Additional Evidence for Reciprocal Monophyly of Hagfish Subfamilies Myxininae and Eptatretinae: a Class Exercise in Phylogenetics

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The phylogeny of the fishes in family *Myxinidae*, commonly referred to as hagfish, has been reviewed and recent data has supported new interpretations of the traditional taxonomy. A study by Fernholm et al. (2013) undertook the phylogenetic assessment and evaluation of 33 species of hagfish using two genes, 16S ribosomal DNA fragments and cytochrome oxidase subunit 1 (CO1). The goal of our study was to test the two main taxonomic revisions proposed by Fernholm et al. (2013) as a way of teaching phylogenetic reconstruction in an upper-level elective course. We used a subsample of the same data from the original study, and our independent phylogenetic reconstruction supported the reciprocal monophyly of the hagfish subfamilies *Myxininae* and *Eptatretinae*. However, our reconstruction of CO1 placed the genus *Rubicundus* as sister to *Eptatretinae*, which is in contrast to the placement in Fernholm et al. (2013). Through this exercise, the student-authors gained a thorough understanding of phylogenetic reconstruction and were able to contribute to revisions of hagfish phylogenetics. Specifically, the authors robustly support the reciprocal monophyly of these two subfamilies and suggest that further work is necessary with respect to the placement of *Rubicundus*.

INTRODUCTION

Myxinidae is a family of eel-shaped jawless fishes found in all oceans except the Arctic, Southern Ocean, and the Red Sea (Froese and Pauly, 2012). Hagfish are found in benthic environments and feed on carrion and bottom-dwelling invertebrates (Wisner, 1999). The family Myxinidae includes 79 species of hagfish in six genera (Froese and Pauly, 2012). The genus *Myxine* holds 23 species; the genus *Eptatretus* holds 49 species; the genus *Nemamyxine* holds two species; the genus *Neomyxine* holds one species; the genus *Notomyxine* holds one species; and the genus *Rubicundus* holds four species. Consistent with their primitive morphology, molecular genetic studies have identified hagfish as the sister clade to other vertebrates (e.g., Zintzen et al., 2011; Uchida et al., 2013). Until recently, little attention has been paid to how different species of hagfish are related to one another, and the taxonomy of *Myxinidae* was largely based on phenotypic variations in teeth, gill structures, and color patterns (Fernholm, 1998). However, Fernholm et al. (2013) conducted a phylogenetic analysis of how 33 different species of *Myxinidae* are related to one another, resulting in a new subfamily and reclassification of some species to different genera.

Two subfamilies of hagfish, Myxininae and Eptatretinae, are categorized by the number of pairs of external gill openings (Nelson, 2006). Fernholm et al. (2013) hypothesized that these gill openings correlate to differences in gene sequences from the cytochrome oxidase subunit 1 (CO1) and 16S ribosomal DNA fragments. Fernholm et al. (2013) also suggested that *Rubicundus lopheliae*, a species known

from only a few specimens and found in geographically isolated, cold water reefs, was not included in either traditional clade, but rather as an outgroup to the *Eptatretus* and *Neomyxine/Myxine* clades. While each gene tree and the concatenated dataset yielded a majority-rule tree that supported these changes, Bayesian posterior support values were low for some clades, and the authors failed to test for any evidence against reciprocal monophyly, which would be a useful contribution to hagfish phylogenetics. To fill this gap, the authors (who were students in a phylogenetics course) worked independently to use the same DNA sequences with analogous methods to evaluate support for the findings of Fernholm et al. (2013) and then to combine their phylogenetic reconstructions into a single analysis.

In this study, we tested two hypotheses proposed by Fernholm et al. (2013). First, we tested the null hypothesis that a subsample of the species used by Fernholm et al. (2013) would yield a phylogeny for which the genera *Neomyxine* and *Myxine* would form a clade separately from *Eptatretus*, resulting in reciprocal monophyly Second, we tested the placement of *Rubicundus lopheliae*, which was previously thought to reside within the genus *Eptatretus*, but is now represented as a sister clade to the subfamilies Eptatretinae and Myxininae.

METHODS AND MATERIALS

DNA Sequences and DNA Alignment

A total of 27 different species were evaluated (Table 1), 20 species with DNA sequences for CO1 (cytochrome oxidase subunit 1, mtDNA), as well as 15 species with DNA sequences for 16S (ribosomal DNA, rDNA). The data for these gene sequences were downloaded from Genbank. There were ten species in common between the two genes (Table 1). The sets of gene sequences were combined for CO1 and then separately for 16S into the program GeneDoc v. 2.7 (Nicholas, K.B., Nicholas, H.B. Jr., 1997). The sequences for each gene were then aligned by hand, working towards an alignment with the fewest number of gaps and/or required mutations. Once the finalized aligned sequences were achieved for CO1 and 16S, the 16S dataset was automatically aligned through the CLUSTALW2 web portal (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to identify any alternative alignments, given the general difficulty of manually aligning this gene (see Fernholm et al., 2013). We did not evaluate all 33 species used by Fernholm et al. (2013) due to computational constraints, as this was an undergraduate class exercise. However, our subsample did include representative sampling from all critical genera and clades.

Estimating a Model of Nucleotide Evolution

After the manual alignments were finalized and a single alignment for each gene was agreed to by all eight students, jModelTest2 (Darriba et al., 2012; Guindon and Gascuel, 2003) was used to identify the best model of evolution for each gene (16S and CO1). We identified the best model based on differences in AICc scores (Akaike information criterion corrected for small sample size), provided in the results of the jModelTest2. AIC is a measure of the relative fit of a statistical model for a given set of data, and AICc is AIC with a correction for finite sample sizes. The delta AICc between 0 and 4 expresses the top models, while a delta of 4 or greater differentiates considerably inferior models (Burnham and Anderson, 2002).

Phylogenetic Reconstruction

We used the program suite BEAST v.1.8 (Drummond et al., 2014) to find the tree with the highest likelihood within a Bayesian framework. The aligned gene files were used to create a file in BEAUti. We specified the outgroup (stem) as *Petromyzon marinus*. Under the sites tab the results from jModelTest were used to specify the model of evolution for each gene (see Results). The base frequencies were set to "estimated," the site heterogeneity model was changed in accordance with the gene, and all other aspects were left in default settings. The clock model was set to be "Lognormal Relaxed Clock (uncorrelated)" and the box for "estimate" was checked. The tree prior was changed to "Speciation: Yule Process."

The priors for the first run were left at their default values for the initial run of 15-20 million generations. BEAST log files were checked routinely in the program Tracer v1.6 (Rambaut et al., 2014) to check the priors and monitor progress. If multiple runs had to be completed for either gene sequence, the priors were changed in accordance to the values with the highest likelihood from previous runs provided by BEAST and identified by Tracer. After the effective sample size (ESS) values for each of the priors were greater than 200 (as in Bryson et al., 2014; Zhang and Li, 2013; Reece et al., 2010), the files were run again with the same priors to test for consistency. When all runs for each gene were completed (two replicates by four independent students for each gene), the tree files were combined in LogCombiner v1.8.1 (Drummond et al., 2014) and the burn-in was set to ten percent of each run (10% was visually inspected in Tracer as being sufficient burn-in for each run). This completed file was then put into TreeAnnotator v1.8.1 (Drummond et al. 2014) without burn-in to obtain an annotated Bayesian maximum clade credibility tree, which was visualized in the program FigTree v1.4.2 (Rambaut et al. 2014).

Tests of Reciprocal Monophyly

We evaluated the finding of reciprocal monophyly of the subfamilies Myxininae and Eptatretinae (Fernholm et al., 2013) in two ways. First, we modified our initial XML files created in BEAUti for each gene to include a constraint of reciprocal monophyly for Myxininae and Eptatretinae. We executed a second phylogenetic reconstruction that was identical to our unconstrained reconstruction save for the constraint of reciprocal monophyly (hereafter referred to as "constrained" versus "unconstrained"). After the phylogenetic reconstruction in BEAST was completed for both the constrained and unconstrained gene trees, we compared the likelihoods of the post burn-in (10%) tree samples. Likelihood values were normally distributed, and we assumed that if the distribution of likelihoods from the unconstrained trees was overlapping the distribution of the constrained tree by less than 2.5% (equivalent to a two-tailed t-test with an alpha of 0.5), then the distributions were significantly different. We executed this test for both genes independently. Our second test of reciprocal monophyly was to query the number of trees that violated reciprocal monophyly from the post-burn-in set of equally likely trees for each gene. This was executed using the search for non-monophyly tool in the program TNT (Goloboff et al., 2008).

The placement of *Rubicundus* was evaluated by simply comparing our phylogenetic reconstruction to that of Fernholm et al. (2013). The position of *Rubicundus* in the previous study varied depending on the placement of *Neomyxine* in COI and 16S. In our study, we only utilized sequences for COI, and thus report solely on those results.

RESULTS

DNA Sequences and DNA Alignment:

Twenty-one sequences of a 553 bp fragment of the 16S rRNA and sixteen sequences of a 587 bp fragment of the CO1 gene were represented. These sequences were downloaded from GenBank. Because some species were used for both 16S and CO1 sequences, a total of twenty-seven different species were included (Table 1).

Estimating a Model of Nucleotide Evolution:

We determined that the model of evolution for CO1 was best estimated by the HKY+I+G (Hasegawa-Kishino-Yano) model, while 16S was best estimated by GTR+G (General Time Reversible model). The GTR+G model resulted in a Delta AICc value of 3.873 and therefore was chosen to represent the 16s gene, although TIM2+G or TIM2+I+G have an equally good fit. The HKY+I+G model possesses the lowest AICc value and was chosen to represent the CO1 gene. However, the 5 succeeding models had Delta AICc values less than 4. Table 2 lists the top 5 models for each gene and their Delta AICc scores.

Phylogenetic Reconstruction:

Phylogenetic trees for CO1 and 16S genes were each reconstructed independently twice by each of four students for each gene. Between 15-30 million generations were required to reach stationarity for each gene. A ten percent (10%) burn-in was required. All ESS values peaked over the 200 minimum requirement. All combinations from each respective group were then combined using the program LogCombiner. Figure 1 and Figure 2 display the resulting phylogenetic reconstructions.

Tests of Reciprocal Monophyly:

The number of generations used to reach stationarity was determined by taking the longest number of generations executed during the unconstrained phylogenetic reconstruction, which was 15 million (sampling every 1000 generations) for COI and 30 million (sampling every 1000 generations) for 16S. The species constrained for each gene are noted in Table 1. Both constrained reconstructions reached stationarity well within the allotted generations and had strong ESS values (>200) for all parameters. Figure 3 displays the distribution of likelihood values post-burn-in for each constrained tree. Both the unconstrained gene trees resulted in reciprocal monophyly of the subfamilies. The constrained version of the COI gene tree resulted in a likelihood that was lower and non-overlapping with the likelihood of the unconstrained tree. The 16S gene trees had broadly overlapping likelihoods. Our search for examples of non-monophyly of either subfamily yielded zero examples from the distribution of trees for either gene. These results suggest that both gene trees support the reciprocal monophyly of Eptatretinae and Myxininae, despite some relatively low posterior probabilities at the nodes that encompass each subfamily (Figures 1 and 2).

Our results for COI did not place *Rubicundus* in the same position as the COI reconstruction in Fernholm et al. (2013). Instead of being sister to the Eptatretinae and Myxininae, our phylogenetic reconstruction placed *Rubicundus* as sister only to *Eptatretinae*, although support for these relationships was very weak in both our results and those of Fernholm et al. (2013).

DISCUSSION

Our results show support for the two hagfish subfamilies Eptatretinae and Myxininae to be reciprocally monophyletic. We support designation of the subfamily Rubicundinae (*Eptatretus lophelia*) being a sister clade to the *Eptatretinae* clade, as represented in Figure 1 and Figure 2. A previous phylogenetic analysis of hagfish by Fernholm et al. (2013) found that the two subfamilies *Eptatretinae* and *Myxininae* are reciprocally monophyletic after moving and renaming one Eptatretus species (*Eptatretus fernholmi*) to the Myxinae clade.

The Fernholm et al. (2013) study also proposed a new subfamily, which was previously thought to reside within the genus *Eptatretus*, Rubicundinae as a sister clade to the two existing clades, which is not what we found in our analysis (Figure 2). To ensure the relationships estimated in our initial unconstrained trees were well supported, we estimated a constrained tree with reciprocal monophyly, under the assumption that the constrained and unconstrained topologies would have equivalent likelihoods. In addition, we searched the post-burn-in trees from the unconstrained analysis and found no topologies that violated the reciprocal monophyly of *Eptatretinae* and *Myxininae*. For both genes, we found no evidence for violations of reciprocal monophyly. Therefore, we fail to reject the null hypothesis for 16S and CO1, supporting the reciprocal monophyly of the *Eptatretinae* and *Myxininae* families with a statistical framework that was lacking in the original analysis by Fernholm et al. (2013).

Our analysis of the CO1 gene also supported the newly proposed subfamily, Rubicundinae, as a sister clade only to the Eptatretinae clade as shown in Figure 2. Fernholm et al. (2013) proposed Rubicundinae as being sister to both Eptatretinae and Myxininae (with low posterior support), which is not supported by our findings. There is not any data sampled from the 16S gene for this species in our dataset, and, consequently, the relationship of this subfamily was analyzed using only the CO1 gene. Further research using additional independent loci may resolve the placement of this subfamily.

For the 16S gene, there was difficulty estimating the coefficient of variation (Table 2a). The coefficient of variation determines if the mutations are occurring in a predictable clock-like fashion or if they are occurring randomly. This ESS value is not crucial to this study because mutation rate is not a parameter of interest. However, all other ESS values were estimated with high probability consistently throughout the study for both CO1 and 16S with values greater than 200.

Based on the study by Fernholm et al. (2013), and similar to problems we encountered, the aligning of the 16S genes proved to be more challenging than expected and other alignment software was used. In contrast to our study, Fernholm et al. (2013) specifically used taxa for which both 16S and CO1 were available. For our purposes, we subsampled the taxa for each of the genes. Doing this likely resulted in gaps in our data that may not have been present if we had used the full species complement (which would have been computationally challenging to do in a lab-course setting). The CO1 data presented by Ferholm et al. (2013) was partitioned based on three codon positions and a 4th partition for the 16S data. The 16S sequences are made up of RNA that included loops in contrast to the CO1 codons which are all transcribed. The fact that we did not use a partition of codons may have resulted in slightly different results than those of Fernholm et al.

(2013), which is important because codon partitioning holds certain positions constant while changing others with higher frequency. Regardless, both approaches support the findings that Eptatretus and Myxininae are indeed monophyletic.

Our study examined a subsample of the taxa evaluated by Fernholm et al. (2013) and yielded results to further support the reciprocal monophyly of the two hagfish families, Eptatretinae and Myxininae. The findings from the CO1 gene also supported the subfamily, Rubicundinae, as being a sister clade to Eptatretinae and Myxininae. The methods used in our study differ slightly from those used by Fernholm et al. (2013), but our methods are tests of reciprocal monophyly that strengthen the taxonomic revisions proposed by the previous authors. As discussed in Maddison (1997), sampling only one gene can lead to an incorrect representation of the phylogeny, and sampling multiple genes provides more support for the generated gene tree. It is important to note that Fernholm et al. (2013) did analyze both CO1 and 16S separately, and also concatenated the two genes into one "super gene" for analysis, but in neither case did they test for support for the non-monophyly of either subfamily.

Overall, the student-authors who participated in this study gained an appreciation for phylogenetic reconstruction methods, the various and often equivalent approaches that one can take to reconstruct a phylogeny, and the computational complexity involved in identifying the "best" topology from hundreds of millions of potential topologies. In the course of learning these lessons, they also provided additional support for the taxonomic revisions recently proposed by hagfish researchers.

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FIGURES & TABLES

FIGURE 1. Maximum clade credibility phylogenetic tree of the observed hagfish species using 16s ribosomal DNA.

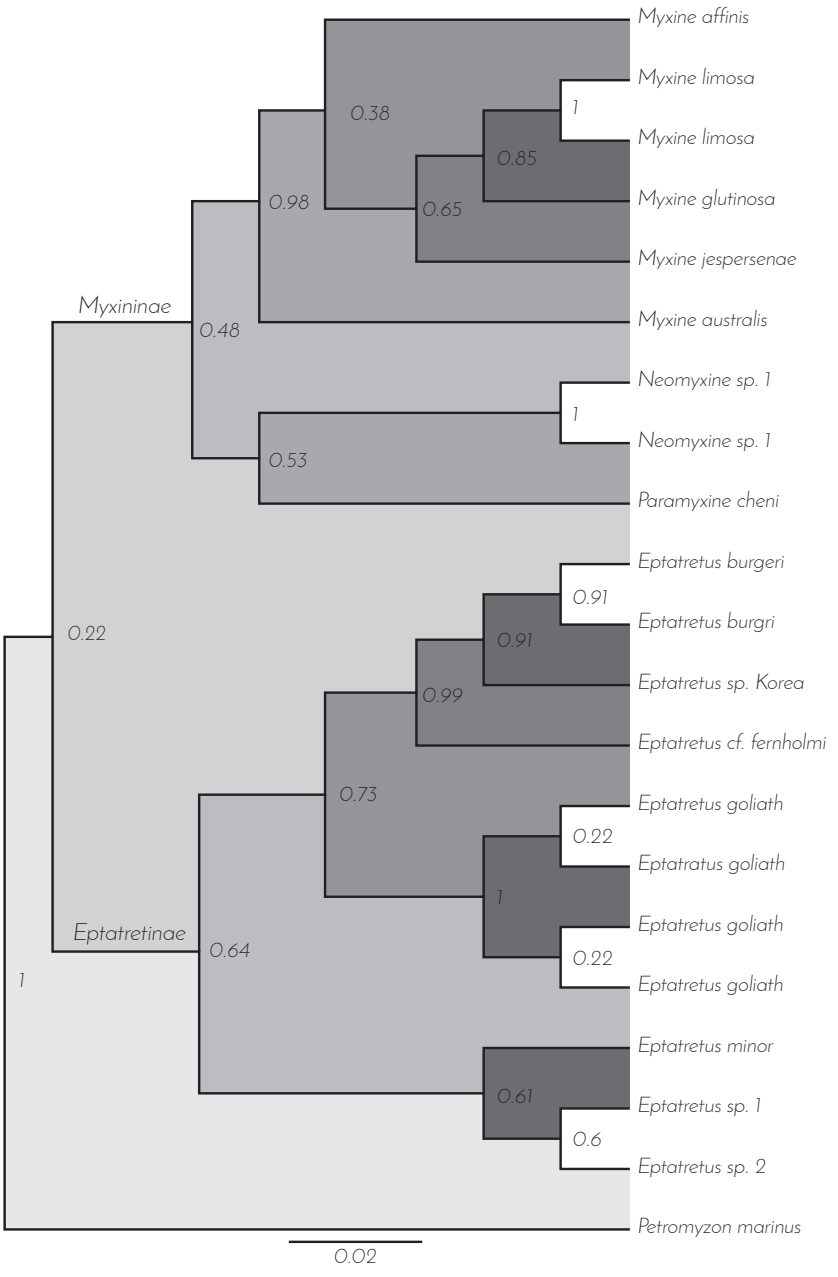

FIGURE 2. Maximum clade credibility phylogenetic tree of the observed hagfish species using CO1 mitochondrial DNA.

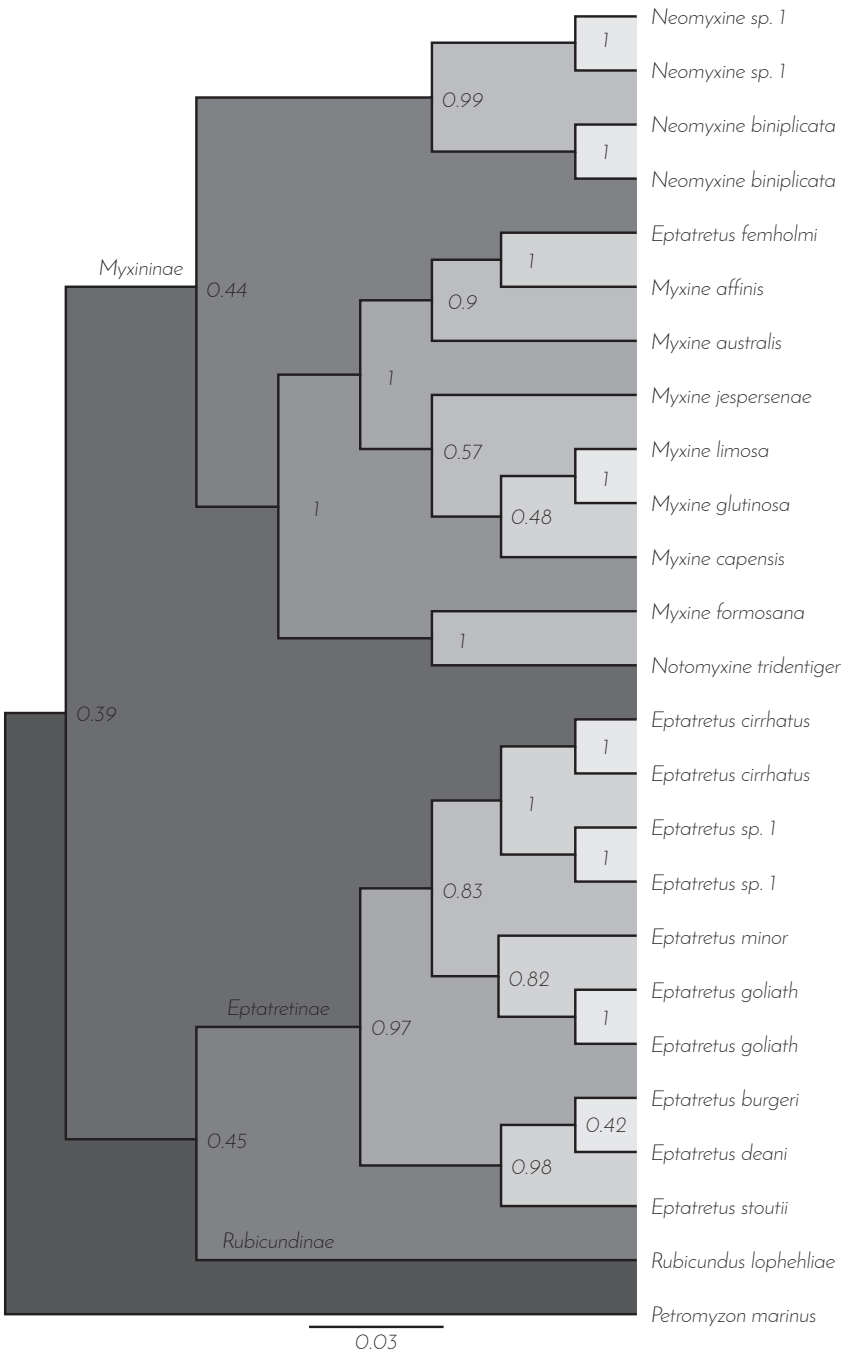

TABLE 1. Species represented in DNA Alignment, their corresponding Genbank number for 16s and/or CO1, along with the constraint of being monophyletic for *Eptatretus* or monophyletic for *Neomyxine*, *Myxine*, and *Paramyxine*.

SPECIES
Eptatretus burgeri 2
Eptatretus cirrhatus
Eptatretus cirrhatus 2
Eptatretus cf. fernholmi
Eptatretus deani
Eptatretus fernholmi
Eptatretus goliath
Eptatretus goliath 2
Eptatretus goliath 3
Eptatretus goliath 4
Rubicundus lopheliae
Eptatretus minor
Eptatretus sp. 1 LDS-2013 *voucher* NMNZ P037108
Eptatretus sp. 1 LDS-2013 *voucher* NMNZ P045318
Eptatretus sp. 1 LDS-2013 *voucher* NMNZ P045319
Eptatretus sp. 2 LDS-2013 *voucher* NMNZ P045332
Eptatretus sp. 'Korea'
Voucher NRM 50590
Eptatretus stoutii
Myxine affinis
Myxine australis
Myxine capensis
Myxine formosana
Myxine glutinosa
Myxine jespersenae
Myxine limosa
Myxine limosa 2
Neomyxine biniplicata
Neomyxine biniplicata 2
Neomyxine sp. 1 LDS-2013 *voucher* NMNZ P044014
Neomyxine sp. 1 LDS-2013 *voucher* NMNZ P044153
Neomyxine sp. 1 LDS-2013 *voucher* NMNZ P044215
Neomyxine sp. 1 LDS-2013 *voucher* NMNZ P044216
Notomyxine tridentiger
Paramyxine cheni
Petromyzon marinus

*: Monophyly for *Eptatretus*
#: Monophyly for *Neomyxine*, *Myxine*, and *Paramyxine*
OG: Outgroup

GENEBANK #16s	GENEBANK #C01	CONSTRAINT
JX442459.1	JF952730.1	*
JX442460.1	-	*
-	KF144341.1	*
-	KF144342.1	*
JX442463.1	-	*
-	KF929858.1	*
-	KC807350.1	*
KF144271.1	KF144306.1	*
KF144269.1	Kf144307.1	*
KF144268.1	-	*
KF144270.1	-	*
-	KC807325.1	*
JX442456.1	KC807331.1	*
-	KF144298.1	*
-	KF144299.1	*
KF144252.1	-	*
KF144253.1	-	*
JX442462.1	-	*
-	GU440317.1	#
JX442471.1	KC807353.1	#
JX442470.1	KC807355.1	#
-	JF493944.1	#
-	JN027323.1	#
JX442476.1	KF930165.1	#
JX442474.1	KC807335.1	#
JX442480.1	KC807332.1	#
JX442479.1	-	#
-	KF144285.1	#
-	KF144286.1	#
-	KF144280.1	#
KF144244.1	-	#
-	KF144281.1	#
KF144243.1	-	#
-	EU074500.1	#
AF364620.1	-	#
KJ128854.1	KF930255.1	OG

TABLE 2A. Effective Sample Size (ESS) and mean parameter values for combined phylogenetic reconstructions from sequences of the 16S gene.

STATISTIC	MEAN	ESS
Posterior	-2178.97	2158
prior	58.275	1262
likelihood	-2237.245	5892
treeModel.rootHeight	0.153	1954
tmrca(outgroup)	0	-
Yule.birthRate	19.82	17472
ac	0.346	2835
ag	0.499	3050
at	0.334	2408
cg	0.16	1719
gt	0.129	2832
frequencies1	0.349	795
frequencies2	0.196	1284
frequencies3	0.164	1145
frequencies4	0.291	1121
alpha	0.224	1892
ucld.mean	4.525	26430
ucld.stdev	2.425	869
meanRate	6.564	1770
coefficientOfVariation	4.2	536
covariance	-0.01132	3045
treeLikelihood	-2237.245	5892
speciation	39.063	1229

TABLE 2B. Effective Sample Size (ESS) and mean parameter values for combined phylogenetic reconstructions from sequences of the CO1 gene.

STATISTIC	MEAN	ESS
Posterior	-3995.282	1372
prior	-15.662	1321
liklihood	-3979.619	3307
treeModel.rootHeight	2.177	1937
tmrca(untitled1)	0	-
Constant.popSize	0.9	1735
kappa	11.83	1179
frequencies1	0.316	645
frequencies2	0.247	842
frequencies3	0.09579	865
frequencies4	0.341	768
alpha	0.895	435
plnv	0.486	346
ucld.mean	1.675	1605
ucld.stdev	0.398	985
meanRate	1.696	1626
coeffiecientOfVariation	0.4	948
covariance	-0.03918	9282
treeLikelihood	-3979.619	3307
coalescent	-9.365	1323

TABLE 3. Aikake Information Criterion, corrected for finite sample sizes (AICc) values and models of nucleotide evolution for the 16S and CO1 genes. The best model has a value of 0, and is considered statistically superior to other models with AICc values that are 4 or more AICc units greater than the best model.

	MODEL	Delta AICc
16S	TIM2+G	0
	TIM2+I+G	1.058
	GTR+G	3.873
	GTR+I+G	5.525
	TPM2uf+G	6.049
	TPM2uf+G	7.389
CO1	HKY+I+G	0
	TPM1uf+I+G	1.796
	TIM2+I+G	1.821
	TPM2uf+I+G	1.914
	TrN+I+G	2.277
	TPM3uf+I+G	2.347