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Improved resolution and a novel phylogeny for the Neotropical triplefin blennies (Teleostei: Tripterygiidae) ☆

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ABSTRACT

The triplefin blennies (Teleostei: Tripterygiidae) are a diverse group of small-bodied benthic fishes associated with rocky or coral reefs. The Neotropics contain four genera and 26 species, many of which have only been recently described. A recent molecular phylogeny (Lin and Hastings, 2013) contrasts with previous phylogenies based on morphology in recovering the four Neotropical genera as a single clade with respect to the Indo-Pacific genera; however, relationships within and among genera were poorly resolved. This study reports a novel topology based on an expanded seven-loci molecular dataset. Individual gene trees have poor resolution, but concatenated analyses show strong support for most nodes, likely due to emergent support from concatenation. Consistent with Lin and Hastings (2013), three of the Neotropical genera, *Axoclinus*, *Enneanectes*, and *Crocodilichthys*, form a well-supported clade, but relationships of the fourth (*Lepidonectes*) are not confidently resolved. The monophyly of *Axoclinus* is well supported, but *Enneanectes* is paraphyletic with the inclusion of *Axoclinus* and *Crocodilichthys*. Improved resolution allows for reinterpretation of the biogeography of the Neotropical Tripterygiidae. Broader taxon sampling is still necessary for resolving the relationships within Tripterygiidae globally.

1. Introduction

The Tripterygiidae is a diverse blennioid family that includes 32 genera and over 164 species (Fricke, 2009). Triplefins are found worldwide, in both temperate and tropical oceans, where they are often associated with rocky or coral reef habitats. Their peak diversity is in New Zealand, where 20 of the 26 endemic species have resulted from an adaptive radiation (Wellenreuther et al., 2007; Hickey et al., 2009). In addition, two Indo-Pacific genera, *Enneapterygius* and *Helcogramma*, are highly diverse with over 53 and 39 species, respectively (Fricke, 2009). Due to their diversity, abundance and benthic lifestyle, tripterygiids have been the focus of studies on marine radiations (Carreras-Carbonell et al., 2005; Wellenreuther et al., 2007), breakup of mitochondrial lineages (Victor, 2013), life history (Longenecker and Langston, 2005; Riginos and Victor, 2001) and isolation by distance (Riginos and Nachman, 2001; Hickey et al., 2009).

The Neotropics includes four genera and 26 species of triplefins, 14 of which have only been described in the past 25 years (Allen and Robertson, 1991, 1992; Rosenblatt et al., 2013; Victor, 2013). Of the 26, seven are endemic to single islands (Robertson and Allen, 2008; Hastings, 2009; Rosenblatt et al., 2013). Three genera are endemic to the Tropical Eastern Pacific (TEP): *Axoclinus* (six species), *Lepidonectes* (three species), and *Crocodilichthys* (one species). The fourth and most diverse genus, *Enneanectes*, contains 15 species distributed in both the TEP and Western Atlantic (Rosenblatt, 1960; Robertson and Allen, 2008). Although their ecological contribution is unclear due to their cryptic nature (Smith-Vaniz et al., 2006), tripterygiids are among the most dominant members of the ichthyofauna of TEP rocky reef communities, especially in the Gulf of California (Aburto-Oropeza and Balart, 2001; Thomson and Gilligan, 2002; Galland, 2013).

Most previous hypotheses of the generic relationships within the Tripterygiidae have been based solely on morphology. Rosenblatt (1959) placed the four Neotropical genera in a clade containing Indo-Pacific genera in his unpublished dissertation (Fig. 1). Fricke (1994, 2009) later revised the family, recognizing eight tribes, and proposed different placements for the Neotropical

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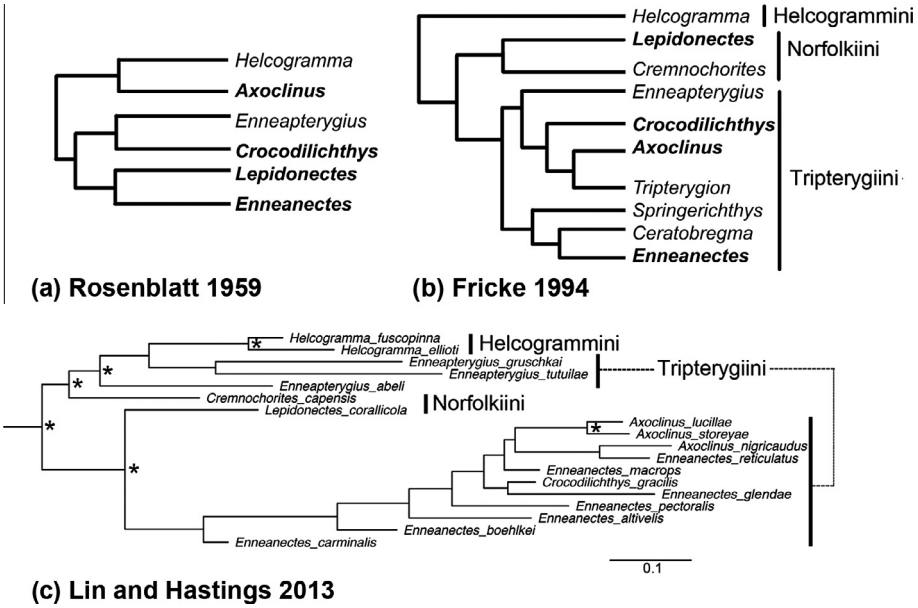

Fig. 1. A–C: Previously published phylogenetic hypotheses for relationships of the Neotropical tripterygiids, modified from their original sources. * = bootstrap support over 70 in maximum likelihood analysis.

genera implying multiple colonizations of the Neotropics. *Lepidonectes* was placed in the tribe Norfolkini with the South African genus *Cremnochorites*, and the other three genera were placed in the tribe Tripterygiini with the Mediterranean genus *Tripterygion.*

Lin (2009) and Lin and Hastings (2013) constructed a five-loci molecular phylogeny that recovered the four Neotropical genera as a monophyletic group with strong support, a result contrasting with previous non-phylogenetic hypotheses based on similarity in morphology (Fig. 1). That analysis only included seven of 29 genera (including the four Neotropical genera), and so it is difficult to make robust conclusions on their relationships to triplefins from other regions. An interesting, but unanswered question concerns the biogeographic origins of the Neotropical triplefins (Hastings, 2009): do they represent a monophyletic group or were there multiple invasions of the region from the Indo-Pacific? Frequent long-distance dispersal seems unlikely given their semi-sessile lifestyle, demersal eggs, short pelagic larval duration, and nearshore development (Lin, 2009). Indeed, other blennioid families with more resolved phylogenetic relationships show strong geographic restriction (Lin and Hastings, 2013).

The species-level relationships of triplefins studied by Lin and Hastings (2013) are unresolved, with most nodes in that study poorly supported (Fig. 1). Curiously, both *Enneanectes* and *Axoclinus* were found to be paraphyletic, with *E. reticulatus* and *A. nigricaudus* placed as sister species. This result is in conflict with traditional taxonomy defining the genera based on possession of a continuous (*Axoclinus*) or discontinuous (*Enneanectes*) lateral line, among other morphological features (Rosenblatt, 1959; Fricke, 1994; Smith and Williams, 2002). Lin and Hastings (2013) attributed the poor resolution within the Tripterygiidae to poor taxon sampling, missing sequence data, and several indels in sampled nuclear genes. Additionally, there are thought to be several rapid speciation events within the family (Carreras-Carbonell et al., 2005; Wellenreuther et al., 2007), resulting in many short branches, which can complicate phylogenetic reconstruction (Townsend et al., 2011; Corl and Ellegren, 2013; Patel et al., 2013; Lambert et al., 2015).

This study expands Lin and Hastings' (2013) molecular dataset to more fully resolve the species-level relationships within the four Neotropical genera. The expanded molecular dataset joins three nuclear markers from their previous work with extended mitochondrial sampling of three new loci, for a total of seven loci.

2. Materials and methods

2.1. Taxon sampling

Tissues from 17 triplefin species from seven genera (Supp. Table 1) were taken from voucher specimens stored in the Scripps Institution of Oceanography Marine Vertebrate Collection (SIO) and the University of Kansas Natural History Museum (KU). Of the four Neotropical genera, one of three *Lepidonectes* species, seven of 15 *Enneanectes* species, and three of six *Axoclinus* species were included (Table 1). Taxon sampling is incomplete primarily because tissues were unavailable for six of seven species endemic to remote islands, as well as several Western Atlantic *Enneanectes* species. Three Indo-Pacific triplefin species were also included: *Enneapterygius gruschkai*, *Cremnochorites capensis*, and *Helcogramma fuscopinna.* The following outgroups were included based on Lin and Hastings (2013): the blennies *Alloclinus holderi* (Labrisomidae), *Ophioblennius steindachneri* and *Hypsoblennius brevipinnis* (Blenniidae), and the clingfish *Gobiesox pinniger* (Gobiesocidae).

2.2. Molecular data and sequence assembly

A total of four mitochondrial markers (12S, 16S, Cytochrome C Oxidase 1 (CO1) and Cytochrome b) and three nuclear markers (Rag-1, Rhodopsin, and TMO-4C4) were included in this study. Novel sequence data for 12S, 16S, CO1, Cytochrome b, Rhodopsin, and TMO-4C4 were generated in this study. When available, we sequenced additional individuals not included in Lin and Hastings (2013). We recognize that mitochondrial loci are sampled from a single non-recombining genome and are often treated as a single locus in phylogenetic analyses, as we do in our *BEAST analyses detailed below. Thus we are analyzing four independent loci as opposed to five in Lin and Hastings (2013), who sequenced CO1, Rag1, Rhodopsin, TMO-4C4, and Histone H3 for a total of 3562 bp. However, we will refer to seven loci to emphasize the additions made by our study. Our study places more emphasis on mitochondrial loci because of the expected younger divergences of our focal species as opposed to Lin and Hastings (2013), who were analyzing the relationships across the suborder Blennioidei. Mitochondrial loci continue to provide resolution for studies across

Table 1
Distribution of species sampling effort within the Neotropics.

Genus	# Sampled/Total described (%)	Pacific: # Sampled/Total	Atlantic: # Sampled/Total
Lepidonectes	1/3 (33%)	1/3	–
Enneanectes	7/15 (47%)	4/5	3/10
Axoclinus	3/6 (50%)	3/6	–
Crocodilichthys	1/1 (100%)	1/1	–

short time scales, even in the age of broader genomic resources (Bowen et al., 2014).

Total genomic DNA was extracted from muscle tissue using a Qiagen (Chatsworth, CA) DNeasy Blood and Tissue kit by following the manufacturer's instructions. When available, multiple individuals per species were used. Primers used to obtain all seven markers are described in Supp. Table 3. PCR was performed under the following conditions: 94 °C for one minute for initial denaturing, 34–35 cycles of 94 °C for 30 s, 50–56 °C for 45 s, and 72 °C for 45 s, followed by 72 °C for five minutes as the final extension. PCR products were purified using a Sephadex gel matrix (Sigma–Aldrich), and sequenced in both directions using the amplifying primers via Retrogen, Inc (San Diego, CA).

Sequences were assembled and edited in Sequencher 5.2 (Gene Codes, Ann Arbor, MI), and imported into Mesquite v. 3.01 (Maddison and Maddison, 2014), where they were aligned using MAFFT v. 7 (Katoh and Standley, 2013). To eliminate poorly aligned portions, we checked each gene alignment using the Gblocks v. 0.91b web server (Castresana, 2000; Talavera and Castresana, 2007) under strict conditions for 12S, 16S, and TMO-4C4 (no gaps allowed) and allowing gaps for the remaining genes. For the protein-coding markers (all but 12S and 16S), codon position was assigned by minimizing stop codons in Mesquite, and translating sequences to ensure that no stop codons were present.

2.3. Test of saturation

Due to the documented high rates of molecular evolution in blennies (Eytan, 2010; Lin and Hastings, 2011; Near et al., 2013), each locus was tested for saturation using Xia's test (Xia et al., 2003, Xia and Lemey, 2009) implemented in DAMBE 5 (Xia, 2013), with protein-coding genes separated by 1 + 2 and 3rd codon positions. In addition, we plotted the number of transitions vs. transversions against corrected genetic distances using the GTR model of evolution. The 3rd codon positions of Cytochrome b were determined to be saturated by both measures (see Results for interpretation of diagnostic measures), guiding downstream analyses.

2.4. Phylogenetic analyses

In order to confirm species identity of sequences and identify potential contamination, we constructed gene trees of individual locus partitions using maximum likelihood (Felsenstein, 1981) implemented in RAxML v7.4.2 (Stamatakis, 2006) via raxmlGUI v1.3 (Silvestro and Michalak, 2012). We removed individual sequences that appeared erroneous due to odd placement compared to others of the same species. After pruning, gene trees were again constructed separately in RAxML using the rapid bootstrapping algorithm and 500 bootstrap replicates, and treated as a single partition. Based on the PartitionFinder results (below, and Supp. Table 2), 12S and 16S were concatenated and a single tree was built.

We concatenated the following datasets: mitochondrial only with all codon positions, mitochondrial only without the 3rd codon positions of Cytochrome b, nuclear only, total (seven-gene) with all codon positions, and total with saturation removed. The best-fit partitioning scheme and appropriate substitution models for the seven-loci datasets was determined in PartitionFinder v.1.1.1 (Lanfear et al., 2012) using the AIC criterion, linked branch lengths, and the "greedy" heuristic algorithm. We ran a total of five separate analyses: three analyses with the dataset containing all codon positions, with (1) all models, (2) models restrained to those implemented in MrBayes (Ronquist and Huelsenbeck, 2003), and (3) models restrained to those implemented in BEAST (Drummond et al., 2012); and two analyses with the dataset with saturation removed, with (4) all models and (5) only models restrained to those in MrBayes.

We built trees from the five concatenated datasets using Maximum likelihood analyses (ML) implemented in RAxML. We conducted 10 independent runs using the thorough bootstrapping algorithm and 1000 bootstrap replicates. All analyses were partitioned according to the PartitionFinder results with all models included, and the model GTR + GAMMA was assigned to all partitions. In addition, trees were also constructed from the two seven-loci datasets using Bayesian Metropolis coupled Markov chain Monte Carlo (MCMC) analyses in MrBayes v. 3.2.2 (Ronquist and Huelsenbeck, 2003). Bayesian inference was performed with two independent MCMC runs with three heated and one cold chains, for 10 million generations, sampling every 1000 generations, and conservatively discarding the first 30% of samples as burn-in.

Species-tree methods using coalescent models are believed to be more accurate than concatenation when incomplete lineage sorting is present, which may result from rapid speciation during the history of a clade (Liu et al., 2009; Townsend et al., 2011; Corl and Ellegren, 2013; Patel et al., 2013; Lambert et al., 2015). Since rapid radiations have been observed in other triplefin clades (Carreras-Carbonell et al., 2005; Wellenreuther et al., 2007), we also inferred a species tree under the coalescent model implemented in *BEAST (Heled and Drummond, 2010). We did this with the caveat that for the following species, only a single individual was available: *Axoclinus lucillae*, *Enneanectes pectoralis* and *E. altivelis*, all Indo-Pacific triplefins, and all outgroups. Although coalescent methods are more accurate with many loci and several individuals (Heled and Drummond, 2010; Corl and Ellegren, 2013), recent studies suggest that *BEAST can be robust to missing data and taxa (Hovmöller et al., 2013; Lambert et al., 2015). We imported the seven-loci dataset with all codon positions into BEAUTi (distributed through the BEAST v.1.8.1 package, Drummond et al., 2012), partitioned according to codon position for protein coding genes (Supp. Table 2). Substitution models of all partitions were unlinked, while clock and tree models were linked by individual genes. Mitochondrial markers were treated as a single gene, and the ploidy type was set to "mitochondrial." We used the Yule species model (Yule, 1924) as species-tree prior, random starting tree, and lognormal relaxed clock model for all genes. We ran four independent runs for 500 million generations each, with parameter and tree sampling every 10,000 generations. The resulting parameter files were combined using LogCombiner, and a maximum clade credibility tree was produced in TreeAnnotator after discarding 30% of trees as burn-in.

For all MrBayes and *BEAST analyses, we confirmed convergence of runs based on plots of ln*L* scores versus generation time, as well as ESS values, visualized in Tracer (Drummond et al., 2012). In addition, we used plots generated by the web-based program Are We There Yet (AWTY, Nylander et al., 2008) to confirm convergence of topology and posterior probabilities. Concatenated trees were rooted with the clade containing *Alloclinus*, *Ophioblennius*, *Hypsoblennius*, and *Gobiesox* because it is unclear if the sister clade to the Tripterygiidae are clingfishes or other blenniiformes (Lin and Hastings, 2013; Near et al., 2013).

Table 2
Number of characters contributed by each marker, and included in each dataset. P-U = Parsimony uninformative, P-I = Parsimony informative.

Dataset	# Sites	# Constant	# P-U	# P-I	% P-I	%Spp./%Individual coverage[a]
12S	321	225	10	86	26.8	81/58
16S	533	398	46	89	16.7	57/45
CO1	569	332	18	219	38.5	95/90
Cytochrome B, all	786	445	21	320	40.7	76/58
Cytochrome B, sat. removed	524	441	17	66	12.6	76/58
Rag1	1503	961	257	285	19.0	52/28
Rhodopsin	737	565	38	134	18.2	95/73
TMO-4C4	424	260	46	118	27.8	95/75
mtDNA, all codons	2209	1400	95	714	32.3	100/97
mtDNA, sat. removed	1947	1396	91	460	23.6	100/97
nuDNA	2664	1786	341	537	20.2	100/82
All genes, all codons	4873	3186	436	1251	25.7	100/100
All genes, sat. removed	4611	3182	432	997	21.6	100/100

[a] Out of 21 species and 40 individuals.

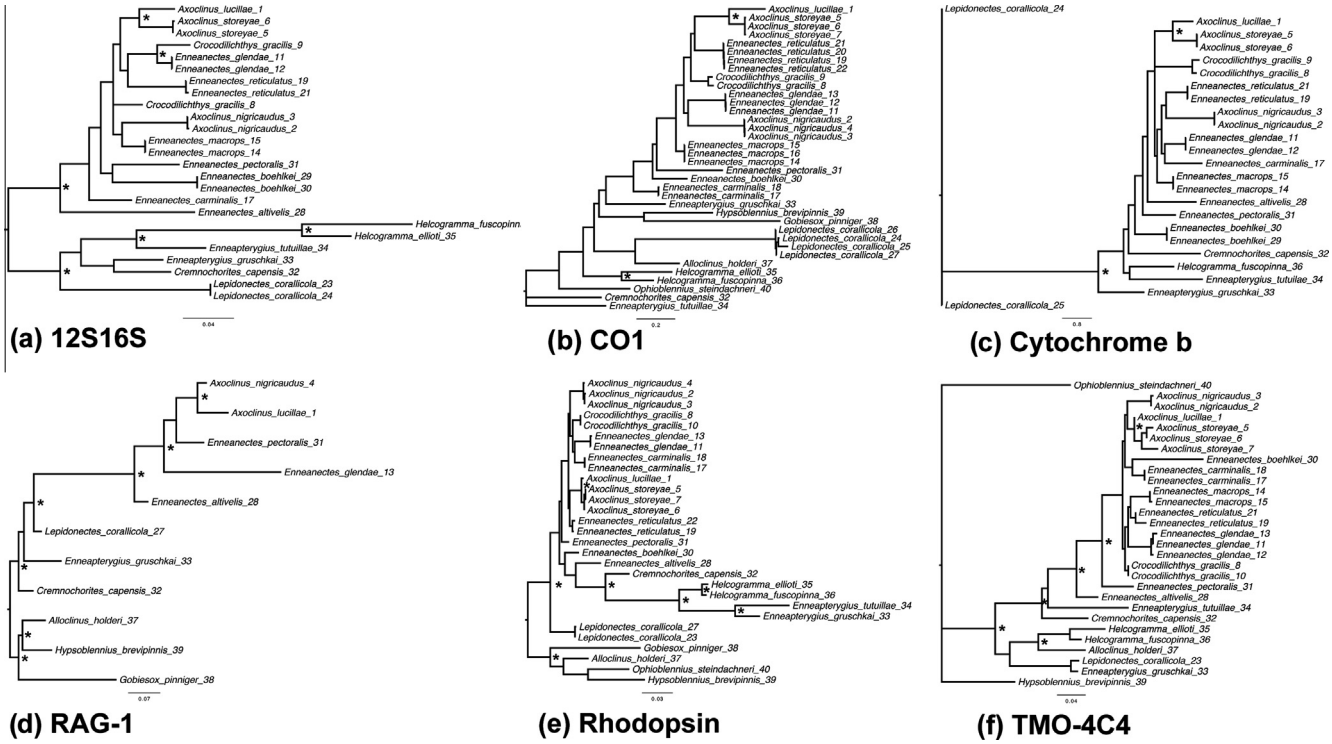

Fig. 2. Individual gene trees created in RAxML: (a) 12S + 16S, (b) CO1, (c) Cytochrome b, (d) RAG-1, (e) Rhodopsin, (f) TMO-4C4. * = node was supported by bootstrap values ⩾70.

3. Results

3.1. Sequencing results

We obtained a total of 4873 base pairs for analysis (2209 from mitochondrial genes and 2664 from nuclear genes, Table 2), expanded from Lin and Hastings (2013) who sequenced 3562 bp (an addition of 1311 bp). Several indels were observed in the TMO-4C4 and RAG-1 alignments. Sequences are deposited in GenBank (Supp. Table 1). Due to the conglomerate nature of our sampling efforts (with some individuals represented in Lin and Hastings, 2013 and some new to this study), many individuals are missing from individual gene partitions (Table 2, Supp. Table 1), with CO1, TMO-4C4, and Rhodopsin with the highest coverage (each with 95% of species and 90%, 75%, and 73% of individuals respectively) and RAG-1 with the lowest coverage (28% of individuals and 52% of species). This is in part also due to the difficulty of amplifying several markers for triplefins compared to other blenniiforms (Lin and Hastings, 2013).

We considered the results of Xia's test of saturation as well as visual plots of transitions versus transversions when deciding to remove saturated codon positions. The 1 + 2 codon positions of TMO-4C4 and Rhodopsin were suggested to be saturated by Xia's test (Supp. Table 4); however, there was no graphical evidence of saturation (graphs of 1 + 2 codon positions not shown) and the results of Xia's test are likely an artifact of the high number of constant sites in these partitions. 12S and the 3rd codon positions of CO1, Cytochrome b, and RAG-1 were potentially saturated based on Xia's test, but only Cytochrome b was considered to be saturated both in cases of symmetrical and asymmetrical trees and by a plateau in the number of transitions versus transversions (Supp. Fig. 1). For these reasons, we only chose to exclude the 3rd codon positions of Cytochrome b due to saturation.

3.2. Support and congruence between phylogenetic analyses

The PartitionFinder results (Supp. Table 2) were largely congruent with each other, each recommending 15 partitions based on codon positions. With all codons included, the second codon positions of RAG-1 and TMO-4C4 were considered a single partition, but they were separated when saturation was removed. 12S and 16S were joined in a single partition for all analyses following these results. Models ranged from simple (F81) to complex (GTR + I + G) indicating rate heterogeneity in the dataset.

Phylogenies based on individual mitochondrial genes were poorly resolved, except with some internal nodes having high support (i.e. bootstrap values $\geqslant$70 in ML trees; Fig. 2). Trees from nuclear genes had a higher number of deeper nodes with high support (Fig. 2). Removing saturation seemed to have mixed effects, with some nodes having stronger support at the expense of other nodes (Fig. 3, Supp. Fig. 2). There are a few notable changes in the relationships recovered, however. The position of *Enneanectes reticulatus* has overall low support, except in the Bayesian tree when saturation is removed (with 99% posterior probability). In addition, the position of *Lepidonectes corallicola* as the sister to the remaining Neotropical triplefins is recovered in both seven-loci concatenated ML trees, but only has high support when saturation is removed. The same position is not recovered in the Bayesian tree except when saturation is removed. In general, removing saturation increased congruence of the Bayesian tree with the ML tree (Fig. 3, Supp. Fig. 2).

Interestingly, although most individual genes had poor resolution, the resulting topology from concatenation had high support for most nodes (Figs. 2 and 3). Some highly supported nodes in the concatenated tree did not have a single gene partition recovery of the same node. However, few conflicting nodes in the gene trees were highly supported (Fig. 2). These highly-supported conflicting nodes suggested paraphyly of *Enneapterygius* and association of *Lepidonectes* with the root of the tree, relationships that were also recovered in the MrBayes trees but not the seven-loci ML trees.

The *BEAST species tree was largely congruent with the concatenated tree within the relationships of the Neotropical triplefin blennies, although with lower support for most nodes (Fig. 4, Supp. Fig. 2). Outgroups and Indo-Pacific triplefins were mixed together as a single clade, with internal nodes having poor support.

3.3. Phylogenetic relationships and systematics

The monophyly of the Tripterygiidae is well supported in all concatenated analyses (Fig. 3, Supp. Fig. 2). The relationships within the Neotropical triplefin blennies were well resolved in the concatenated analyses for the first time in any molecular analysis. We recovered different relationships than previously described (Fig. 1). The most basal members of the genus *Enneanectes* are the Western Atlantic species, which do not form their own clade (Figs. 3 and 4). Within TEP members, the widespread *Enneanectes carminalis* is the most basal, and the genus *Enneanectes* as currently defined is paraphyletic with the inclusion of the monophyletic genus *Axoclinus* and the monotypic *Crocodilichthys*, both of which are endemic to the TEP. The relationships of the Neotropical genus *Lepidonectes* are unclear; in concatenated ML analyses it forms a clade with the rest of the Neotropical genera,

Node	All Codon Positions	Saturation Removed
1	**100/1**	**100/1**
2	**83/1**	**79/1**
3	**83/1**	**80/1**
4	32/0.87	41/**0.99**
5	**73/1**	**72/1**
6	**88/1**	**76**/0.87
7	**82/1**	68/1
8	31/0.67	32/0.88
9	**100/1**	**100/1**
10	**100/1**	**100/1**
11	63/-	71/0.75
12	**100/-**	**100/1**
13	**79/1**	**82/1**
14	**95/-**	**96/-**
15	**75**/-	**80**/-
16	**100/1**	**100/1**
17	**89/0.99**	**89/1**
18	**71**/0.69	**72**/0.8
19	**100/1**	**100/1**

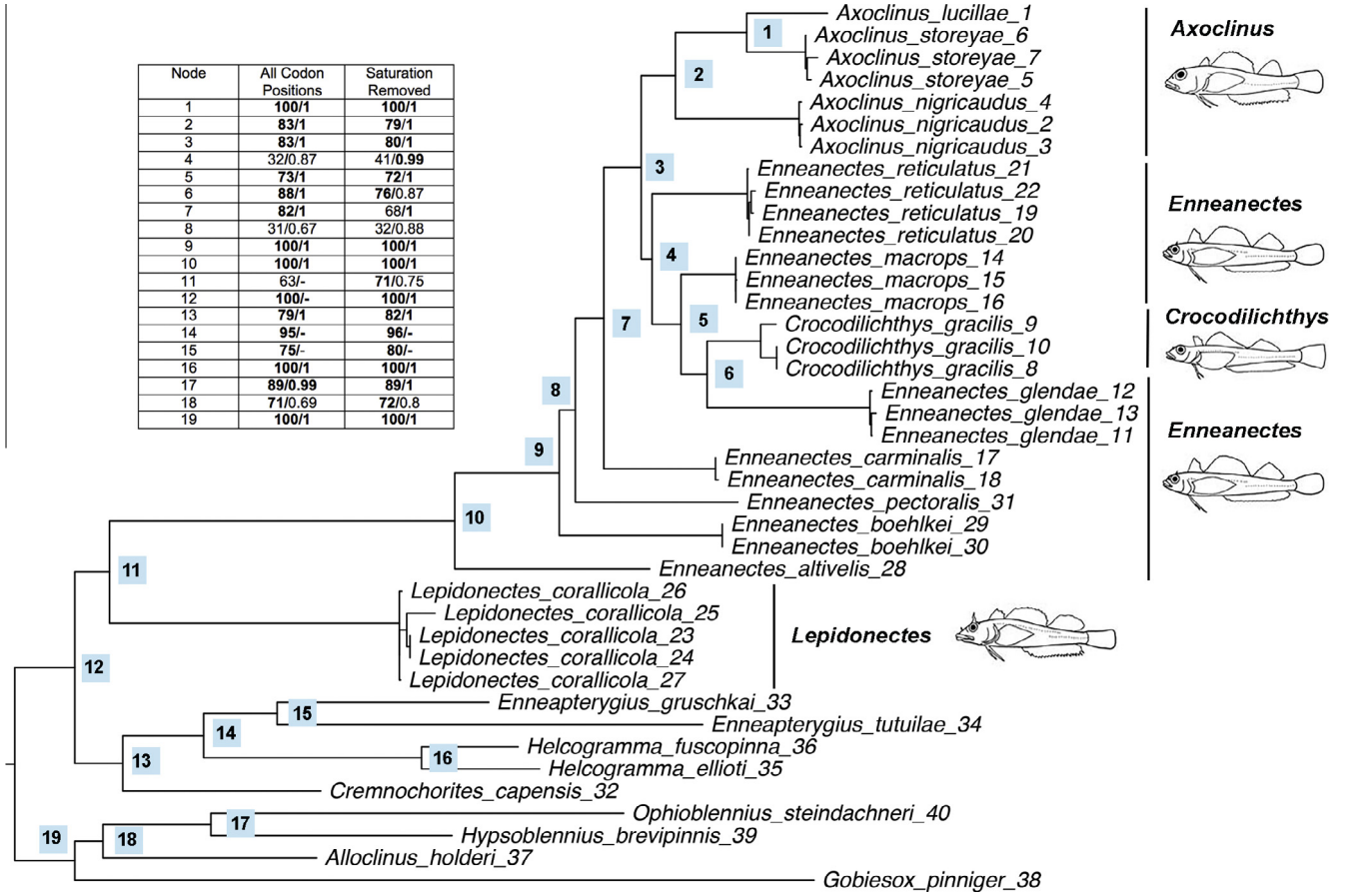

Fig. 3. Maximum likelihood tree of concatenated genes created in RAxML, with saturation removed. Table shows bootstrap support values for: ML RAxML tree/MrBayes Bayesian inference tree. Values in bold are considered highly supported. – = Node not recovered in Bayesian tree. Line drawings were retrieved from the Shorefishes of the Tropical Eastern Pacific Online Information System (Robertson and Allen, 2008).

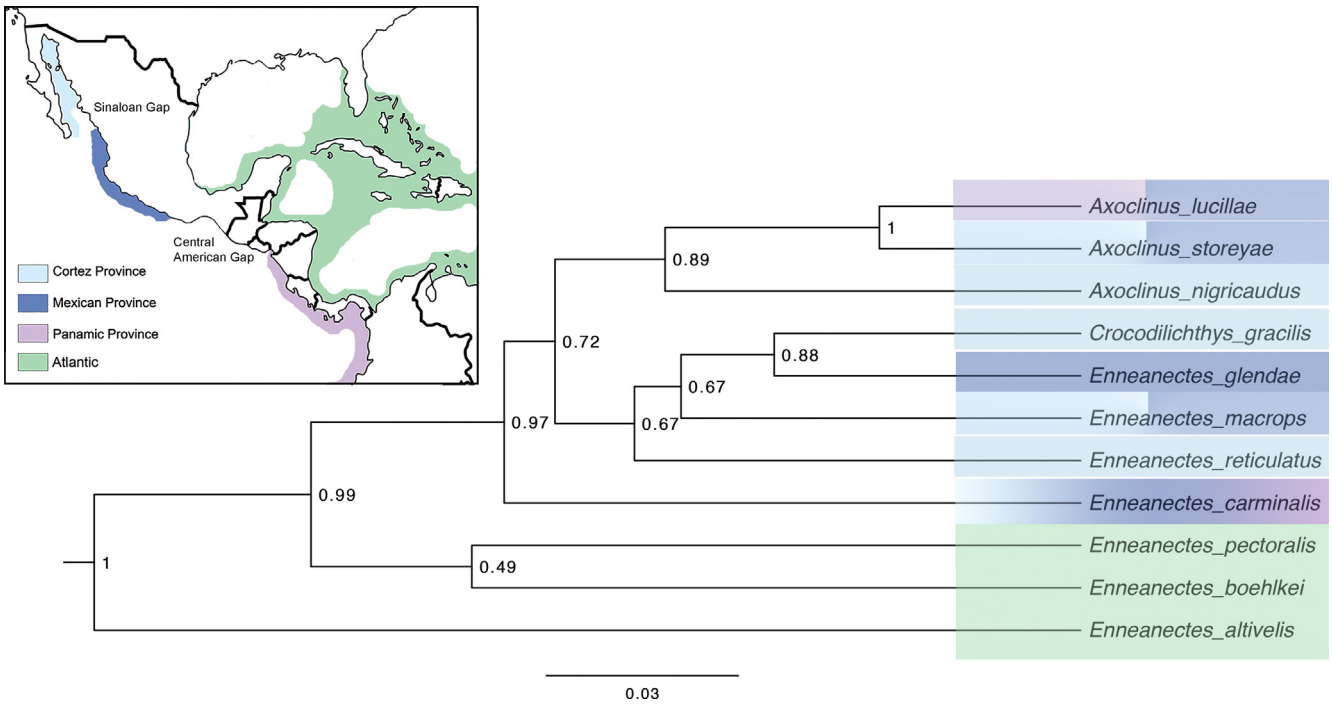

Fig. 4. Range mapped on topology of *BEAST analysis, with map of distribution among TEP provinces (Hastings, 2000). Outgroups, Indo-Pacific triplefins, and *Lepidonectes* are excluded for clarity. Tree with all taxa included can be viewed in Supp. Fig. 2.

but in Bayesian analyses and individual genes it is consistently found near the base of the tree among the Indo-Pacific triplefins (Supp. Fig. 2).

4. Discussion

4.1. Phylogenetic analyses

Mitochondrial loci tended to contribute towards shallow nodes, while nuclear loci contributed to both shallow and deep nodes (Supp. Fig. 2). Traditionally, the greater number of variable sites in mitochondrial loci is thought to swamp the phylogenetic signal from nuclear genes (Eytan, 2010; Pabijan et al., 2012). However, our results are consistent with a recent review of concatenated datasets that did not find support for mitochondrial dominance, instead finding that nuclear-only trees more closely resemble total-evidence trees, and that nuclear genes contribute more to resolution of deeper nodes (Fisher-Reid and Wiens, 2011).

Although individual genes had overall poor resolution and support (Fig. 2), the concatenated trees recovered clades with high node support (Fig. 3, Supp. Fig. 2). This observation is consistent with emergent "hidden support," defined as increased support due to concatenation relative to the additive support of individual partitions (Gatesy et al., 1999). This occurs because common historical signal in individual genes may be locally swamped by idiosyncratic homoplasy, but becomes amplified when genes are concatenated while homoplasy unique to each partition is dispersed (Thompson et al., 2012). Hidden support is a common phenomenon that is a justification of concatenation methods (de Queiroz and Gatesy, 2007; Gatesy and Springer, 2014), and most likely contributed towards our improved resolution compared to Lin and Hastings (2013).

The effects of saturation on phylogenetic reconstruction are not fully understood (Heath et al., 2008). In theory, saturated sites can bias support and topology by introducing homoplasy (Xia and Lemey, 2009); however, in practice removing saturation may have little effect (Arroyave et al., 2013). Although we only removed 262 bp due to potential saturation (5% of all sites), removal resulted in small changes with important systematic implications. In general, some nodes increased support at the cost of other nodes, with no clear trend for preference in the shallow or deep regions of the tree (Fig. 3). However, in the MrBayes analysis support for two contentious placements changed: the posterior probability for the placement of *Enneanectes reticulatus* crossed the threshold from "poor" to "high" support ($\geqslant$95% posterior probability), while *Lepidonectes corallicola* changed position to be congruent with the ML tree (Supp. Fig. 2). For reasons discussed below, the systematic placement of both species is controversial (Fig. 1).

The *BEAST tree was most congruent with the ML and Bayesian concatenated trees within the Neotropical triplefins, but resulted in strange nesting of the Indo-Pacific triplefins and *Lepidonectes* within the outgroups (Fig. 4, for outgroups see Supp. Fig. 2). The most incongruence occurred with species that only had one individual sampled, which may contribute to decreased accuracy of coalescent methods (Heled and Drummond, 2010; Corl and Ellegren, 2013; Hovmöller et al., 2013; Lambert et al., 2015). Our results are also consistent with the empirical observation that estimating a species tree under a coalescent framework is difficult when individual genes have low resolution (Townsend et al., 2011; Pabijan et al., 2012; Gatesy and Springer, 2014). Coalescent approaches may be important for understanding broader relationships of the Tripterygiidae due to rapid speciation in the family (Carreras-Carbonell et al., 2005; Degnan and Rosenberg, 2009; Townsend et al., 2011), and their accuracy should greatly improve with broader taxon sampling, multiple individuals per species included, and additional genes.

4.2. Systematics of the Neotropical Tripterygiidae

We recovered a novel phylogeny for the Neotropical triplefin blennies with high support for most nodes. Our topology is similar to Lin and Hastings (2013), but more closely resembles traditional taxonomy by supporting the monophyly of *Axoclinus* (Fig. 1).

A single clade was recovered containing the genera *Enneanectes*, *Axoclinus*, and *Crocodilichthys* (Fig. 3). Our study is the first to provide molecular support for the monophyly of the genus *Axoclinus*, and is congruent with Lin and Hastings (2013) in recovering a paraphyletic *Enneanectes*. Traditionally, *Axoclinus* and *Enneanectes* were distinguished by the former having a continuous lateral line (Smith and Williams, 2002). Based on our results, the discontinuous lateral line is not a useful diagnostic for the monophyly of *Enneanectes*. This trait appears to be labile among the Tripterygiidae (Rosenblatt, 1959; Fricke, 1994, 1997).

The Western Atlantic *Enneanectes* do not form their own clade, but instead are the most basal members of the genus and are serial sister groups to the TEP *Enneanectes* (Fig. 3). However, they have never been hypothesized to be monophyletic based on morphology (Rosenblatt, 1959). Unlike Lin and Hastings (2013), we recovered *Enneanectes carminalis* (Delicate Triplefin) as sister to the remaining TEP species. *Crocodilichthys gracilis* and *E. glendae* were recovered as sister species with high support across several markers (Figs. 2 and 3). This is consistent with Lin and Hastings (2013), as well as morphological similarities including a long, slender body and high meristic counts (Rosenblatt, 1959; Rosenblatt et al., 2013). Rosenblatt (1959) previously hypothesized *Enneanectes reticulatus* (Reticulated Triplefin) to be closely affiliated with *Enneanectes macrops* (Mexican Triplefin), consistent with our study, although with poor support in the concatenated analyses (Fig. 2). The tendency for *E. reticulatus* to form a clade with *Axoclinus* in several gene trees may reflect introgression, or incomplete lineage sorting, or it may result from incomplete taxon sampling (Fig. 2).

The position of *Lepidonectes* is uncertain based on our analyses. Fricke (1994, 2009) placed *Lepidonectes* and *Cremnochorites* in the tribe Norfolkiini, although these two are not sister taxa in any of our analyses. In the concatenated analyses, *Lepidonectes* is either the sister group to the other Neotropical triplefins (ML trees) or in a clade with the Indo-Pacific species (MrBayes trees, Fig. 3, Supp. Fig. 2). In mitochondrial trees and the *BEAST species tree, *Lepidonectes* is pulled to the base along with the non-triplefin outgroups. Based on very long branches separating *Lepidonectes* from the rest of the tree (Figs. 2 and 3), its tendency to be pulled to the root in some analyses, its change in position when saturated codon positions are removed, and its morphological distinctiveness from other sampled taxa, its varying placement in our analyses is likely due to long branch attraction that may be alleviated with greater taxon sampling (Bergsten, 2005).

In congruence with Lin and Hastings (2013), the Indo-Pacific triplefin species sampled form a clade independent of the Neotropical triplefins (with the possible exception of *Lepidonectes*). We are limited in discussing their relationships due to poor taxon sampling of these species-rich groups.

4.3. Biogeography

Our novel topology allows for reinterpretation of the biogeography of the Neotropical triplefins. As suggested for the Blenniiformes as a whole (Lin and Hastings, 2013), the Indo-Pacific seems to be a source of diversity within the Tripterygiidae, from which the Neotropical species are derived. It is unclear whether there was more than one dispersal event into the Neotropics, due to the ambiguous placement of *Lepidonectes*, which is found in the Galapagos and Panamic provinces.

Within the Neotropics, our relationships provide support for the biogeographic provinces described by Hastings (2000) for other benthic fishes. The barriers most relevant to benthic fishes within the Neotropics include the Isthmus of Panama separating the West Atlantic from the East Pacific, and two gaps in the otherwise continuous rocky shoreline of the TEP, which contain sandy bottom and mangrove habitats unsuitable for triplefins (Hastings, 2000). The most basal relationships within the clade containing *Axoclinus*, *Enneanectes* and *Crocodilichthys* are in the West Atlantic (Fig. 4). There are no putative trans-isthmian geminate pairs in this group, despite the occurrence of several other examples of transisthmian geminates among Neotropical blennies (Hastings, 2009; Eytan et al., 2012). Interestingly, the widespread *E. carminalis* is sister to the remaining Pacific members of this clade. The ranges of these species fall within the three biogeographic provinces discussed by Hastings (2000): the Cortez Province including the Gulf of California and southern Baja California peninsula, the Mexican Province corresponding to Mazatlán, Sinaloa to the Isthmus of Tehuantepec in southern Mexico, and the Panamic Province extending from the Gulf of Fonseca in Nicaragua to the Gulf of Guayaquil, Peru. There are no instances of sister species pairs occupying the same province within the TEP (although this is not true for triplefins in other regions, see Hickey et al., 2009 and Rabone et al., 2015), a pattern also seen in chaenopsid blennies (Hastings, 2000), consistent with an allopatric speciation model. Tissue samples of several TEP triplefins endemic to various oceanic islands were unavailable. Their inclusion in a phylogenetic analysis would help to further clarify the biogeography of eastern Pacific triplefins.

4.4. Conclusion and future directions

In conclusion, we provide a novel topology with improved resolution of the Neotropical triplefin relationships from earlier studies based on molecular (Lin and Hastings, 2013) and morphological evidence (Rosenblatt, 1959; Fricke, 1994, 1997). This improved resolution results from the inclusion of additional mitochondrial loci, new nuclear sequence data for additional individuals per species, and concatenation of gene sequence data. Unlike Lin and Hastings (2013), this study supports the monophyly of the genus *Axoclinus*, and *Enneanectes* is made paraphyletic given that *Axoclinus* and *Crocodilichthys* are nested within. The genus *Axoclinus* is defined by the autapomorphy of a continuous lateral line ending at the level of the third dorsal fin (Smith and Williams, 2002), and *Crocodilichthys* is distinctive by its relatively high meristics and elongate shape compared to members of *Enneanectes* (Allen and Robertson, 1991). However, given the variable morphology and uncertainty of relationships within the species currently allocated to *Enneanectes*, we do not recommend nomenclatural changes at this time. A thorough re-evaluation of the morphology and taxonomy of the Neotropical Tripterygiidae is warranted in the future.

The Tripterygiidae is an ideal group for studying marine speciation and biogeography due to their circumglobal distribution and life history characteristics that limit dispersal. However, a molecular phylogeny with taxon sampling spanning all geographic regions and hypothesized lineages has not been attempted (Fricke, 2009). The limitations to this goal include the inaccessibility of tissues suitable for molecular analysis, and the necessity of broad collaboration to collect these tissues. Key taxa include the genera *Tripterygion* (Mediterranean, 6 species), *Ceratobregma* (Indo-Pacific, 2), *Springerichthys* (Northwest Pacific, 2), and *Enneapterygius* (Indo-Pacific, 53) that Fricke (1994) included in the Tripterygiini along with *Axoclinus*, *Crocodilichthys* and *Enneanectes* (Table 2). Study of the genus *Helcogrammoides* from Peru, Chile, and Antarctica (Williams and Springer, 2001) may be important for elucidating additional possible dispersal events into the eastern Pacific, along with clarifying the affinity of *Lepidonectes*. In addition, due to the power of increasing the number of characters in resolving rapid radiations, genomic approaches will be useful in providing the necessary phylogenetic resolution for triplefin blennies (Jarvis et al., 2014).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.12.003.

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