UNIVERSITY OF CALIFORNIA, SAN DIEGO

A novel phylogeny for the Neotropical triplefin blennies (Teleostei: Tripterygiidae): Contrasting molecules and morphology.

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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This thesis will be submitted for publication. The thesis author was the primary investigator of this material. In addition to P.A. Hastings, H.C. Lin will be listed as a co-author for her contribution of sequences for analysis.

ABSTRACT OF THE THESIS

A novel phylogeny for the Neotropical triplefin blennies (Teleostei: Tripterygiidae): Contrasting molecules and morphology

by

Elizabeth Christina Miller

Master of Science in Biology

University of California, San Diego, 2014

Professor Phil Hastings, Chair

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The triplefin blennies (Teleostei: Tripterygiidae) are a diverse group of cryoptobenthic fishes found globally, though usually associated with rocky or coral reefs. The Neotropics contain four genera and 25 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-gene molecular dataset. The monophyly of *Axoclinus* is well supported, but *Enneanectes* is paraphyletic with the inclusion of *Axoclinus* and *Crocodilichthys*. A morphological dataset was constructed, but did not provide resolution. Apparent sister species appear have disjunct ranges, and allopatric speciation due to oceanographic breaks is a likely driver of speciation in the Tropical Eastern Pacific. Improved taxa sampling within the Neotropical species, in addition to sampling additional genera outside of the Neotropics, is necessary to further improve resolution and confirm the monophyly of the Neotropical triplefins.

INTRODUCTION

The Tripterygiidae are a diverse blennioid family, composed of 29 genera and 171 species (Fishbase). 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 cryptobenthic lifestyle, tripterygiids have been the focal species for studies on marine radiations (Carreras Carbonell et al 2005, Wellenreuther 2007), cryptic speciation (Victor 2013), life history (Longnecker and Langston 2005, Riginos and Victor 2001) and isolation by distance (Riginos and Nachman 2001, Hickey et al 2009).

The Neotropics contain four genera and 25 species of triplefins (Figure 1), many of which have only been described in the past 25 years (Allen and Robertson 1991, Allen and Robertson 1992, Rosenblatt et al 2013, Victor 2013). Of the 25, seven are island endemics. 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, *Enneanectes*, contains 15 species distributed in both the TEP and Western Atlantic. Although their ecological contribution is unclear, tripterygiids comprise a significant portion of the ichthyofauna of rocky reef communities in the TEP, especially in the Gulf of California (Aburto-Oropeza and Balart 2001, Thomson and Giligan 2002, Galland 2013).

Previous phylogenetic 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 Indopacific genera in his unpublished dissertation (Figure 2). Fricke (1994) later revised the family, recognizing eight tribes, and proposed different placements for the Neotropical genera. *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.* Both studies hypothesized each genus to be monophyletic. However, the four Neotropical genera do not form a monophyletic clade in either study, implying multiple colonizations of the Neotropics.

In contrast, Lin (2009) and Lin and Hastings (2013) constructed a 5-gene molecular phylogeny that recovered the four Neotropical genera as monophyletic, with implications of a single colonization event from the Indo-Pacific. 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) given their apparently poor dispersal ability, a consequence of their benthic, territorial adult behavior, demersal eggs, short pelagic larval duration, and near-shore 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 included in Lin and Hastings (2013) are unresolved. Although the four Neotropical genera were recovered as monophyletic

with strong support, most nodes within genera had very low support. Curiously, both *Enneanectes* and *Axoclinus* were paraphyletic, with *E. reticulatus* and *A. nigricaudus* falling out as sister species. They attributed the poor resolution within the Tripterygiidae to poor taxon sampling, missing sequence data, and several indels in sampled nuclear genes (Lin and Hastings 2013). Additionally, there are thought to be several rapid speciation events within the family (Carreras-Carbonell *et al* 2005; Wellenreuther 2007), causing many short branches, which can complicate phylogenetic reconstruction.

This study expands the Lin and Hastings (2013) molecular dataset to more fully resolve the species-level relationships within the four Neotropical genera. The expanded molecular dataset contains seven markers, including new sequence data for three mitochondrial genes. In addition, a morphological dataset was constructed in an attempt to reconcile the disparity between previously published morphology and molecular phylogenies.

Submission for Publication

This thesis will be submitted for publication. The thesis author was the primary investigator of this material. In addition to P.A. Hastings, H.C. Lin will be listed as a co-author for her contribution of sequences for analysis.

MATERIALS AND METHODS

Taxon Sampling

For molecular analysis, tissues from 12 species from four genera were taken from voucher specimens stored in the Scripps Institution of Oceanography Marine Vertebrate Collection (SIO), with the exception of three specimens from the University of Kansas Natural History Museum (KU). *Lepidonectes corallicola* was selected as an outgroup based on Lin and Hastings (2013). Species not included are predominantly island endemics for which tissues were not available. For morphological analysis, *Lepidonectes clarkhubbsi* was substituted for *L. corallicola,* and the additional outgroups *Helcogrammoides cunninghami* and *Helcogramma ellioti* were examined. See Tables 1, 2, and 3 for a description of included taxa.

Molecular Data

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, compared to five total markers in Lin and Hastings 2013 (CO1, Rag1, Rhodopsin, TMO-4C4, and Histone H3). New sequences from CO1, Rhodopsin, and TMO-4C4 were obtained and compared with sequences from Lin and Hastings (2013) to confirm identity. The following sequence data from Lin and Hastings (2013) were included in this dataset: Rag1 (HQ168761-68) and TMO-4C4 (HQ169008, 9, and 12). Histone H3 sequences were not included because the marker seems to evolve more slowly than the others and were not informative (Lin and Hastings 2011, 2013).

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 7 markers are described in Table 4. 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).

Morphological Data

Whole specimens were examined and observations were made on meristics, osteology, sensory pores, and squamation (Table 5). Most characters were chosen *a priori* based on previous studies on triplefins (Fricke 1994, Rosenblatt 1959, Wellenreuther 2010, Jawad 2005, 2008). To assist examination of osteology, specimens were cleared in aqueous 1% KOH and trypsin, and stained using alcian blue and alizarin red following methods of Dingerkus and Uhler (1997) and Taylor and Van Dyke (1985). Sensory pores were examined by drying specimens with a small air pump.

Phylogenetic Analysis

Sequences were assembled and edited in Sequencher 5.2 (Gene Codes, Ann Arbor, MI), and imported into Mesquite 2.75 (Maddison and Maddison 2011), where they were aligned using MUSCLE (Edgar 2004). Alignments were checked by eye. For

the protein-coding markers (all but 12S and 16S), codon position was assigned by minimizing stop codons, and translating sequences to ensure that no stop codons were present.

Due to the documented high rates of mtDNA evolution in blennies (Eytan 2010), each gene was tested for saturation, with protein-coding genes separated by 1+2 and 3rd codon positions in DAMBE 5 (Xia 2013). The number of transitions and transversions versus genetic distance was plotted to look for plateaus in accumulated changes. The Xia test for saturation (Xia 2003) was implemented as well, providing two measures to determine saturation.

To compare the impact of partitioning scheme on topology and support (Dornburg 2008), the seven-gene concatenated dataset was analyzed according to two partitioning schemes: by gene (seven partitions) and according to the best-fit scheme selected by PartitionFinder (Lanfear 2012). The best-fit model of evolution for individual markers was determined in JModelTest 2 using the AIC criterion (Posada 2008). The best-fit partitioning scheme and appropriate substitution models for the entire dataset and individual codon positions were determined in PartitionFinder using the AIC criterion and the "greedy" heuristic algorithm (Table 6). A second PartitionFinder analysis was run with the number of models restrained to only those recognized by MrBayes (Ronquist and Huelsenbeck 2003).

Individual gene trees were analyzed separately, as well as seven concatenated datasets: mtDNA (four markers), mtDNA with saturated sited removed, nuDNA (three markers), and all seven markers partitioned either by gene or via PartitionFinder, with or without saturated sites. Bayesian Metropolis coupled Markov chain Monte Carlo

(MCMC) analyses were conducted in Mr. Bayes v. 3.2.2 (Ronquist and Huelsenbeck 2003). Bayesian inference was performed with four 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. Convergence of runs was confirmed based on plots of lnL scores versus generation time, as well as ESS values, visualized in Tracer (Drummond and Rambaut 2007). Maximum likelihood analyses (Felsenstein 1981) were conducted in the program RAxML v7.4.2 (Stamatakis, 2006) via raxmlGUI v1.3 (Silvestro and Michalak, 2012), using the thorough bootstrapping algorithm and 100 replicates. The model GTR+GAMMA was assigned to all partitions. Maximum parsimony analyses were conducted in PAUP 4.0b10 (Swofford 2003) using 1000 heuristic search replicates with random addition, and jackknife node support with 10 additional replicates.

Morphological characters were scored assigned binomial or numerical values. The character matrix was constructed in Mesquite. Phylogenetic inference was conducted in PAUP and Mr. Bayes under the same conditions, with the exception of sampling every 100 generations in the Bayesian analysis.

Submission for Publication

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RESULTS

Molecular Data

Sequence data from seven markers totaling 4198 bp were obtained and analyzed (Table 7). The number of parsimony-informative sites was 776 (19%)for the total dataset with all codons, and 376 (10%) when saturated sites were removed. Nodes with bootstrap values above 70 and posterior probabilities above 95% were considered well-supported.

The 3rd codon positions of both CO1 and Cytochrome b were saturated as demonstrated both graphically and statistically (Table 8, Figure 3). The plot of transitions vs. transversions for Rag1 also plateaued; however, saturation was rejected according to Xia's test. Therefore, the 3rd codon positions of CO1 and Cytochrome B were removed in three of the datasets, but all codon positions of Rag1 were included. After removing saturated sites, both CO1 and Cytochrome b had a very low number of parsimony-informative sites (11 and 26 respectively).

Each individual gene tree had poor resolution overall, and few nodes were supported until genes were concatenated (Figures 4, 5, and 6). The ideal partitioning scheme determined by PartitionFinder combined 12S and 16S into one partition, and partitioned the remaining markers by codon position for a total of 16 partitions (Table 6). Models assigned to individual codon positions ranged from simple (F81+G) to complex (GTR+I+G), revealing great heterogeneity in substitution rates within the dataset.

Mitochondrial and Nuclear Trees

Overall, any conflicts between the mitochondrial and nuclear trees, and the mtDNA and nuDNA trees with the total seven-gene trees had low node support. Node

support was low overall for both mtDNA datasets (Figures 7 and 8). As expected, removing saturation resulted in much shorter branch lengths in the ML tree. The mtDNA trees recovered the three Atlantic species as a clade including *E. carminalis*, a result not seen in the other datasets. Instead of *E. carminalis, A. nigricaudus* and *E. reticulatus* were the outgroups to the remaining species. The same (*E. macrops (E. glendae-C. gracilis*)) clade as the total seven-gene dataset was recovered with fair support, with *A. lucillae* and *A. storeyae* as a sister clade.

There was overall strong congruence between ML, parsimony, and Bayesian nuclear trees (Figure 9). The ML and Bayesian nuclear trees recovered the same placement of *E. carminalis* and the Atlantic species as the total seven-gene dataset. *Axoclinus* is recovered as monophyletic with high support. The only deviation from the seven-gene topology is in the placement of *E. macrops* and *E. reticulatus,* with low support.

Effects of Saturation and Partitioning

The mtDNA dataset with saturated sites included (saturated sites comprised about 20% of this dataset) recovered a similar topology as the dataset without saturation, with the exception of *E. pectoralis* and *E. boehlkei* changing positions (Figures 7 and 8). Removing saturation resulted in greater congruence between ML, parsimony, and Bayesian trees. Bootstrap support was comparable in both mtDNA ML trees, but removing saturation improved bootstrap support in the Bayesian trees (not shown).

Both removing saturation and increasing partitioning of the total seven-gene dataset had minor effects on node support and topology (Figures 10-14). Partitioning by

codon did improve support for basal nodes in the ML trees, but the impact on shallower nodes was ambiguous. The only changes in topology between these four datasets concerned placements with low support in all trees. For the total dataset partitioned by gene, removing saturation resulted in comparable node support in both ML and Bayesian trees as the dataset with saturated sites. Within each dataset, ML and Bayes trees recovered the same topology except with *E. reticulatus* in a polytomy at the base of the remaining Pacific species. The dataset with saturation recovered *E. boehlkei* and *E. pectoralis* as sister species, while removing saturation produced no sister species pairs among the three Atlantic species, all with low support. . Removing saturation had similar effects in the total dataset partitioned by codon. In both, *E. boehlkei* and *E. pectoralis* were sister species. Node support was slightly higher for shallower nodes when saturation was left in and slightly lower when it was removed. The reverse was true for deeper nodes.

The only difference in topology between the total dataset partitioned by gene and partitioned by codon was that in the ML trees, *E. reticulatus* was the sister species to *Axoclinus* in the dataset partitioned by codon. In all Bayesian trees, it was placed in a polytomy with *Axoclinus* and the clade containing *E. macrops, E. glendae,* and *C. gracilis*.

Morphological Data

The morphological dataset included 41 parsimony-informative characters and two autapomorphies (Tables 5 and 10). The following characters were constant for all taxa and were excluded from the analysis: two elements supported on last anal fin

pterygiophore (Fishbase), autogenous bony stay present on last anal fin pterygiophore (Fishbase), scapula and cleithrum in close proximity (Jawad 2008), pelvic girdle without sutured halves (Jawad 2008), and two anal-fin spines (Rosenblatt 1959).

According to the maximum parsimony reconstruction, *Axoclinus* and *Helcogramma* were distinct from the remaining genera (Figure 16). Characters supporting this are related to the lateral line position and squamation (13, 19, 21, 24, 31), lack of scales on the anal and first dorsal fin base (17, 18) and lack of branched rays in the 3rd dorsal fin (32; see Table 5). Unfortunately, the dataset was unable to resolve relationships any further than this. The Bayesian analysis did not recover any resolution at all, and is not shown.

Phylogenetic relationships

According to the seven-gene concatenated datasets, *Axoclinus* is a monophyletic genus, with *A. storeyae* and *A. lucillae* as sister species with high support from almost every gene (Figures 6, 10-14). *Enneanectes* is paraphyletic with the inclusion of *Axoclinus* and *Crocodilichthys.* After *Lepidonectes, E. carminalis* is the outgroup to the remaining species. The three Atlantic species are not monophyletic; *E. boehlkei* and *E. pectoralis* are sister species, but with low support. The remaining seven Pacific species form a clade with high support. Within this clade, *E. glendae* and *C. gracilis* are sister species, with *E. macrops* as a sister group to them. Finally, the placement of *E. reticulatus* within this clade is unresolved.

Submission for Publication

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DISCUSSION

Phylogenetic relationships

This study proposes a novel topology for the Neotropical tripterygiids. Lin and Hastings (2013) reported *A. nigricaudus* and *E. reticulatus* as sister species, making both *Axoclinus* and *Enneanectes* paraphyletic (Figure 2). The present study is consistent with previous morphological phylogenies (Rosenblatt 1959, Fricke 1994) in recovering a monophyletic *Axoclinus,* but like Lin and Hastings (2013), did not support the monophyly of *Enneanectes*. While *Axoclinus* is well defined morphologically, sharing the synapomorphies of a continuous lateral line and blunt head shape, members of *Enneanectes* are more varied. *Crocodilichthys gracilis* and *E. glendae* were recovered as sister species with high support across several markers (Figure 6). This is consistent with Lin and Hastings (2013), as well as morphological similarities including a long, slender body and high meristic counts (Rosenblatt 2013).

In both this study and previous phylogenetic reconstructions (Rosenblatt 1959, Lin and Hastings 2013), the Atlantic species are not monophyletic but they are not closely related to any eastern Pacific species. These Caribbean species are found primarily on coral reefs (Rosenblatt 1960), while TEP triplefins are associated with rocky reefs (Hastings 2009). This pattern is similar to other groups of blennioids that include coral specialists. For example, the chaenopsid genus *Emblemariopsis* is a coral specialist and restricted to the Caribbean basin. However, relationships among the Atlantic species of *Enneanectes* are unresolved. This is most likely due to poor sampling, with only 3 of 10 Atlantic *Enneanectes* sampled (Victor 2013).

The placement of *E. reticulatus* is unresolved in this study. In the Bayesian analyses, it is placed either as the outgroup to *Axoclinus* or as the sister species of *E. macrops* (Figure 13). The latter result is consistent with its placement by Rosenblatt (1959) based on morphological similarity. Interestingly, the mtDNA data set recovers *E. reticulatus* and *A. nigricaudus* as outgroups to all remaining *Enneanectes* species (Figures 7 and 8). This is possibly due to the rapid evolution of mitochondrial markers, resulting in long branch attraction. A likely solution is to improve taxon sampling in this group to break up these long branches.

Clade support

It is interesting that although each individual gene tree had overall low support, support for most nodes was high when genes were concatenated (Figures 4, 5, and 6). This is especially true for basal nodes, many of which were collapsed into polytomies in individual gene trees. Low basal node support is indicative of rapid radiation early in this group's history. Rapid radiation events have been proposed for other triplefin clades (Carreras-Carbonell et al. 2005, Wellenreuther 2007), as well as other blennioids (Lin and Hastings 2011, Eytan 2010). In addition, this result may be an example of "hidden support," where individual genes actually contain consistent support for nodes, but due to a low number of parsimony-informative sites, support is not apparent until all genes are concatenated (Townsend 2011). In this case, concatenation is an appropriate and effective method for phylogenetic reconstruction, especially when attempting to resolve relationships by increasing the number of loci.

Removing saturated sites had a stronger impact on the mtDNA dataset, for which these sites comprise about 20% of base pairs (Figures 5 and 6). However, removing saturation had minor effects on the total concatenated set, where saturated sites are only 10% of base pairs. Interestingly, including 3rd codon positions may have improved support for some nodes (Figures 7-14). This may be because these codon positions may indeed include some sites with phylogenetic signal, and the inclusion of nuclear genes may overwhelm conflicting signal from the saturated sites.

The effects of partitioning a dataset in phylogenetic analyses is controversial; some argue that over-parameterizing can increase error (Burnham and Anderson 2003), while empirically the impacts may actually be slight (Dornburg 2008). The PartitionFinder analysis revealed a large degree of heterogeneity in codon substitution sites within the molecular data (Table 6) that is not taken into account when models are applied to whole genes. In practice, partitioning by codon position improved the support for basal nodes in the ML trees. However, there did not seem to be an effect on shallower nodes due to partition scheme. The fact that neither removing substitution nor changing partitioning scheme had strong effects on topology (except for poorly supported relationships) indicates that portions of the topology recovered in this study are robust.

Morphology

Traditionally, as with other groups, phylogenetic relationships in tripterygiids were inferred exclusively from morphology, and without the use of algorithms that are widespread in modern phylogenetic analyses (Figure 2). Despite including several morphological characters that Rosenblatt (1959) used to distinguish species-level

relationships within the Neotropical species, the morphological dataset in this study provided almost no resolution. This is most likely due to the small number of parsimony-informative characters, but may also reflect limited taxon sampling and a high degree of homoplasy. Rosenblatt's species-level relationships within *Enneanectes* and *Axoclinus* are exclusively based on external characteristics such as squamation and spination patterns (1959). He hypothesized that there was a great degree of convergence in these characters, which he thought had complicated phylogenetic inference in his study (1959). The Neotropical triplefins appear to have less variation in osteological characters as some New Zealand tripterygiid species (Jawad 2008), providing relatively few osteological characters for analysis.

Despite the inability of morphological characters to provide resolution in this study, some morphological characters reported here provide support for the clades recovered by molecular data (Table 5). In congruence with previous studies (Rosenblatt 1959, Fricke 1994), the monophyly of *Axoclinus* is supported by the presence of 1 upper unbranched pectoral-fin ray unattached to the scapula (3, 33), broad shape of the 4th pterygiophore (6), and a continuous lateral line (13) with position and squamation distinct from that of *Enneanectes* (19, 21, 24, 31). Some of these characters are shared with *Helcogramma ellioti,* which resulted in their placement in a clade in the parsimony trees (Figure 16) as well as in Rosenblatt's phylogeny (Figure 2). The monophyletic Pacific clade containing *Axoclinus, Crocodilichthys,* and some members of *Enneanectes* is supported by the thin shape of the coracoid (4).

Sister-species relationships reported by molecules have ambiguous support from the morphological dataset; for example, morphology supports the sister species pair *A.*

storeyae and *A. nigricaudus* instead of *A.storeyae* and *A. lucillae,* despite high support from gene trees (Figures 6 and 16). This may be due to homoplasy of characters associated with squamation and supination (Rosenblatt 1959).

Biogeography

According to the topology recovered with the seven-gene dataset, the Pacific species do not form a monophyletic group. Instead, *E. carminalis* is the sister group of all remaining species. The so-called "Delicate Triplefin" has the broadest distribution of any TEP triplefin, occurring from the Gulf of California to Panama. It and the similar island endemic *E. exsul* are distinctive among the region's triplefins in having short, robust bodies (Rosenblatt et al. 2013). The Atlantic species of *Enneanectes* included in this study also do not form a monophyletic group. Several additional species, including cryptic species (Victor 2013), are known from the western Atlantic but were unavailable for this study. The remaining Pacific species included in this study, currently allocated among three genera (*Axoclinus*, *Enneanectes* and *Crocodilichthys*), form a monophyletic group, but increased taxon sampling is necessary to verify their monophyly.

Given that most blennies, including triplefins, are poor dispersers with short larval phases (Lin and Hastings 2011, Riginos and Victor 2001), allopatric speciation caused by oceanographic or vicariant barriers are often a driving force in their diversification (Hastings 2000, 2009). The ranges of the Pacific species in this study appear to 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. Interestingly no transisthmian geminate species pairs were recovered in this study, despite the occurrence of several other examples of transisthmian geminates among Neotropical blennioids (Hastings 2009). Within the Pacific species, the sister species pair of *C. gracilis* and *E. glendae*, are allopatric, separated by the Sinaloan Gap, a region of coastline largely devoid of rocky reefs (Hastings, 2000). A second species pair, *A. storeyae* and *A. lucillae*, occur allopatrically in the Cortez and Panamic provinces, respectively, but their ranges overlap in the intervening Mexican province. 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.

Concluding Remarks

This study supports the monophyly of the genus *Axoclinus,* but not of *Enneanectes*. Given the uncertainty of relationships within the species currently allocated to *Enneanectes*, we do not recommend nomenclatural changes at this time. In addition, the monophyly of this entire group of Neotropical triplefins cannot be adequately tested without more thorough taxon sampling, despite the fact that triplefins are limited in their dispersal. Key taxa include the genera *Tripterygion* (6 species), *Ceratobregma* (2), *Springerichthys* (2), and *Enneapterygius* (53) that Fricke (1994) included in the Tripterygiini along with *Axoclinus*, *Crocodilichthys* and *Enneanectes* (Table 2). Given the morphological variation among the species currently included in *Enneanectes,* as well as the paraphyly revealed in this study, it is possible that the

included species have affinities with different lineages of triplefins from other regions and that these Neotropical triplefins represent multiple invasions from other parts of the world.

Submission for Publication

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TABLES

Table 1: Summary of species sampled

Species	Catalogue # of Exemplar Individual	Locality of Exemplar Individual	Range (FishBase and STRI Shorefishes Database)
Lepidonectes corallicola	SIO 02-82	Isla Pinzon, Ecuador	Pacific: Galapagos Islands
Enneanectes carminalis	SIO 01-170	Golfo de Fonseca, El Salvador	Pacific: Central Baja to Gulf of California to Panama
Enneanectes boehlkei	KU 166	Carrie Bow Cay, Belize	Atlantic: Florida, Bahamas, Yucatan Peninsula, to Venezuela
Enneanectes pectoralis	KU 167	Carrie Bow Cay, Belize	Atlantic: Florida, Bahamas, Yucatan Peninsula to Venezuela
Enneanectes altivelis	KU 225	Carrie Bow Cay, Belize	Atlantic: Florida, Bahamas to Brazil
Enneanectes reticulatus	SIO 11-355	Isla Espiritu Santo, Mexico	Pacific: Lower Gulf of California
Enneanectes glendae	SIO 01-182	Puerto Vallarta, Mexico	Pacific: Central Mexico and surrounding islands
Enneanectes macrops	SIO 01-182	Puerto Vallarta, Mexico	Pacific: Gulf of California, Central and southern Mexico
Axoclinus nigricaudus	SIO 07-50	Punta Chivato, Mexico	Pacific: Western and northern Gulf of California
Axoclinus storeyae	SIO 09-267	Isla Tortuga, Mexico	Pacific: Southern Baja California and central Gulf of California to central Mexico
Axoclinus lucillae	SIO 01-164	Isla Taboquilla, Panama	Central Mexico to Colombia
Crocodilichthys gracilis	SIO 06-55	Bahia de Los Angeles, Mexico	Gulf of California

Table 2: Distribution of species sampling effort.

Genus	# Sampled/Total Described (%)	Pacific: # Sampled/Total	Atlantic: # Sampled/Total
Lepidonectes	1/3 (33%)	1/1	0
Enneanectes	7/15 (47%)	4/5	3/10
Axoclinus	3/6 (50%)	3/6	0
Crocodilichthys	1/1 (100%)	1/1	0

Table 3: Additional material examined for morphological analysis

Species	Collection Number	Locality
Crocodilichthys gracilis	SIO 09-264	Isla Espirutu Santo, Southern Gulf of California, Mexico
	SIO 65-323	Isla Carmen, Central Gulf of California, Mexico
	SIO 08-120	Bahia de los Angeles, Central Gulf of California, Mexico
Axoclinus nigricaudus	SIO 61-265	Isla Espirutu Santo, Southern Gulf of California, Mexico
	SIO 65-330	Isla Ildefonso, Central Gulf of California, Mexico
Axoclinus storeyae	SIO 04-145	Isla Dazante, Central Gulf of California, Mexico
Axoclinus lucillae	SIO 67-36 SIO 67-38-61	Pearl Islands, Panama
Enneanectes reticulatus	SIO 65-331	Isla Ildefonso, Central Gulf of California, Mexico
	SIO 65-261-61	Isla San Jose, Southern Gulf of California, Mexico
Enneanectes carminalis	SIO 62-56	Tres Marias Islands, Mexico
Enneanectes glendae	SIO 70-155	Zihuatanejo, Mexico
	SIO 70-163	Bahia Navidad, Mexico
Enneanectes macrops	SIO 70-155 SIO 11-399	Zihuatanejo, Mexico Isla Venado, Mexico
Enneanectes altivelis	LACM 6727-9	Lagoen, Curacao, Netherland Antilles
	LACM 8938-17	Montego Bay, Jamaica
Enneanectes boehlkei	SIO 06-64 LACM 54087	Navassa Island, Caribbean, U.S.A.
Enneanectes pectoralis	LACM 6162 LACM 6160 LACM 5943	Lime Cay, Jamaica Pedro Cays, Jamaica Runaway Bay, Jamaica
Lepidonectes clarkhubbsi	SIO 67-39	Pearl Islands, Panama
Helcogrammoides cunninghami	SIO 65-678	La Ventana, Valparaiso, Chile
Helcogramma ellioti	CAS 217490	Tiapea Point, American Samoa

Table 4: Summary of genes and primers.

Gene	Primer Set	Primer Sequences	Primer Reference	Model by JModelTest
12S	12SFor 12S Rev	AACTGGGATTAGATACCCCAC GGGAGAGTGACGGGCGGTGTG	Almada et al 2005	GTR+I+G
16S	16SAR 16SBR	CGCCTGTTTATCAAAAACAT CCGGTCTGAACTCAGATCACGT	Palumbi et al., 1991	GTR+I
CO1	Fish-F1 Fish-R2	TCAACCAACCACAAAGACATTGGCAC ACTTCAGGGTGACCGAAGAATCAGAA	Ward et al., 2005	GTR+I+G (1+2 codons only: TrN+I)
Cytochr-ome b	ExoCBFwd ExoCBRev	GGACTTATGAYTTGAAAAACCATCGTTG AACCTTCGACGTTCGGCTTACAAG GCCG	Sevilla et al., 2007	GTR+I+G (1+2 codons only: TrN+I+G)
Rhodopsin	RhodF RhodR Rhod-BleF Rhod-BleR	CCGTCATGGGCGCCTAYATGTTYYT CAGCACAGGGTGGTGATCATRCARTG CGTCACCCTCGAACACAAGAA GTTGTAGATGGAGGAACTCTT	Taylor & Hellberg, 2005; Lin and Hastings 2013	TrN+I
Rag-1	Rag-F1 Rag-R2	AGCAGGCTCATCCTGTCCAT GGGTGATGGAGTGCAGCACCATGTT	Lin and Hastings 2011	GTR+G
TMO-4C4	TMO-F2 TMO-R2 TMO-F3 TMO-R3 TMO-F4 TMO-R4	GAKTGTTTGAAAATGACTCGCTA AAACATCYAAMGATATGATCATGC GTGAAGTGGTTCTGCAA GTGTACTCNGGRATRGT GGTGAAGTGGTTCTGCAAC GCYGTGTACTCNGGRATRGT	Near et al. 2004, Lin and Hastings 2011, Lin and Hastings 2013	GTR+I+G

Table 5: List of morphological characters.

Number	Description	Character States	PI?	Reference
1	# Epurals	2 or 3	No	Fricke 1994
2	Palatine teeth	Present (1) or absent (0)	Yes	Fishbase
3	Dorsal-most pectoral rays free from scapula	0, 1, or 2	Yes	Jawad 2008
4	Coracoid shape	Thin (1) or squat (0)	Yes	Jawad 2008
5	Pterygiophore formula	Variations 1, 2, and 3*	No	Jawad 2008
6	Shape of 4th dorsal pterygiophore	Broad (1), moderate (2), thin (3)	Yes	This study
7	Overall size of head pores	Small (1), moderate (2), large (3)**	Yes	This study
8	Overall number of head pores	Many (1), Moderate (2), Few (3)	Yes	This study
9	Paired on mandibular	2+ pairs (1), one pair (2), none (3)	Yes	This study
10	Paired on preopercle	Yes (1) or no (0)	Yes	This study
11	Paired on subocular	Yes (1) or no (0)	Yes	This study
12	Mandibular pit	Present (1) or absent (0)	Yes	This study
13	Lateral line	Continuous (1) or discontinuous (0)	Yes	Rosenblatt 1959
14	Belly scalation	Present (1) or absent (0)	Yes	Rosenblatt 1959, Fricke 1994
15	Operculum scalation	Present (1) or absent (0)	Yes	Rosenblatt 1959, Jawad 2005
16	Pectoral fin base scalation	Present (1) or absent (0)	Yes	Rosenblatt 1959, Jawad 2005
17	First dorsal base scalation	Present (1) or absent (0)	Yes	Rosenblatt 1959, Jawad 2005
18	Anal fin base scalation	Present (1) or absent (0)	Yes	Rosenblatt 1959, Jawad 2005
19	# Scale rows above anterior lateral line	3, 4, 5 or more	Yes	Jawad 2005
20	Scale cteni	Present (1) or absent (0)	Yes	Jawad 2005
21	Shape of pored lateral line scales	Rounded (1) or angular (0)	Yes	Jawad 2005
22	Shape of lateral line canals	Straight (1) or constricted (0)	Yes	Jawad 2005
23	Length of lateral line canals	Long (1) or short (0)	Yes	Jawad 2005
24	Location of lateral line canal	Center (1) or angled (0) on scale	Yes	This study
25	Scales above lateral line larger than lateral line scales	Yes (1) or no (0)	Yes	Rosenblatt 1959

Table 5, continued.

Number	Description	Character States	PI?	Reference
26	Scales on cheek	Present (1) or absent (0)	Yes	Rosenblatt 1959
27	Spinules on nasal bone	Present (1) or absent (0)	Yes	Rosenblatt 1959
28	Spinules on anterior orbital margin	Present (1) or absent (0)	Yes	Rosenblatt 1959
29	Spinules on top of head	Present (1) or absent (0)	Yes	Rosenblatt 1959
30	Spinules on supraorbital margin	Present (1) or absent (0)	Yes	Rosenblatt 1959
31	Lateral line location	Middle (1) or upper half (0) of body	Yes	Rosenblatt 1959
32	Branched rays in 3rd dorsal	Present (1) or absent (0)	Yes	Rosenblatt 1959
33	Upper unbranched pectoral rays	1, 2, or 3+ (3)	Yes	Rosenblatt 1959
34	Membrane connecting spinous dorsals	Present (1) or absent (0)	Yes	Rosenblatt 1959
35	Pelvic rays united by membrane	Yes (1), very small (2), no (0)	Yes	Rosenblatt 1959
36	Supraorbital cirrus	None (0), Simple (1), Flaplike (2), Frilled (3)	Yes	Rosenblatt 1959
37	Head shape	Rounded (0), Pointed (1), Strongly pointed (2)	Yes	Fricke 1994
38	Edge of cheek with spinules	Present (1) or absent (0)	Yes	Rosenblatt 1959
39	First spine of first dorsal with spinules	Present (1) or absent (0)	Yes	Rosenblatt 1959
40	Second dorsal spines***	Low (0), moderate (1), high (2)	Yes	This study
41	Pectoral fin rays***	Low (0), moderate (1), high (2)	Yes	This study
42	Anal fin rays***	Low (0), moderate (1), high (2)	Yes	This study
43	Third dorsal rays***	Low (0), moderate (1), high (2)	Yes	This study

* Pterygiophore variations: 3-N-0-0-1-1-1 (1); 3-N-0-1-1-1-1 (2); 3-N-0-1-0-1-1 (3)
** If variable in size from moderate to large, scored as moderate
*** Meristics scored as follows:

	Anal fin rays	Second Dorsal Spines	Third Dorsal Rays	Pectoral Fin Rays
Low	14-16	11	7 to 8	14
Moderate	17-18	12 to 13	9	15
High	19+	14+	10+	16+

Note: If variation was recorded, scored as moderate if range values included those in the “moderate” score.

Table 6: Best partition scheme and models determined by PartitionFinder.
(All Models: Scheme AIC= 23172.66; lnL= -15944.33;

MrBayes Models: Scheme AIC= 32192.08; lnL= -15941.04)

Partition #	Partitions	Best Model	Best Model (MrBayes)
1	12S, 16S	GTR+I+G	GTR+I+G
2	CO1_1	GTR+G	GTR+G
3	CO1_2	F81+I	F81+I
4	CO1_3	TrN+I+G	GTR+I+G
5	CytB_1	TrNef+I+G	SYM+I+G
6	CytB_2	TVM	GTR
7	CytB_3	TIM+I+G	GTR+I+G
8	Rag1_1	TrN+G	F81+G
9	Rag1_2	TVMef+G	K80+G
10	Rag1_3	HKY	HKY
11	Rhod_1	TrN+I+G	GTR+I+G
12	Rhod_2	K81uf+I	HKY+I
13	Rhod_3	TVM+G	GTR+G
14	TMO_1	GTR+G	GTR+G
15	TMO_2	TrN+G	GTR+G
16	TMO_3	K81uf+G	GTR+G

Table 7: Summary of sites sampled.

Gene	Codon Position	Size (bp)	Constant Sites	P-I Sites
12S	Non-coding	368	43	43
16S	Non-coding	549	62	62
CO1	1	190	162	11
	2	190	190	0
	1+2	380	352	11
	3	189	11	151
	All	569	363	162
Cytochrome b	1	283	229	25
	2	283	272	1
	1+2	566	501	26
	3	284	6	249
	All	850	507	275
Rag 1	1	256	207	28
	2	257	257	12
	3	256	104	119
	All	769	515	159
Rhodopsin	1	243	230	6
	2	243	239	3
	3	243	173	30
	All	729	642	39
TMO-4C4	1	122	110	4
	2	121	112	2
	3	121	46	30
	All	364	268	36

Table 8: Summary of Xia's test of saturation in DAMBE. Only those positions that had evidence of saturation are shown.

Gene	Iss	Iss.c (symmetrical tree)	P-value (symm. Tree)	Iss.c (asymmetrical tree)	P-value (asymm. Tree)	Conclusion
CO1 3rd codon	0.6112	0.6919	.0097	.5540	.0653	"Useless sequences" if asymm. tree
CytB 3rd codon	0.6904	0.6906	.99	0.5274	0.00	"Useless sequences" if asymm. tree
Rag1 3rd codon	0.5037	0.7361	0.000	0.6539	0.000	"Little saturation"

Table 9: Summary of datasets.

Dataset	Number of Genes/ Number of Partitions	Size (bp)	Parsimony-Informative Sites	Score of Best Tree in PAUP	lnL Score of ML Tree	lnL Score range of MrBayes runs
mtDNA,	4/7	2336	542	2191	-10521.03	-11380 to -11340
mtDNA, saturation removed	4/5	1863	142	575	-5150.58	-5250 to -5205
nuDNA	3/9	1862	234	653	-5288.43	-5340 to -5290
Total, partition by gene	7/7	4198	776	2867	-17217.87	-17190 to -17135
Total, partition by gene, sat. removed	7/7	3725	376	1239	-11073.46	-11090 to -11030
Total, partition by codon	7/16	4198	776	2867	-15961.21	-16030 to -15960
Total, partition by codon, sat. removed	7/14	3725	376	1239	-10565.48	-10620 to -10565
Morphology	-	43 (characters)	41	144	-	

Table 10: Morphological matrix analyzed in this study. (-) represents missing or ambiguous data.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
A. lucillae	2	0	1	1	1	1	1	1	1	0	1	1	1	0	0	0	0	1	4	1	1	1	1	0	0	0	0	1
A. nigricaudus	2	0	1	1	2	1	2	2	3	0	0	0	1	0	0	0	0	0	5	1	1	1	1	0	0	0	0	0
A. storeyae	2	0	1	1	2	1	2	3	2	1	0	1	1	0	0	0	0	0	5	1	1	1	1	0	0	0	0	0
C. gracilis	2	0	2	1	2	2	2	3	3	0	0	1	0	0	0	0	1	1	5	1	0	1	1	1	0	0	0	1
E. altivelis	2	0	0	0	2	2	3	3	3	1	1	0	0	1	0	1	1	1	3	1	0	1	0	1	1	0	0	0
E. boehlkei	2	0	2	0	3	1	3	3	3	-	-	1	0	0	0	0	0	1	3	1	0	0	0	1	0	0	0	1
E. carminalis	2	0	2	0	2	3	2	2	3	1	1	1	0	1	0	0	1	1	2	1	0	1	1	1	0	0	0	0
E. glendae	2	0	2	1	2	1	1	3	3	0	1	1	0	0	1	0	1	1	3	1	1	1	1	1	0	0	0	0
E. macrops	2	0	2	1	2	3	1	3	2	0	1	1	0	0	0	0	1	1	3	1	0	1	1	1	0	0	1	1
E. pectoralis	2	0	1	0	2	3	1	2	2	0	1	0	0	1	1	1	1	1	3	0	0	1	0	1	1	1	0	1
E. reticulatus	2	0	2	1	2	3	1	3	2	1	1	1	0	0	1	0	1	1	3	1	1	0	0	1	0	0	0	1
Helcogramma ellioti	-	1	0	0	2	-	2	2	2	-	0	0	1	0	0	0	0	0	-	0	1	1	1	1	0	0	0	0
L. clarkhubbsi	3	1	1	1	2	3	2	1	1	1	1	1	0	1	1	1	1	1	4	1	0	1	1	1	0	1	1	1
Helcogrammoides cunnnghami	2	-	2	1	2	1	2	2	3	1	1	1	1	0	1	0	1	1	4	1	0	1	0	0	0	1	1	1

Species	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
A. lucillae	1	1	1	0	2	0	0	1	1	1	1	1	0	0	1
A. nigricaudus	0	1	1	1	2	0	0	0	0	0	0	1	0	1	2
A. storeyae	1	1	1	0	2	0	0	0	0	1	0	1	0	1	1
C. gracilis	0	0	0	1	2	0	0	0	2	0	0	2	1	2	2
E. altivelis	0	1	0	1	3	0	2	2	0	0	1	0	1	1	0
E. boehlkei	1	0	0	0	-	0	2	2	1	-	0	1	1	1	1
E. carminalis	1	1	0	1	2	1	0	2	0	1	1	1	1	0	0
E. glendae	1	1	0	1	2	1	2	0	1	0	1	1	2	2	2
E. macrops	1	1	0	1	1	1	2	2	0	1	1	1	1	1	1
E. pectoralis	1	1	0	1	1	0	0	2	1	1	0	1	1	1	1
E. reticulatus	1	1	0	1	1	0	0	2	1	1	1	1	1	1	2
H. ellioti	0	1	1	0	1	1	1	0	2	0	1	2	2	2	1
L. clarkhubbsi	1	1	0	1	2	1	2	1	1	1	1	2	2	2	2
H. cunnnghami	1	1	1	0	1	0	1	3	0	1	1	1	1	2	2

Submission for Publication

This thesis will be submitted for publication. The thesis author was the primary investigator of this material. In addition to P.A. Hastings, H.C. Lin will be listed as a co-author for her contribution of sequences for analysis.

FIGURES

Figure 1: Photographs of genera included in this study. Top left: *Enneanectes reticulatus*. Top right: *Lepidonectes corallicola*. Bottom Left: *Axoclinus lucillae*. Bottom Right: *Crocodilichthys gracilis*. Photographs by Ross Robertson and Gerald Allen, STRI Shorefishes of the Tropical Eastern Pacific Database.

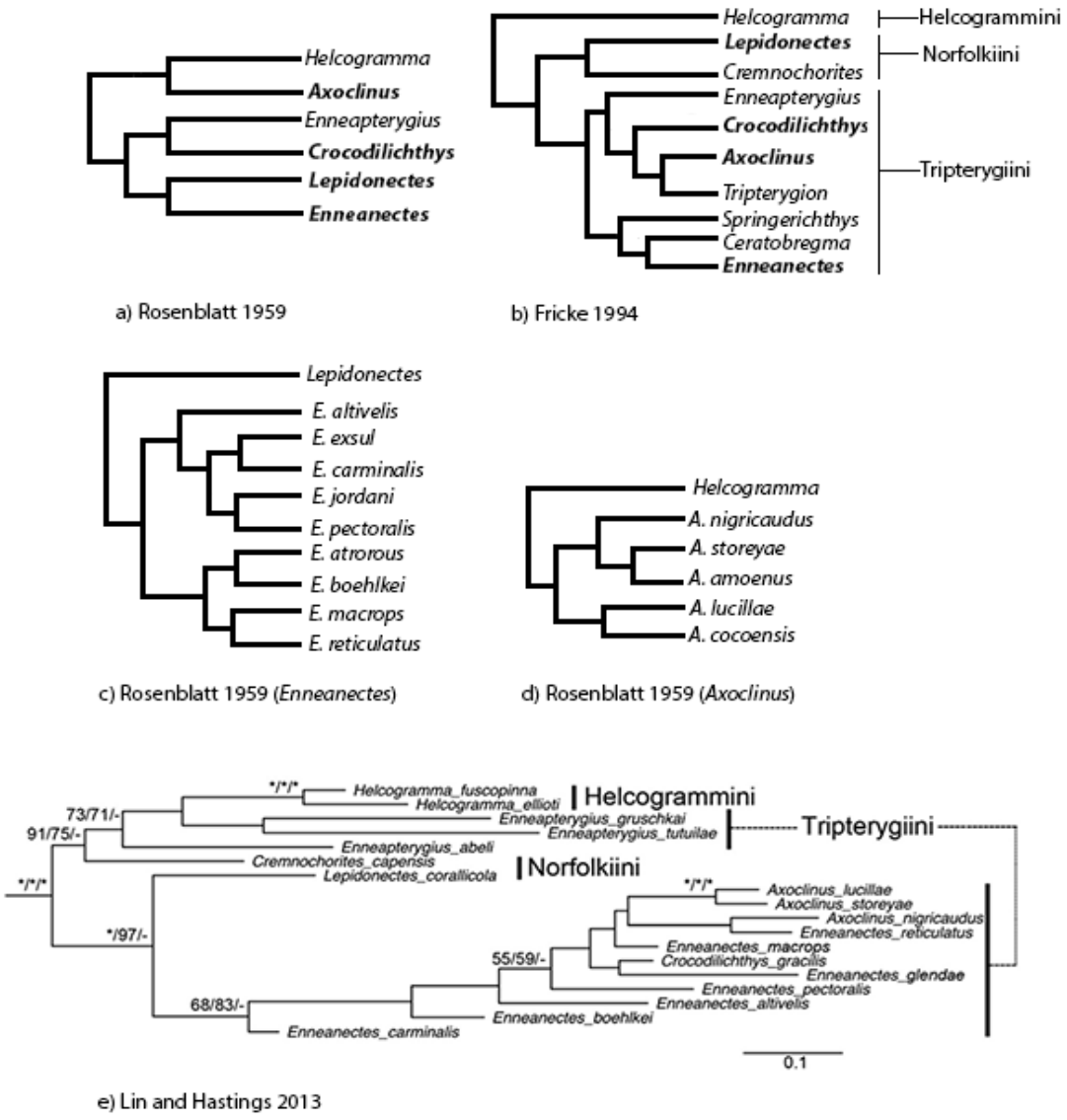


Figure 2: Previous hypotheses of relationships among the Tripterygiidae. a) Rosenblatt 1959 family-level phylogeny based on morphology. Neotropical genera in bold; b) Fricke 1994 family-level phylogeny based on morphology. Neotropical genera in bold; c) Rosenblatt 1959 phylogeny of *Enneanectes* species based on morphology; d) Rosenblatt 1959 phylogeny of *Axoclinus* species based on morphology; e) Lin and Hastings 2013 family-level phylogeny based on 4 nuclear and 1 mitochondrial markers. Support values indicate: ML bootstrap, parsimony bootstrap, and Bayesian posterior probabilities.

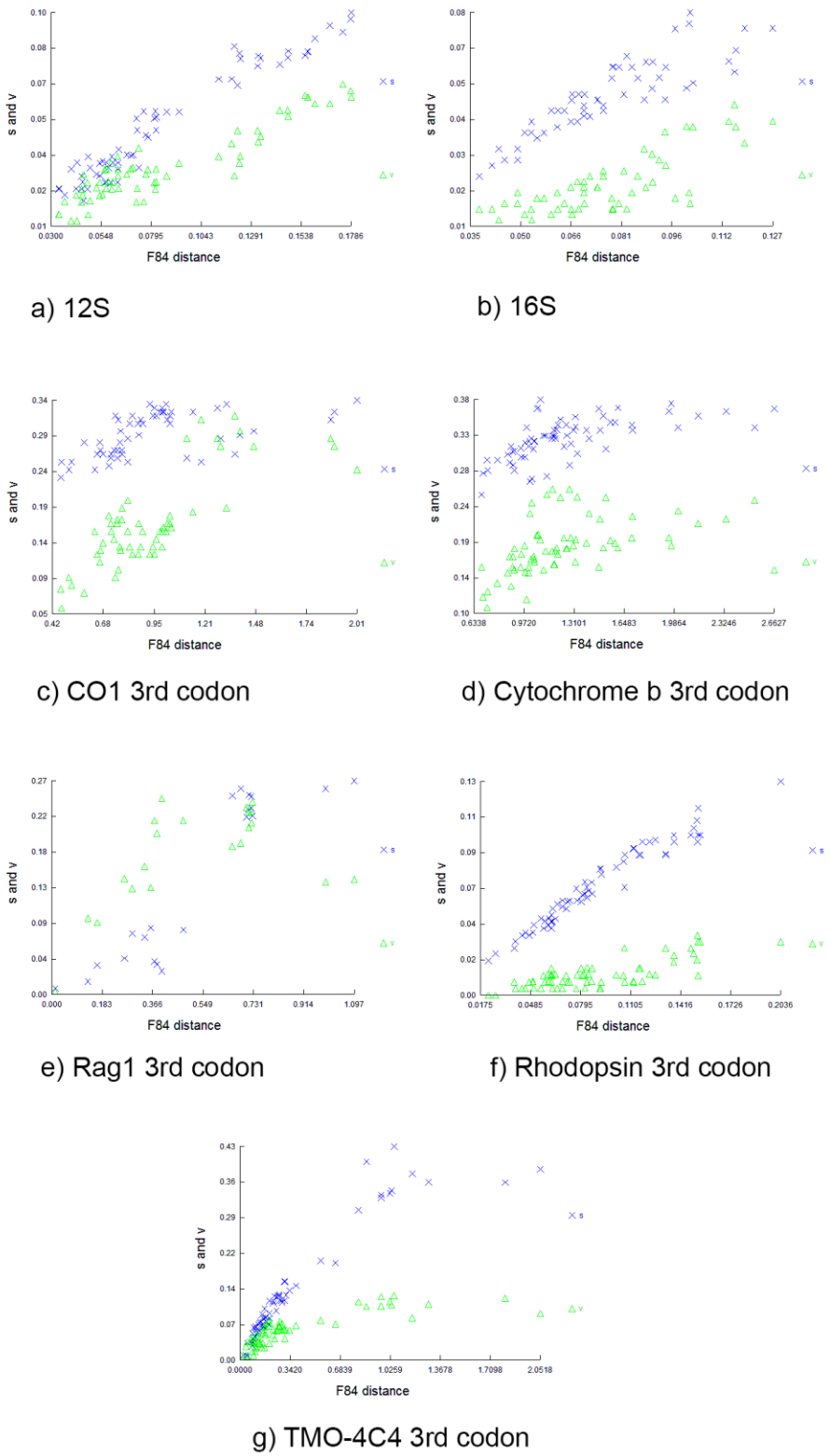


Figure 3: Plots of transitions vs. transversions for each marker.

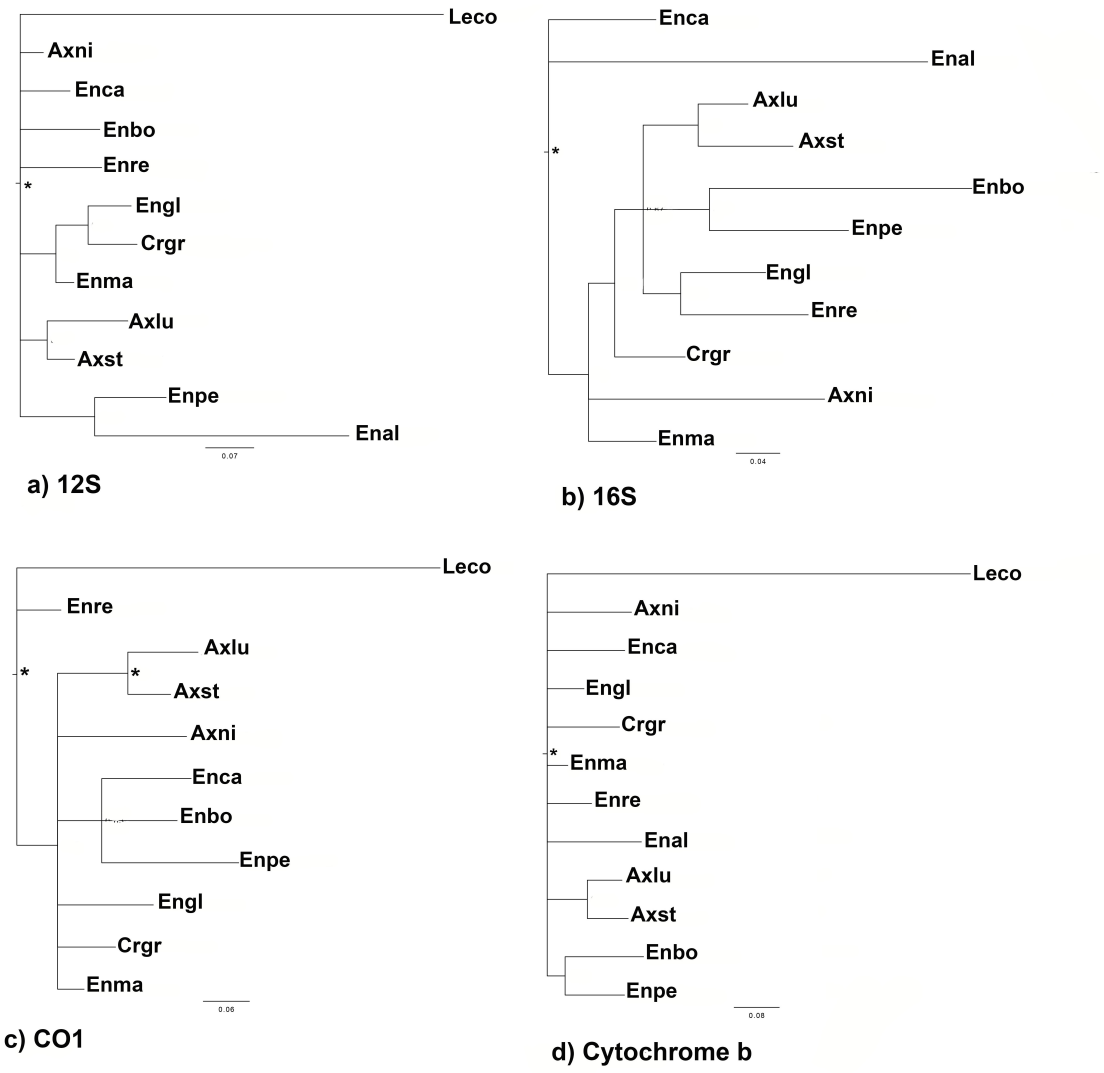


Figure 4: Bayesian gene trees for mitochondrial markers. a) 12S; b) 16S; c) CO1; d) Cytochrome b. Stars represent well-supported nodes (>0.95 posterior probabilities).

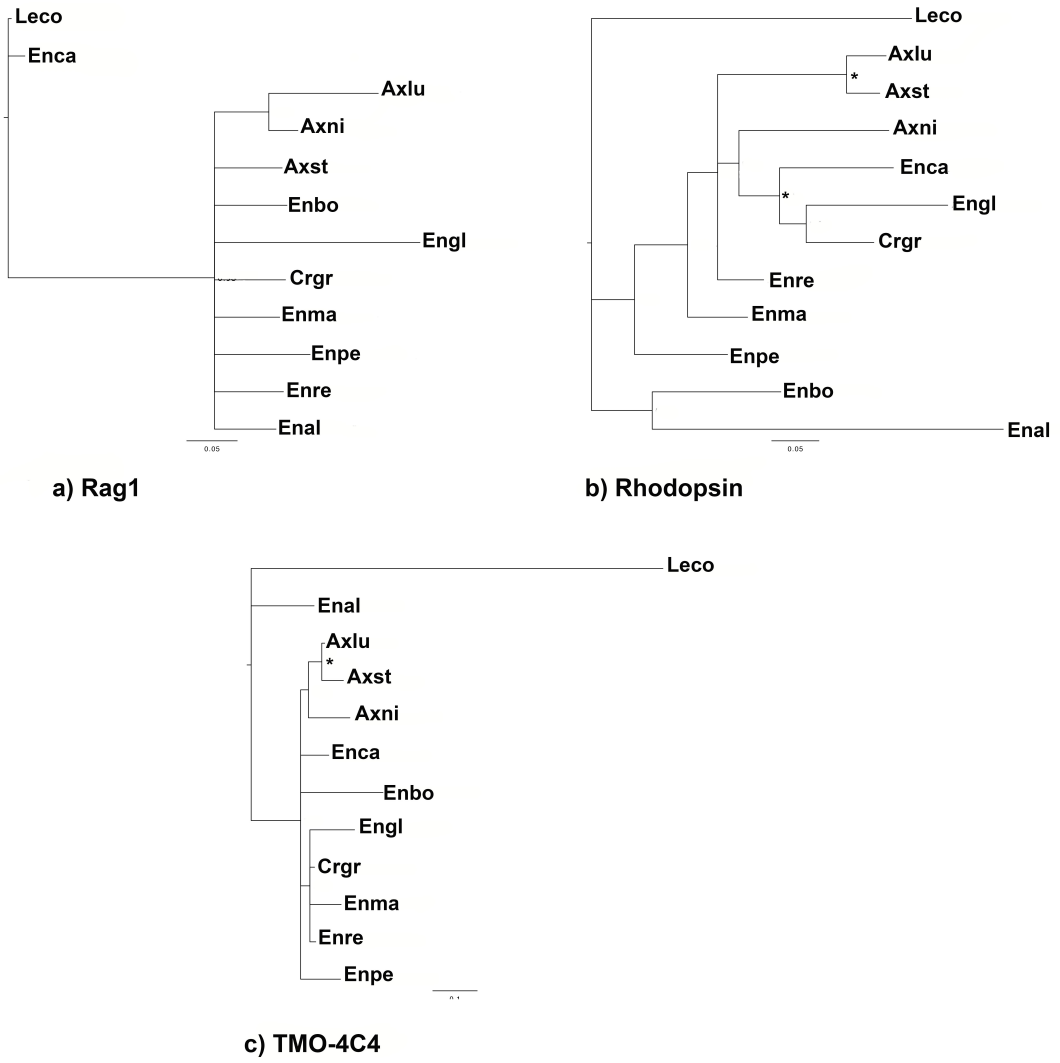


Figure 5: Bayesian gene trees for nuclear markers. a) Histone H-3, b) Rag-1, c) Rhodopsin, d) TMO-4C4. Stars represent well-supported nodes (>0.95 posterior probabilities).

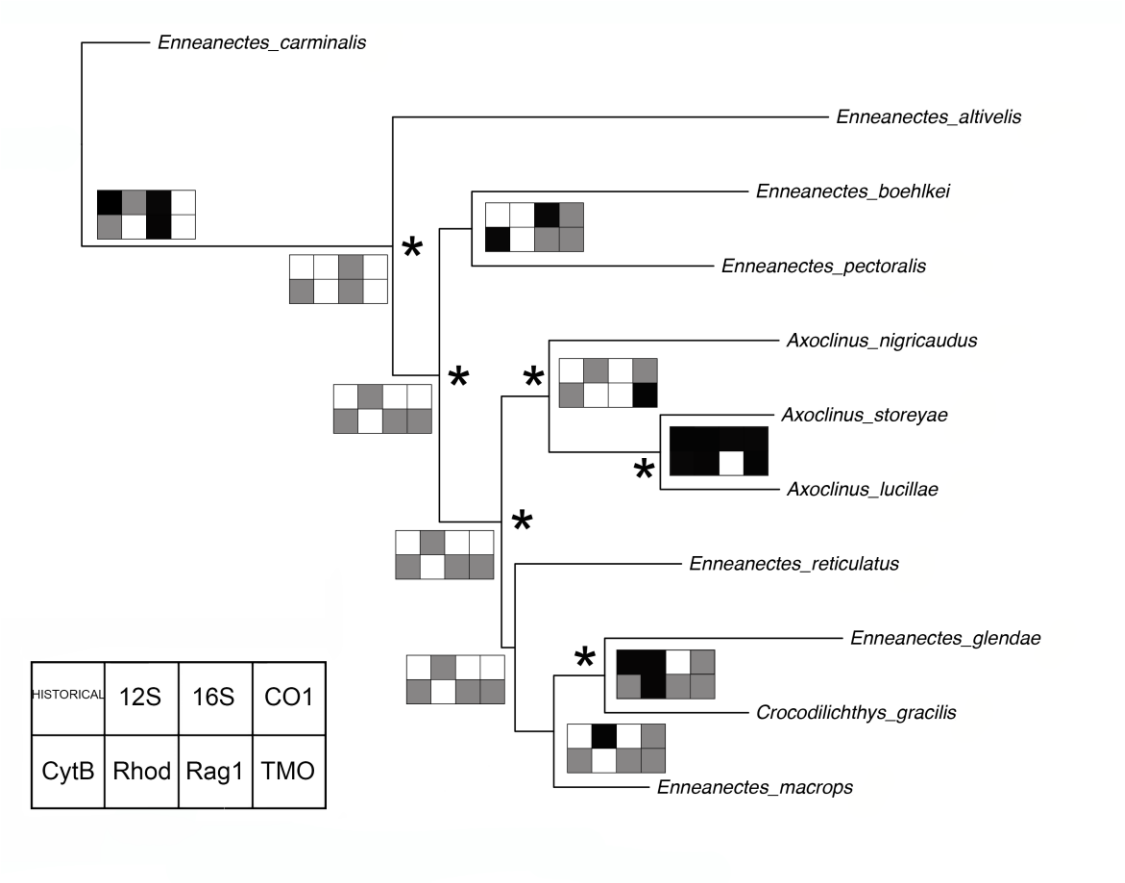


Figure 6: Topology of 7-gene dataset, showing support from individual sources. Black shading indicates the node is supported; grey indicates node not supported due to polytomies or missing data, but not in conflict; white indicates conflicting node was supported. "Historical" means the node was recovered by Lin and Hastings 2013; none of the nodes recovered by this study were shared with previously published morphological phylogenies. * = Node was well supported (>70) in ML analysis of 7-gene dataset partitioned by PartitionFinder. *Lepidonectes* outgroup is excluded for clarity.

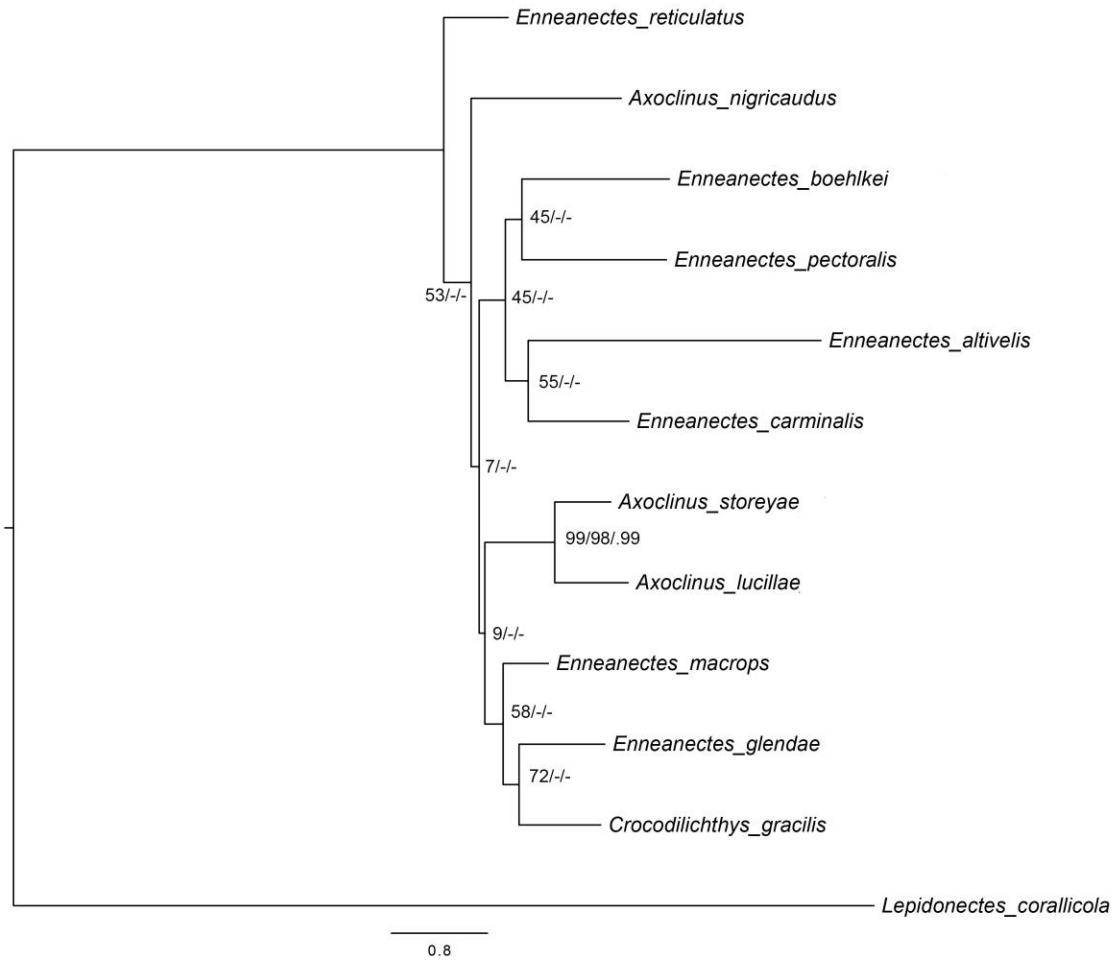


Figure 7: Maximum likelihood tree for mtDNA dataset with all codons. Support values are: ML bootstrap, Parsimony jackknife, and Bayesian posterior probabilities.

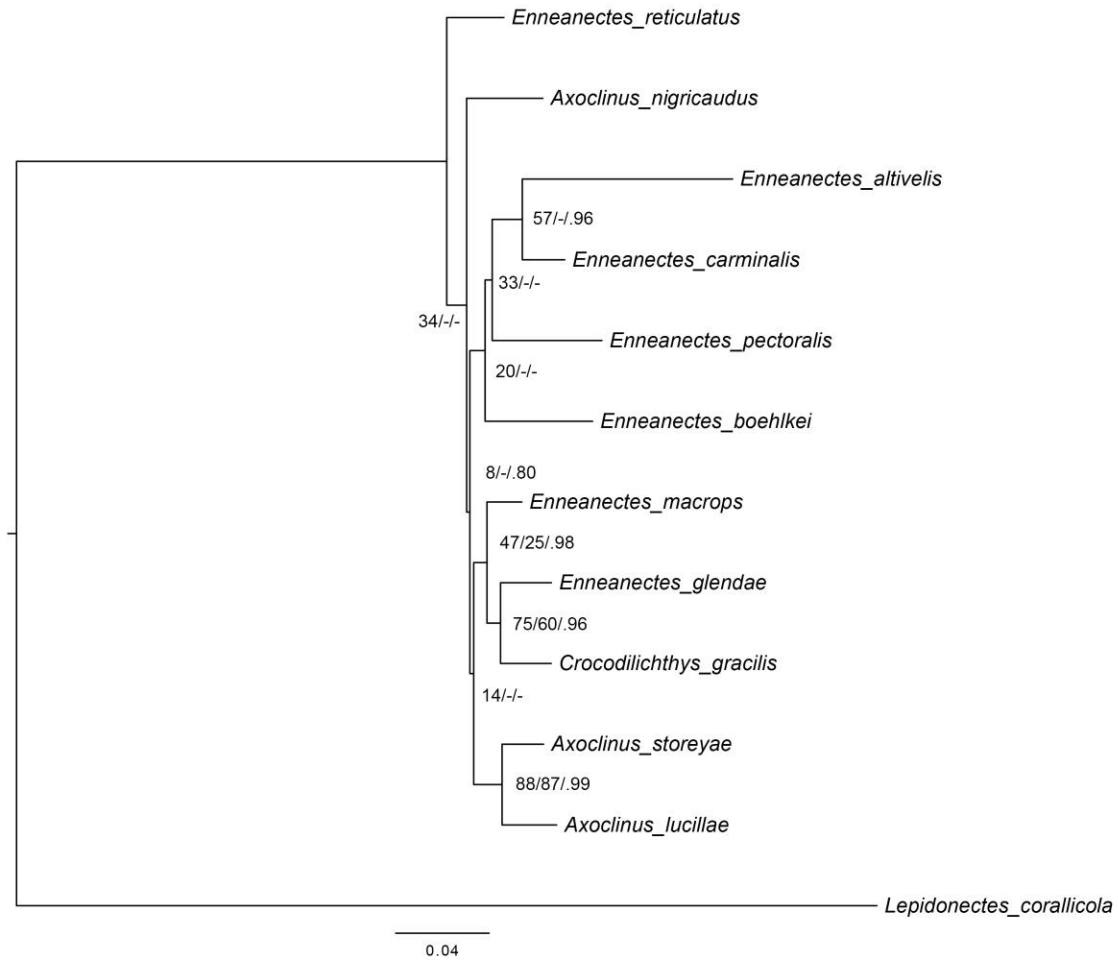


Figure 8: Maximum likelihood tree for mtDNA dataset with saturation removed. Support values are: ML bootstrap, Parsimony jackknife, and Bayesian posterior probabilities.

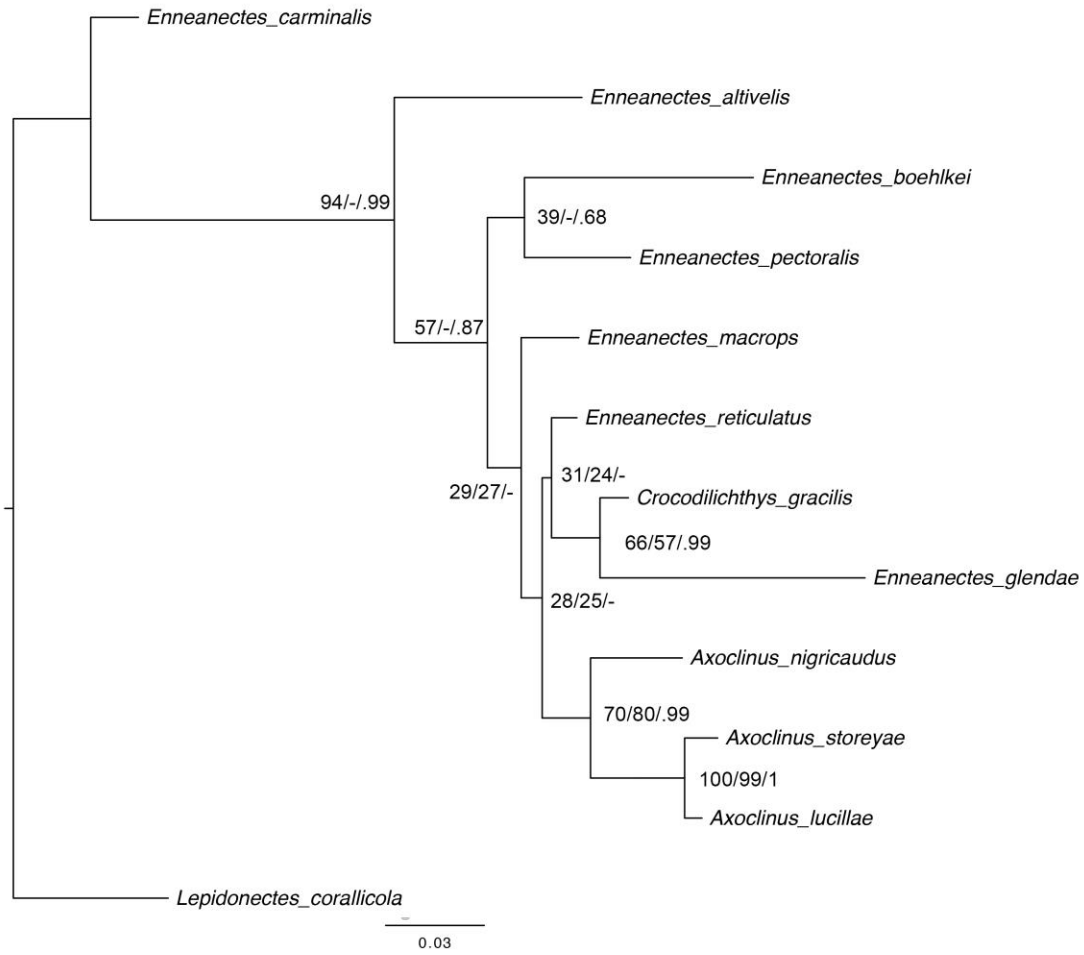


Figure 9: Maximum likelihood tree for nuclear dataset. Support values are: ML bootstrap, Parsimony jackknife, and Bayesian posterior probabilities.

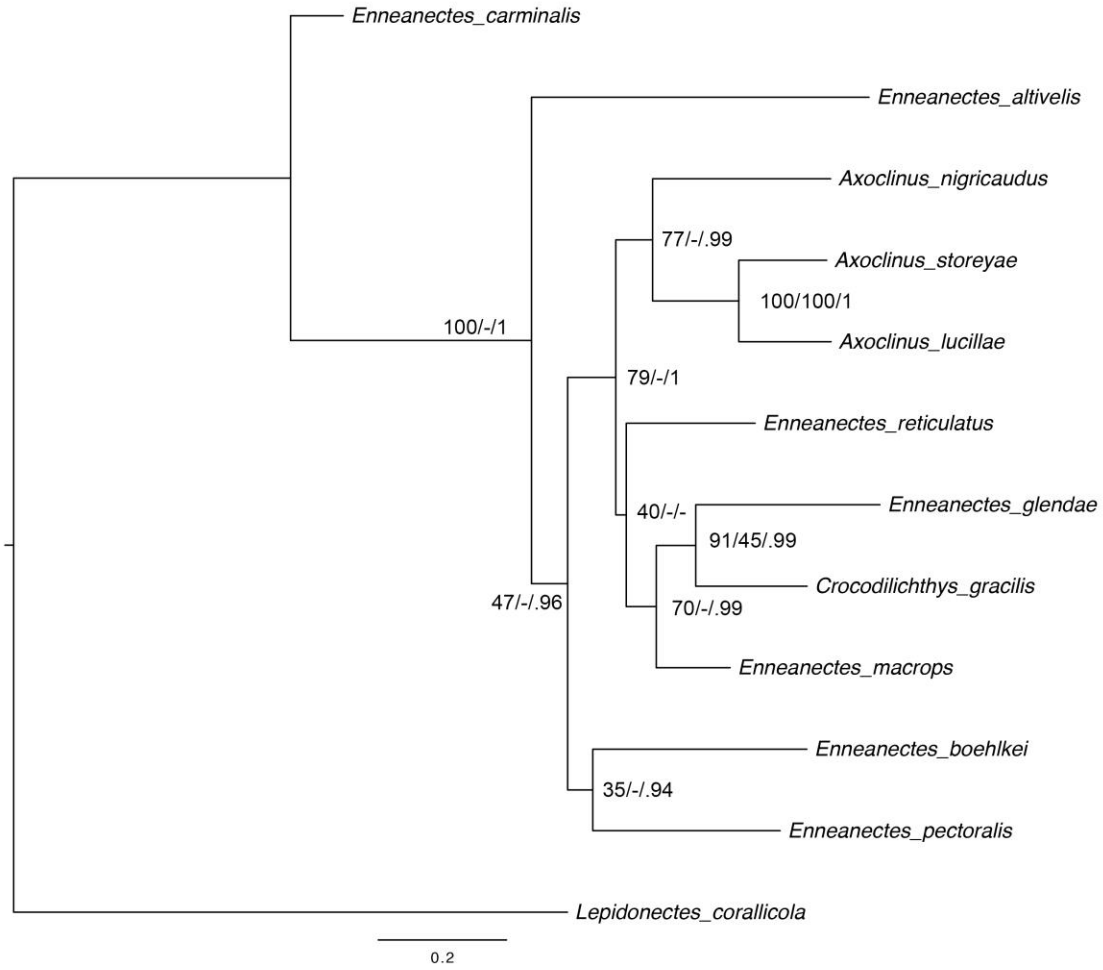


Figure 10: Maximum likelihood tree for total dataset, partitioned by gene, with all codons. Support values are: ML bootstrap, Parsimony jackknife, and Bayesian posterior probabilities.

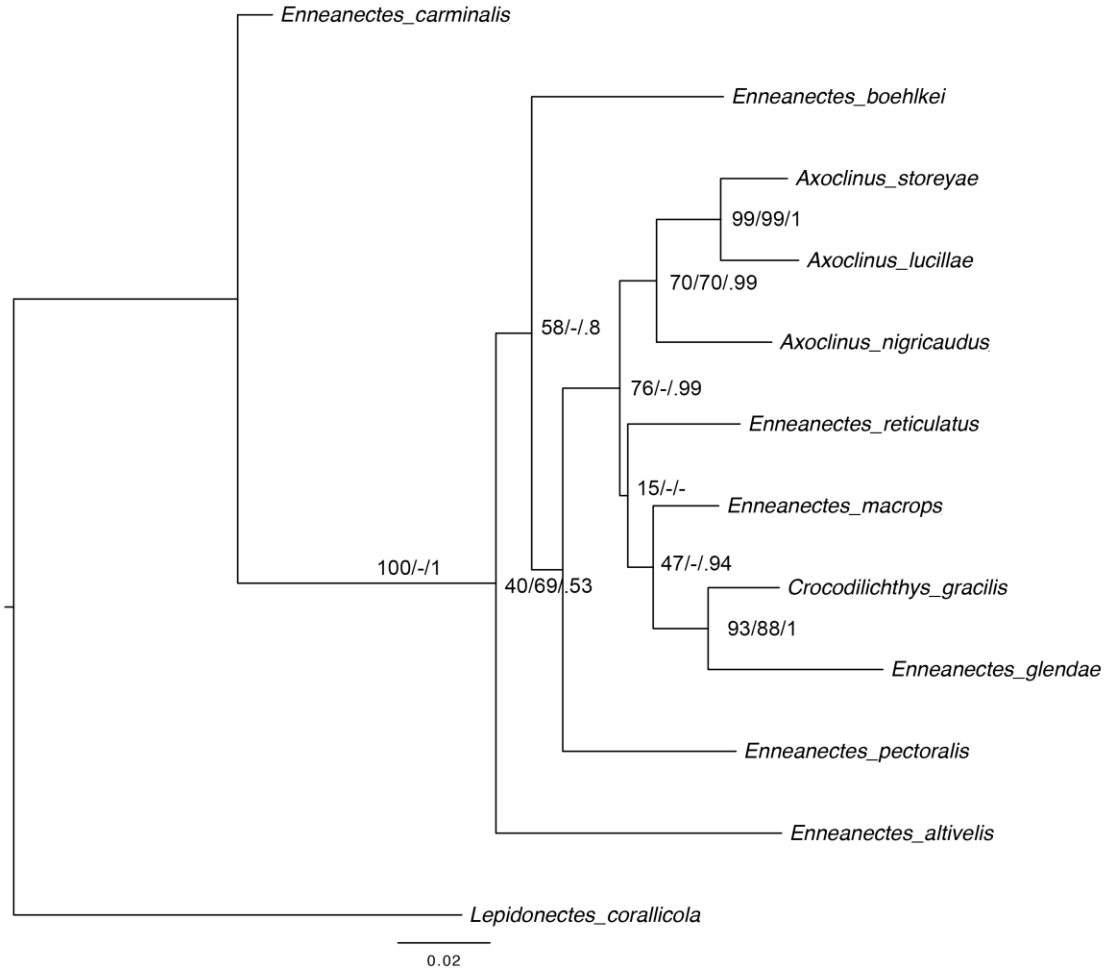


Figure 11: Maximum likelihood tree for total dataset, partitioned by gene, with saturation removed. Support values are: ML bootstrap, Parsimony jackknife, and Bayesian posterior probabilities.

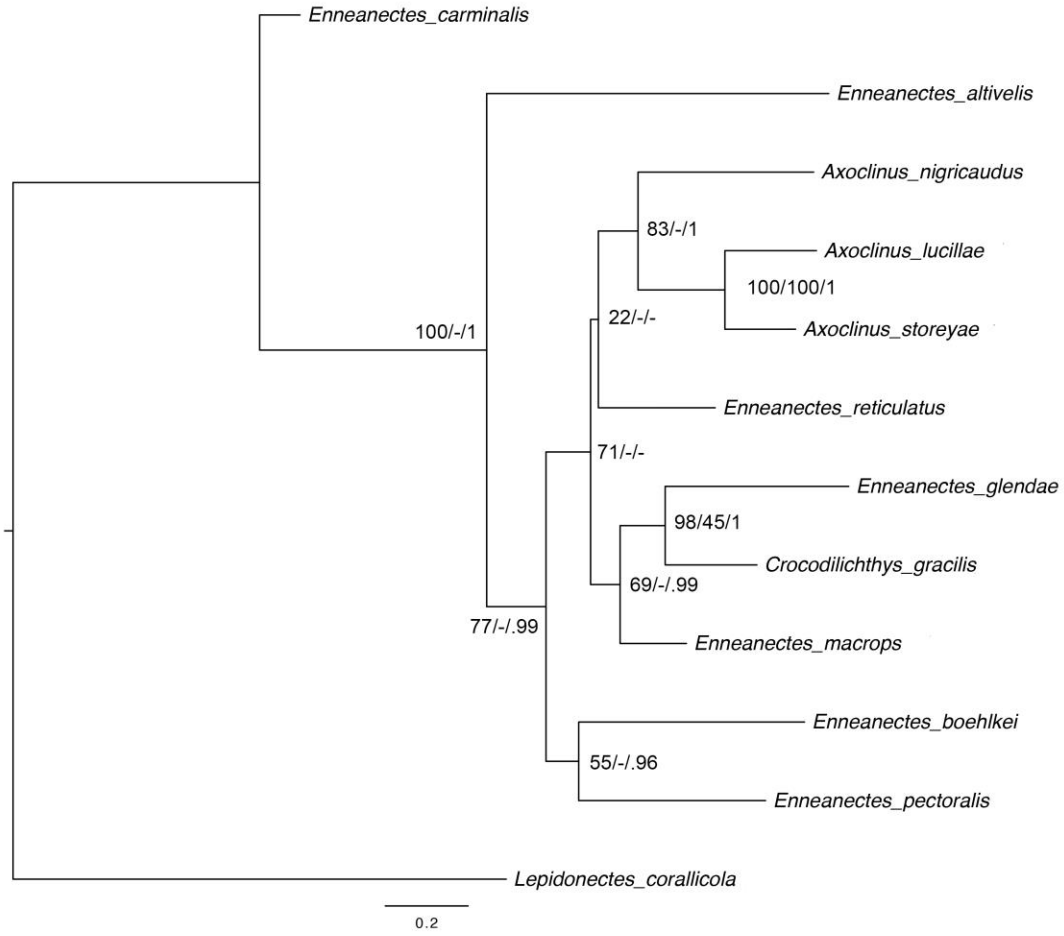


Figure 12: Maximum likelihood tree for total dataset, partitioned by codon position, with all codons. Support values are: ML bootstrap, Parsimony jackknife, and Bayesian posterior probabilities.

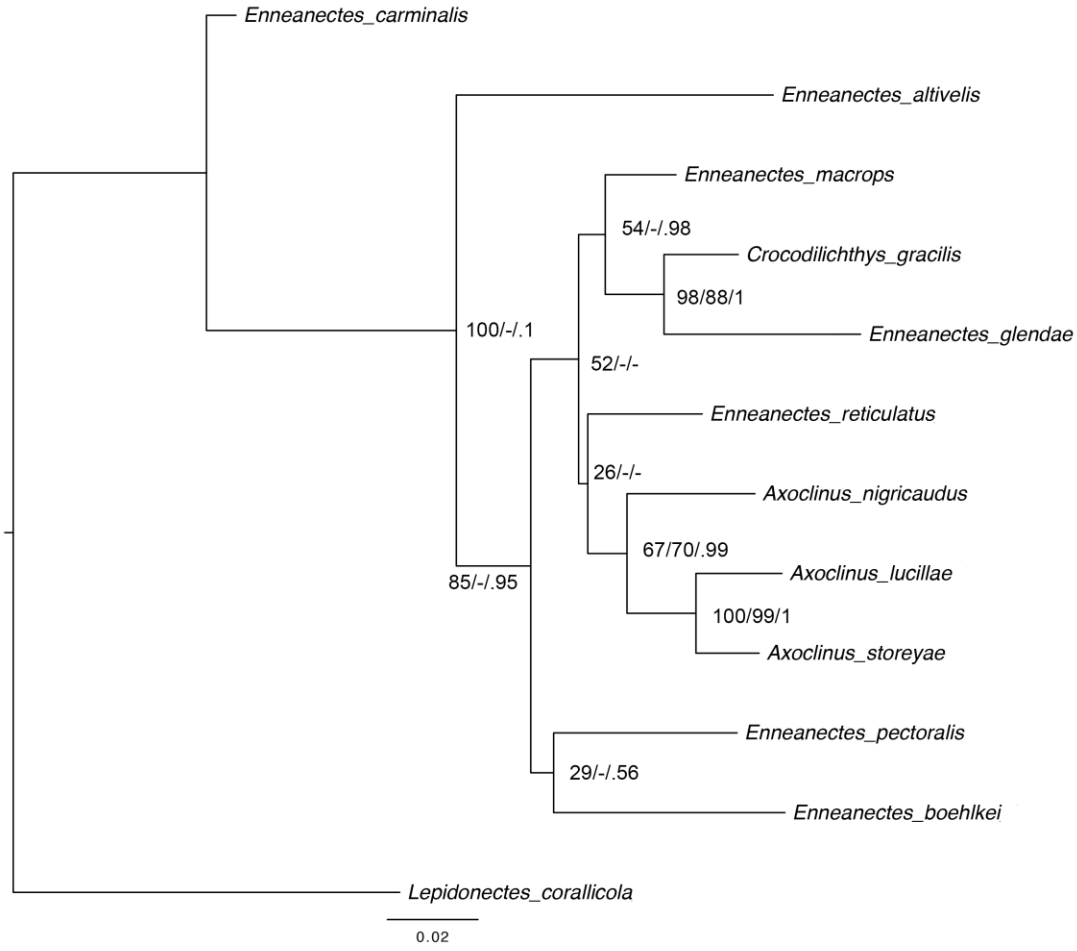


Figure 13: Maximum likelihood tree for total dataset, partitioned by codon position, with saturation removed. Support values are: ML bootstrap, Parsimony jackknife, and Bayesian posterior probabilities.

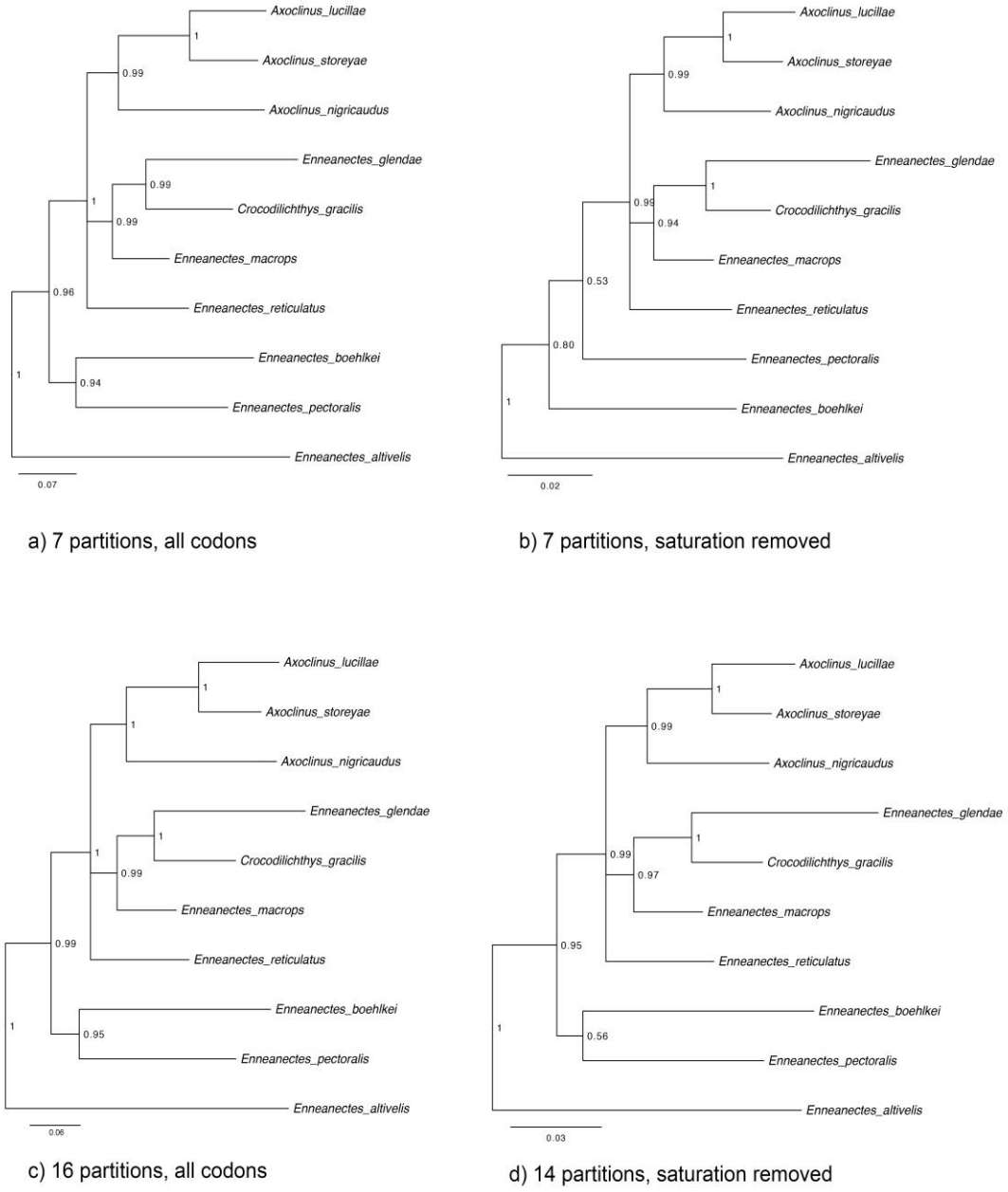
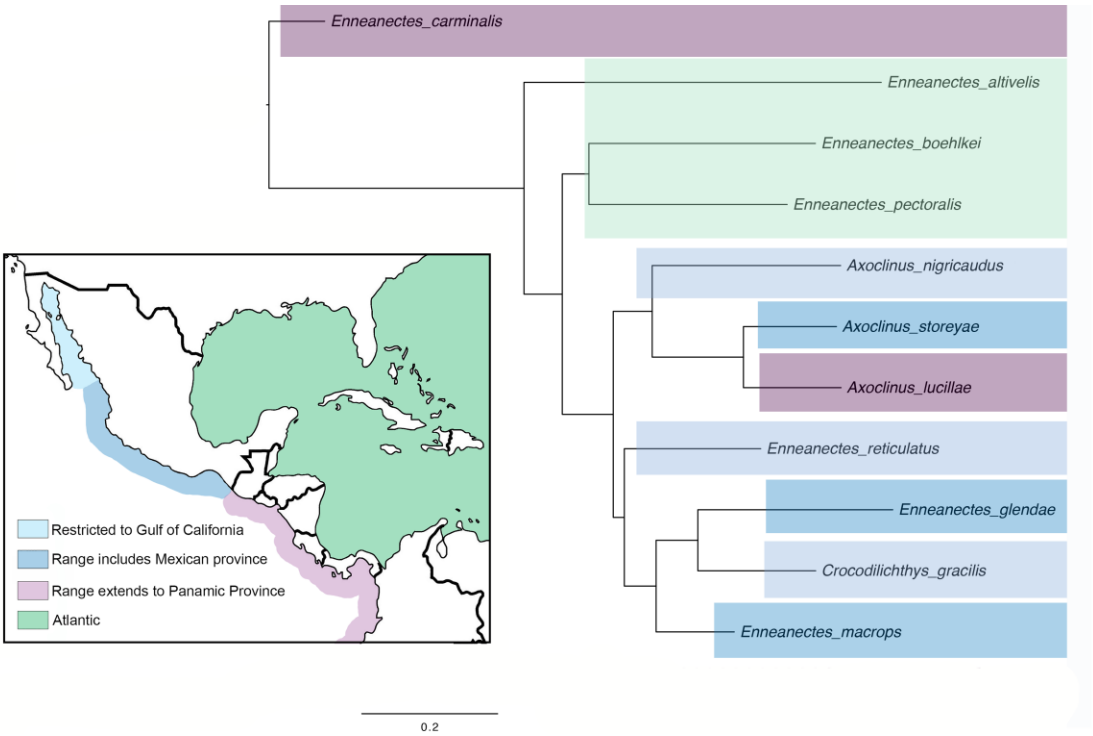


Figure 14: Bayesian trees for total dataset: a) partitioned by gene, all codons; b) partitioned by gene, saturation removed; c) partitioned by codon position, all codons; d) partitioned by codon position, saturation removed. Support values are Bayesian posterior probabilities. *Lepidonectes* outgroup excluded for clarity.



Species	Cortez	Mexican	Panamic
A. lucillae	-	+	+
A. storeyae	+	+	-
A. nigricaudus	+	-	-
E. reticulatus	+	-	-
E. macrops	+	+	-
E. carminalis	+	+	+
E. glendae	-	+	-
C. gracilis	+	-	-

Figure 15: Range mapped on topology from total dataset, with table of distribution among TEP provinces (Hastings 2000)

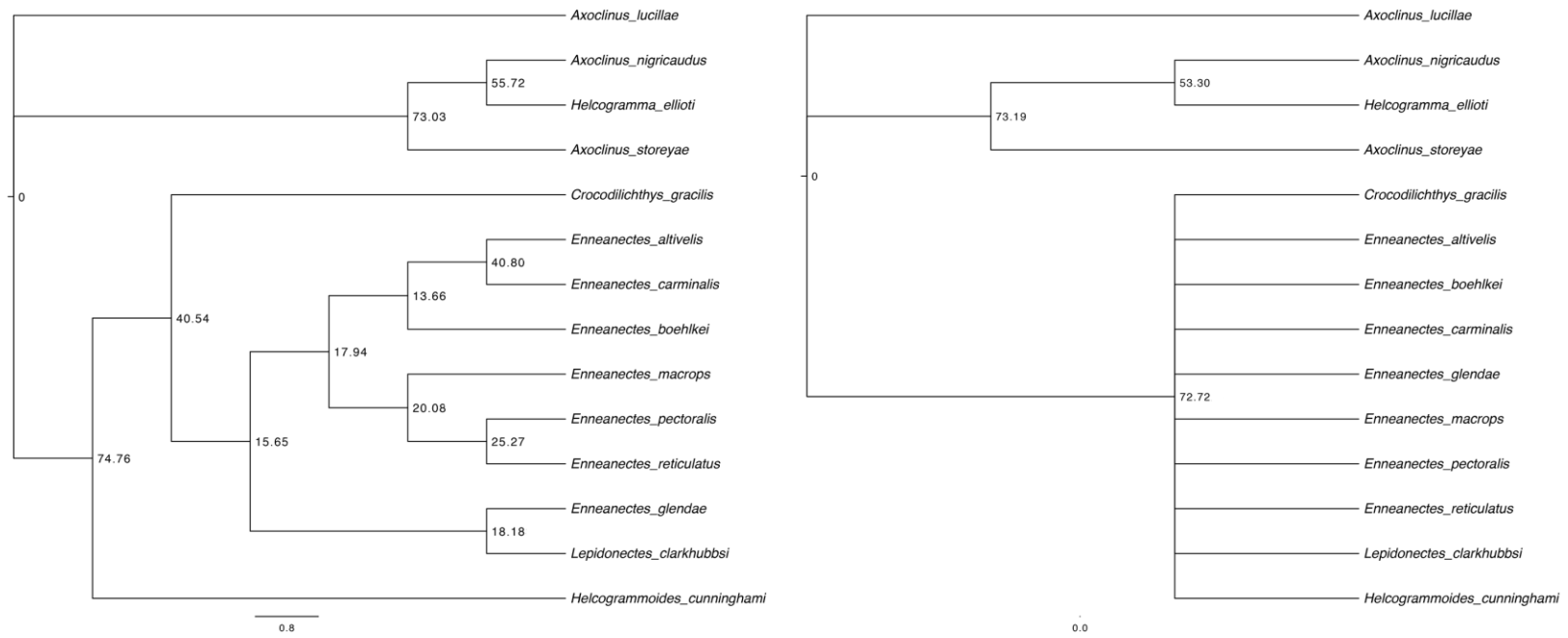


Figure 16: Maximum parsimony trees for morphological dataset. Left: All nodes are shown ("KEEPALL" command); Right: Nodes with jackknife support values <50 are collapsed as polytomies.

Submission for Publication

This thesis will be submitted for publication. The thesis author was the primary investigator of this material. In addition to P.A. Hastings, H.C. Lin will be listed as a co-author for her contribution of sequences for analysis.

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