishes, and are largely characterized by extreme body armor modifications. Body armor has originated multiple times within actinopterygian fishes, with independent origins in lineages as divergent as Siluriformes, Gasterosteiformes, Syngnathiformes, Scorpaeniformes (Nelson, 2006) and fossil tetraodontiforms that include the Cretaceous Plectocretaciodae, the Eocene Spinacanthus and Protobalistum from the Ypresian of Monte Bolca (Italy), and likely Eospinus from the Eocene of Caucasus (Tyler 1973; Tyler and Gregorova, 1991; Tyler and Bannikov 1992; Tyler and Sorbini, 1996; Tyler and Santini, 2002, 2005; Santini and Tyler, 2003, 2004). Yet, despite the multiple origins of body armor across actinopterygians, the degree of carapace shape disparity demonstrated in boxfishes reflects a complex evolutionary history of armor restructuring and modification not common in other armored fish lineages.

The armored carapace in boxfishes is composed of thickened and enlarged scale plates that abut one another (Tyler, 1980; Besseau and Bouligand, 1998), and the evolution of the structure has been facilitated by reductions in both the axial and post-cranial skeleton. Boxfishes lack the first spiny dorsal fin, including all associated pterygiophores, the pelvic fin complex, and pleural ribs (Tyler, 1980). Additionally boxfishes have experienced fusion of several abdominal vertebrae to each other and to the occipital region of the skull, as well as the fusion of several elements of the caudal plate to each other (Tyler, 1962, 1980; Klassens, 1995, 1996; Santini and Tyler, 2003; Britz and Johnson, 2005). The boxfish carapace has long been thought to represent an anti-predatory defense mechanism (Brainerd and Patek, 1998), and the shape of the carapace varies widely.

Boxfishes range from having nearly square transverse sections (e.g., Ostracion), to more triangular (e.g., Tetrasomus) or oblong (e.g., Anoplocapros) shapes. In addition to shape, the surface structure of the carapace is also very variable, with species of boxfishes having either a relatively smooth, uniform surface (e.g., Ostracion, Anoplocapros), or a surface covered with protruding irregularities such as keels, spikes and spines, (e.g., Lactoria, Kentrocapros). A number of recent studies have shown that the carapace shape and structure likely plays a role in swimming by minimizing vortices and drag, as boxfishes have evolved a peculiar style of swimming (ostraciform locomotion) to offset the restrictions to locomotor function imposed by the presence of rigid full body armor (Hove et al., 2001; Bartol et al., 2005; Gordon et al., 2001).
In spite of their peculiar morphology, boxfishes have received relatively scant attention from fish systematists. Based primarily on carapace armoring, boxfishes are currently classified into two families, the Aracanidae (deepwater boxfishes), and the Ostraciidae (boxfishes, cowfishes and trunkfishes) (Tyler, 1980; Santini and Tyler, 2003; Froese and Pauly, 2011). However, only a handful of morphological studies have attempted to resolve the relationships within these families. Tyler (1980) investigated the osteology of representative species within both families, and suggested a hypothesis of relationships among the then valid genera based on an evolutionary taxonomic approach in which the extinct genus Proaracana and the extant genus Strophirhinchus (that was later folded within the genus Anoplacapros), represented the ancestors to all other extant aracanids, while the Eocene Eolactoria represented the ancestor to all ostracids, which were then divided into two groups: one including Acanthostracion, Rhinosomus and Lactophrys, and the other including Lactoria, Tetrasomus, Ostracion and Rhyynchocentrus. Subsequently, Winterbottom and Tyler (1983) investigated the relationships among the genera of aracanids with a cladistic analysis (based on Hennigian argumentation) of a combined myological and osteological dataset (including osteological data from Tyler, 1980), and concluded that the then recently described Polyplacapros together with Kentrocapros formed the sister group to all other aracanids, while the monotypic Capropygia and Caprichthys appeared to be nested deeply within a clade formed by the remaining genera, Aracana and Anoplacapros. These results agreed with Tyler (1980) in the placement of Capropygia and Caprichthys appearing deeply nested within the aracanids, but disagreed in the placement of Anoplacapros. Klassen (1995) analyzed a large osteological dataset for 19 species of ostracid species, and found a clade formed by the genera Acanthostracion and Lactophrys, both characterized by a triangular transverse plane shape, representing the sister group to all other ostracids. A second clade, formed by Ostracion and Rhyynchocentrus, appears as sister to the Lactoria + Tetrasomus clade. Although the monophyly of all genera, except the paraphyletic Lactoria, was highly supported, the relationships within genera appeared to be rather uncertain, with several potential topologies being equally parsimonious (e.g., his Fig. 32).

In this study we present the first molecular phylogeny of the boxfishes providing species level resolution for close to 80% of extant boxfish species. We employ two mitochondrial genes and seven single copy nuclear genes in a suite of Maximum Likelihood parsimonious (e.g., his Fig. 32). DNA was extracted from muscle tissue samples or fin clips previously stored in 70% ethanol using the Qiagen DNAeasy kit (Qiagen, Valencia, CA, USA), following the protocol suggested by the manufacturer. Two mitochondrial genes, cytochrome oxidase subunit I (coxI) and cytochrome b (Cytb), and seven nuclear genes, early growth response gene 1 (EGR1), interphotoreceptor retinoid-binding protein (IRBP); mixed-linked Leukemia-like gene (MLL); cardiac muscle myosin heavy chain 6 alpha (myh6), recombination activating gene 1 (Rag1), rhodopsin (Rh), and zic family member 1 (zic1) were amplified using the polymerase chain reaction (PCR). One to two microliters of genomic template was used per 25-µl reaction, containing 5 µL of 5 × Go-Taq Flexi PCR buffer (Promega), 2 µL MgCl₂ (25 mM), 0.5 µL dNTPs (2.5 mM), 1.25 µL of each primer (10 µM) (Table 2), and 0.125 µL of Promega GoTaq Flexi DNA polymerase (5 U/L). Primers and PCR conditions were obtained from the literature: (Ward et al., 2005) for coxI; (Sevilla et al., 2007) for Cytb; (Chen et al., 2008) for EGR1; (Li et al., 2009) for IRBP and MLL; (Li et al., 2007) for myh6 and zic1; (López et al., 2004) for Rag1; (Chen et al., 2003) for Rh. PCRs were performed on a MJ Research PTC-200 Peltier or Eppendorf Mastercycler ProS thermal cyclers. All products were stored at −20 °C after amplification. We used ExoSap (Amersham Biosciences) to remove the excess dNTPs and unincorporated primers from the PCR products; purified products were then cycle-sequenced using the BigDye Terminator v3.1 cycle sequencing kit (1/8th reaction) (Applied Bioscience) with each gene’s original or additional internal primers (Table 2) used for amplification. The cycle sequencing protocol consisted of 25 cycles with a 10-5 94 °C denaturation, 5-s of 50 °C annealing, and a 4-min 60 °C extension stage. Sequencing was conducted at the Yale University DNA Analysis Facility using an ABI 3730xl DNA Genetic Analyzer (Applied Biosystems).

2. Material and methods

2.1. Sampling

Tissue samples for 26 species of boxfishes, plus two outgroups, were obtained through tissue loans from other university or museum collections or purchases through the pet trade (Table 1). Additional sequence data for two species was downloaded from Genbank (Table 1). Our sampling includes 17 ostracids and 9 aracanids, including representatives from every described genus in the superfamily, with the exception of the aracanid Polyplacapros. Due to uncertainty in higher-level tetraodontiform relationships, we included both a balistid (Rhinecanthus aculeatus) and a triacanthodid (Triacanthodes ethiops) as outgroups. Higher-level relationships among the major tetraodontiform groups are currently highly contentious, and characterized by major disagreement between datasets. Cladistic analyses of osteological and myological data from adult specimens (Winterbottom, 1974; Santini and Tyler, 2003, 2004) agree with evolutionary taxonomic studies (Tyler, 1980) in strongly supporting a sister group relationships between ostracoids and a clade formed by Balistidae (triggerfishes) and Monacanthidae (filefishes), while larval characters (Leis, 1984; Britz and Johnson, 2005) support a close relationships between Ostraciidae, Molidae (ocean sunfishes) and Diodontidae (porcupine fishes). Molecular analyses, however, have so far failed to support either of these topologies. Analyses of the nuclear locus Rag1 and the ribosomal genes 12S and 16S (Holcroft, 2005; Alfaro et al., 2007) support a clade with ostracoids and Molidae + Triacanthidae, while analyses of mitochondrial genomes support a clade with boxfishes, Triacanthoididae (deep sea spikefishes) and Triacanthidae (threetooth puffer) (Yamanoue et al., 2008).

2.2. DNA extraction, PCR amplification, and sequencing

DNA was extracted from muscle tissue samples or fin clips previously stored in 70% ethanol using the Qiagen DNAeasy kit (Qiagen, Valencia, CA, USA), following the protocol suggested by the manufacturer. Two mitochondrial genes, cytochrome oxidase subunit I (coxI) and cytochrome b (Cytb), and seven nuclear genes, early growth response gene 1 (EGR1), interphotoreceptor retinoid-binding protein (IRBP); mixed-linked Leukemia-like gene (MLL); cardiac muscle myosin heavy chain 6 alpha (myh6), recombination activating gene 1 (Rag1), rhodopsin (Rh), and zic family member 1 (zic1) were amplified using the polymerase chain reaction (PCR). One to two microliters of genomic template was used per 25-µl reaction, containing 5 µL of 5 × Go-Taq Flexi PCR buffer (Promega), 2 µL MgCl₂ (25 mM), 0.5 µL dNTPs (2.5 mM), 1.25 µL of each primer (10 µM) (Table 2), and 0.125 µL of Promega GoTaq Flexi DNA polymerase (5 U/L). Primers and PCR conditions were obtained from the literature: (Ward et al., 2005) for coxI; (Sevilla et al., 2007) for Cytb; (Chen et al., 2008) for EGR1; (Li et al., 2009) for IRBP and MLL; (Li et al., 2007) for myh6 and zic1; (López et al., 2004) for Rag1; (Chen et al., 2003) for Rh. PCRs were performed on a MJ Research PTC-200 Peltier or Eppendorf Mastercycler ProS thermal cyclers. All products were stored at −20 °C after amplification. We used ExoSap (Amersham Biosciences) to remove the excess dNTPs and unincorporated primers from the PCR products; purified products were then cycle-sequenced using the BigDye Terminator v3.1 cycle sequencing kit (1/8th reaction) (Applied Bioscience) with each gene’s original or additional internal primers (Table 2) used for amplification. The cycle sequencing protocol consisted of 25 cycles with a 10-5 94 °C denaturation, 5-s of 50 °C annealing, and a 4-min 60 °C extension stage. Sequencing was conducted at the Yale University DNA Analysis Facility using an ABI 3730xl DNA Genetic Analyzer (Applied Biosystems).

2.3. Phylogenetic analysis

The chromatograms were checked and assembled into contigs using Geneious 5.3 (Drummond et al., 2010). The consensus sequences for each individual gene were aligned in Geneious using the MUSCLE software (Edgar, 2004), and the alignments subsequently checked by eye for accuracy. The sequences were trimmed to minimize missing characters, and our final data matrix consisted of 680 bp for coxI, 1088 bp for Cytb, 854 bp for EGR1, 782 bp for IRBP, 704 bp for MLL, 787 bp for myh6, 1353 bp for Rag1, 770 bp for Rh and 726 for zic1, for a total of 7844 nucleotides used in
the concatenated analyses. All sequences generated for this study were deposited in GenBank (Table 1).

We used jModelTest (Posada, 2008) to select the best fitting model of sequence evolution from the candidate pool of models that can be utilized in MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) using AIC (Akaike, 1973). We did not include the proportion of invariant sites parameter in the candidate pool, as this parameter is already taken into consideration by the gamma parameter of invariant sites parameter in the candidate pool, as this parameter

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</table>
2.4. Divergence time estimation

The concatenated alignment was analyzed, both as a single partition or as three unlinked partitions (Mitochondrial loci with HKY + G model of sequence evolution, nuclear genes with HKY + G model, nuclear genes with GTR + G model), with uncorrelated exponential priors in BEAST 1.6.2 (Drummond and Rambaut, 2007). Analyses in which all eight loci were treated as individual partitions, each with the model selected by jModeltest were also attempted, but did not reach convergence, so their results were not further considered. A birth–death prior was assigned to rates of cladogenesis. For each dataset two analyses of 20 million each, with sampling every 10000 generations, were conducted. We used Tracer 1.5 (Rambaut and Drummond, 2009) to inspect the trace files and insure that the chains had reached convergence and the ESS for all parameters was greater than 200. We removed the first 10% of the trees as burnin. We then used LogCombiner to merge the files with the remaining trees, and TreeAnnotator (Drummond and Rambaut, 2007) to obtain a timetree. Due to the potential of clad-specific rate heterogeneity potentially misleading divergence time estimates (Dornburg et al., 2012) and observations that boxfish possess much slower rates of molecular evolution relative to most other tetraodontiforms, as seen by branch lengths in both previously published phylogenetic studies (e.g., Alfaro et al., 2007) and this study, the outgroups were removed before performing the dating analyses in BEAST.

Boxfish have a relatively rich fossil record that spans back to the middle Eocene (Ypresian) deposits of Monte Bolca (50 Ma), enabling us to use two fossil placed calibration points for this study. We dated the split between Ostraciidae and Aracanidae with the fossils of the Eocene Prearacana dubia, a stem aracanid, and Eoalcatoria sorbinii, a stem ostracid (Tyler and Santini, 2002; Santini and Tyler, 2003, 2004). Both of these fossils date from the Ypresian of Monte Bolca (50 Ma), and mark the minimum age for the crown ostracioid clade. We used the age of the Santonian Protriacanthus gortani (Tyler and Sorbini, 1996) to put a soft upper boundary of 85 Ma on the prior age for this node. We note that while the oldest taxon currently assigned to the tetraodontiforms is Plectocretacicus clarae (Francesco Santini, unpublished), for this reason we prefer to use Protriacanthus. We also used the Paleocene Mosclaybalistes danicus, (Santini and Tyler, 2003) a stem balistoid from the late Paleogene of Denmark (58–59 Ma) to put a minimum age prior on the root of the tree. Protriacanthus gortani was again used to set the soft upper bound for the stem age (crown ostracioid: offset = 50, mean = 11; root: offset = 59, mean = 8).

3. Results

3.1. Phylogenetic analyses

Both Maximum Likelihood and Bayesian analyses of the concatenated dataset strongly support the same topology with very high support values present for the inter-specific and generic relationships. Nineteen out of 25 nodes within the ostracioids, including these supporting the monophyly of Ostracioida, Aracanidae and Ostraciidae have a posterior probability (pp) and bootstrap (bsp) support of >0.99 and 100% (Fig. 1 and 2). With the exception of the ostraciid Lactoria, all genera are resolved as monophyletic with high support (pp > 0.99, bsp > 92%).

Within Aracanidae, a clade formed by the monotypic Caprichthys (rigid boxfish) and Capropygia (black banded pigmy boxfish) appear as sister to all remaining species with the two species of Kentrocarps (basketfishes) being the next group to split from the remaining deepwater boxfishes, however these relationships are poorly supported (pp = 0.77; bsp = 44%). The remaining deepwater boxfishes in our tree are split between the reciprocally monophyletic Aracana (ornate boxfishes) and Anoplacopros (smooth boxfishes).

Within Ostraciidae, we recover three highly supported (pp > 0.99; bsp = 100%) clades: (1) Acanthostracion (scrawled and honeycomb cowfishes) + Lactophrys (trunkfishes), (2) Lactoria (cowfishes) + Tetrasomus (triangular boxfishes and turretfishes) and (3) Rhynchostracion (shortnose boxfish) + Ostracion (yellow, whitespotted and horn-nosed boxfish). Acanthostracion + Lactophrys appear to be the sister taxon to all remaining ostraciids, with Lactoria + Tetrasomus sister to Rhynchostracion + Ostracion. We found strong support for the paraphyly of Lactoria, with L. fornasini appearing as the sister taxon to Tetrasomus. The monophyly of all remaining genera is strongly supported in both Bayesian (pp > 0.99) and Likelihood (bsp = 91% or greater) analyses.

The gene-tree species-tree analysis performed with BEAST (not shown) recovers an identical topology for the aracanids, however relationships within the ostraciids differ slightly. When allowing for gene tree heterogeneity, a clade composed of Lactoria cornuta and L. diaphana is sister to a clade with (L. fornasini + Tetrasomus sister to Rhynchostracion + Ostracion). Although the monophyly of most subclades is strongly supported (pp = 0.96 or greater), that of the clade formed by L. diaphana + Tetrasomus sister to Rhynchostracion + Ostracion is not (pp = 0.4).

3.2. Divergence time estimation

The topology of the BEAST analysis (Fig. 3) is highly congruent with that the Maximum Likelihood and MrBayes trees, with the exception of Kentrocarps appearing as sister taxon to all remaining aracanids, though this placement was not highly supported. We estimate the origin of crown ostracioids at 63 Ma. However, aracanids are estimated to be far more recent in origin, with a crown age of 26 Ma and a 95% highest posterior density (HPD) interval between 18 and 36 Ma. Within aracanids, we estimate the split between the Caprichthys + Capropygia and the remaining aracanids at approximately 24 Ma (95% HPD interval 16–33 Ma), and the split between Aracana and Anoplacopros is 13 Ma (95% HPD interval 9–19 Ma). Although our analyses place the initial radiation of crown aracanids in the Miocene, the bulk of the extant species-level diversity likely radiated during the Pliocene, with the three Anoplacopros species originating likely in the last 6 Ma (95% HPD interval 3–9 Ma), Aracana aurita and A. ornata being only 1.8 Ma old (95% HPD interval 0.7–3 Ma) and the Caprichthys + Capropygia clade being only 5 Ma old (95% HPD interval 2–9 Ma).
In contrast to aracanids, we estimate a substantially older origin for ostraciids, with a mean crown age of 56 Ma (95% HPD interval 45–68 Ma), and all extant genera originating by at least the Oligocene. The Acanthostracion + Lactophrys divergence estimated to have occurred approximately 32 Ma (95% HPD interval 21–45 Ma); the Lactoria + Tetrosomus and Rhynchostracion + Ostracion divergence is 39 Ma (95% HPD interval 29–49 Ma); Lactoria cornuta and L. diaphana separated from Tetrosomus (+ L. fornasini) 35 Ma (95% HPD interval 26–45 Ma), and the Rhynchostracion + Ostracion divergence occurring approximately 15 Ma (95% HPD interval 10–21 Ma). In contrast to aracanids, most ostraciid lineages originated by the end of the Miocene (5.3 Ma), with the exceptions
of Ostracion cubicus and O. whiteyi + O. solorensis that diverged in the Pliocene age, approximately 4 Ma (95% HPD interval 2–6 Ma), and the O. whiteyi + O. solorensis divergence approximately 1 Ma (95% HPD interval 0.4–2 Ma).

4. Discussion

4.1. Boxfish relationships

Our study provides strong support for the monophyly of both the Ostraciidae and Aracanidae, agreeing with the previous studies of tetraodontiform relationships (Tyler, 1980; Santini and Tyler, 2003). Within the aracanids both likelihood and Bayesian analyses suggest a topology with the two monotypic genera Caprichthys and Capropygia representing the sister taxon to all remaining aracanids. Although not highly supported, this finding differs from that of Winterbottom and Tyler (1983), who recovered these two genera deeply nested within the Aracana + Anoplocapros clade. Winterbottom and Tyler (1983) indicate six characters to support their topology, however two of these characters (peduncular scale plates and the degree of separation of fibers within the sternobranchialis muscle) are also used to support two subsequent nodes each in their tree (their Fig. 12). Winterbottom and Tyler (1983) used Hennigian argumentation, and not a numerical cladistic approach for data analysis, and thereby did not produce a character matrix. This prevents us from understanding what characters or character states correspond to nodes within their tree. Besides the Caprichthys + Capropygia clade, our hypothesis of genus-level relationships in the Aracanidae match Winterbottom and Tyler (1983).

Our topological inference of ostracid relationships were mostly concordant with Klassen (1995), who analyzed a large osteological dataset of 108 characters for 19 extant species. While the relationships among the genera of ostraciids in Klassen (1995), which were all supported by five or more morphological characters, are fully congruent with both the Bayesian and likelihood based analyses of our molecular dataset, we differ from Klassen in two major regards. First we obtain a strongly supported relationship of Rhyncostracion nasus as sister to Ostracion, while Klassen (1995) placed R. nasus deeply nested within Ostracion. Second, we find strong support for a clade of Lactoria diaphana and L. cornuta, which appears to be the sister taxon to L. fornasini + Tetrasomus, while Klassen (1995) inferred the existence of a clade formed by (L. cornuta (L. fornasini (Tetrasomus))). While Klassen (1995) observed several characters that supported the monophyly of Tetrasomus, and of a Lactoria fornasini + Tetrasomus clade, the only character that supports Lactoria cornuta as the sister taxon to L. fornasini + Tetrasomus is the shape of a pair of ventrolateral processes of the haemal spine of caudal vertebra #6, which join to the base of the last anal basal pterygiophore (character 88–2 in Klassen, 1985). Figs. 28–30 in Klassen (1995), as well as additional illustrations of boxfish osteology in Tyler (1980), reveal a broad variation in this feature, and we could not appreciate the difference between states 88–1 and 88–2 in Lactoria and Tetrasomus illustrated in Fig. 30. We thus assume that this character state may not be a reliable indicator of relationships within this clade.

4.2. The timing of boxfish evolution

Our timetree suggests an early Paleocene origin for the boxfish, with the two families splitting from one another ~63 Ma, and most of the extant boxfish species-richness accumulating in the Miocene and Pliocene. This results broadly agrees with the ages inferred by Alfaro et al. (2007) in their analyses of tetraodontiform higher-level relationships, as well as the pattern observed in other tetraodontiform lineages (Dornburg et al., 2008, 2011; Santini et al. 2009). However, we estimate a much older crown age of the Aracanidae (Ma) compared to Alfaro et al. (2007) (~7 Ma) due to the inclusion in this study of Caprichthys, Capropygia and Kentrocosps.

This late Oligocene/early Miocene (considering the 95% HPD interval) crown age of Aracanidae provides new insights into the radiation of these fishes into cooler temperate waters. With the exception of Kentrocosps, almost all aracanids are restricted to the rocky temperate seas surrounding Australia and New Zealand (Froese and Pauly, 2011). The enigmatic placement of Kentrocosps
4.3. Taxonomic reclassification

All of our phylogenetic analyses reveal Lactoria fornasini (Bianconi, 1846) to be much more closely related to Tetrasomus than to the other species of the genus Lactoria. For this reason we recommend removing fornasini from the genus Lactoria and including it in Tetrasomus. We also recommend changing the generic status of Rhynochostea nassus, to Ostracion nassus due to the fact that the taxonomy of the Ostracion + Rhynochostea clade has been in flux during the past few decades, with species that are alternatively recognized as members of one genus or the other (e.g., R. rhinorhynchos, recognized as R. rhinorhynchos in Klassen, 1995), and that we could not verify any significant morphological difference that can set the two genera apart.

5. Conclusion

Our study provides the first molecular analysis of boxfish interrelationships, providing taxonomic revisions that are consistent with the evolutionary history of these fishes. We find that monophyly of both families, and that of most genera, is strongly supported (pp > 0.99; bps > 91%). We revise the taxonomy of ostracids to eliminate the non-monophyly of the genus Lactoria. By placing the phylogeny of boxfishes into a time calibrated framework, we infer that despite an origination of crown boxfishes by the early Paleocene (~63 Ma) and the formation of the two boxfish families by the Eocene or Oligocene, most of the extant diversity in both families is relatively young, having originated during the Miocene or Pliocene (23 Ma or younger). We hypothesize that the diversification of the boxfishes might have been driven by changes in marine habitats during subsequent Miocene cycles of warming and cooling; while the diversification of the coastal, tropical ostracids might be connected to the expansion of coral reef ecosystems during the Oligocene and Miocene (Wood, 1999).

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