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Short Communication

Evolutionary affinities of the unfathomable Parabrotulidae: Molecular data indicate placement of *Parabrotula* within the family Bythitidae, Ophidiiformes

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ABSTRACT

Fishes are widely diverse in shape and body size and can quite rapidly undergo these changes. Consequently, some relationships are not clearly resolved with morphological analyses. In the case of fishes of small body size, informative characteristics can be absent due to simplification of body structures. The Parabrotulidae, a small family of diminutive body size consisting of two genera and three species has most recently been classified as either a perciform within the suborder Zoarcoidei or an ophidiiform. Classification of parabrotulids as ophidiiforms has become predominant; however the Parabrotulidae has not yet been investigated in a molecular phylogenetic framework. We examine molecular data from ten genetic loci to more specifically place the Parabrotulidae within the fish tree of life. In a hypothesis testing framework, the Parabrotulidae as a zoarcoid taxon is rejected. Previous identity with zoarcoids due to the one fin ray for each vertebra being present, a characteristic for the Zoarcidae, appears to be an example of convergence. Our results indicate that parabrotulids are viviparous ophidiiforms within the family Bythitidae.

1. Introduction

The diversity of fishes spans a wide range of shapes and body sizes from about 10 mm to 12 meters in length (Nelson et al., 2016). The extreme morphological diversity of fishes creates challenges in classification solely from morphology. For example, divergent appearing fishes of the Mirapinnidae, Megalomycteridae and Cetomimidae were shown to be juveniles, males and females of a single family, now the Cetomimidae, through incorporation of molecular data (Johnson et al., 2009). While the life stages and genders of the Cetomimidae look very different from each other, the opposite case where there are few anatomical differences between fishes also occurs. Fishes reduced in body size are often typified by reduction and simplification of anatomical features, leading to numerous difficulties in assigning relationships based on only morphological data (Weitzman and Vari, 1988).

Application of sufficient molecular data can clarify aspects of the phylogenetic placement of diminutive taxa as demonstrated with the some of the world's smallest fish species of the genus *Paedocypris* within the Cypriniformes (Mayden and Chen, 2010; Stout et al., 2016). Prior to sampling by Mayden and Chen (2010) with multiple independent loci, *Paedocypris* was placed either in *Sundadanio* or *Danionella*, two other genera with dimunitive taxa based on a single mitochondrial locus and morphological characteristics (Britz and Conway, 2009; Rüber et al., 2007). Increased molecular sampling by Mayden and Chen (2010) concluding convergence on paedomorphism in *Paedocypris*, *Sundadanio* and *Danionella* may have been insufficient (Britz et al., 2014). However, independent miniaturization within *Paedocypris*, *Sundadanio* and

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Danionella has received further support from a larger molecular dataset (Stout et al., 2016). To refine the phylogenetic placement of the Parabrotulidae, a family of reduced body size, we examine several independent molecular markers.

The Parabrotulidae is a viviparous, bathypelagic family with two genera, *Parabrotula* Zugmayer, 1911 and *Leucobrotula* Koefoed, 1952, and three species (Miya and Nielsen, 1991). Parabrotulids are comparatively small (<60 mm SL) with unclear evolutionary relationships (Figs. 1 and 2). Since 1911, *Parabrotula* and later *Leucobrotula* have been placed in various orders (Gadiformes, Ophidiiformes, Perciformes and Zoarciformes), suborders (Ophidioidei and Zoarcoidei) and families (Brotulidae, Ophidiidae, Parabrotulidae and Zoarcidae). When initially described, *Parabrotula plagiophthalmus* Zugmayer, 1911 was assigned to the Brotulidae as one of three families (Brotulidae, Carapidae and Ophidiidae) in the perciform suborder Ophidioidei (Zugmayer, 1911). The genus most similar to *Parabrotula* is *Leucobrotula*, which was described and assigned to the Brotulidae four decades later (Koefoed, 1952). Major fish classifications of the time kept *Parabrotula* as a member of the Brotulidae (Jordan, 1923; Norman, 1966). The Brotulidae included all viviparous and all oviparous (except for Carapidae and Ophidiidae) genera until the classification by Cohen and Nielsen (1978) in which the viviparous and oviparous genera were separated in two suborders – the Bythitoidei and Ophidioidei respectively.

In Greenwood et al. (1966), the suborders of Ophidioidei and Zoarcoidei were moved from the Perciformes to the Gadiformes. Subsequently, *Leucobrotula* and *Parabrotula* were reassigned from the Brotulidae to Zoarcidae by Nielsen (1968). The reasoning was that the two genera have only one fin ray for each vertebra, which is characteristic for the Zoarcidae, while the fin rays outnumber the vertebrae in all ophidiiform genera. *Leucobrotula* and *Parabrotula* were placed in a new zoarcid subfamily, Parabrotulinae (Nielsen, 1968). The classification of *Leucobrotula* and *Parabrotula* as zoarcoids was supported by various authors (Cohen and Nielsen, 1978; Nelson, 1984; Nielsen and Merrett, 1990; Smith and Heemstra, 1986) and the newest species described, *Parabrotula tanseimaru* Miya and Nielsen, 1991, was assigned to the Zoarcoidei. Re-evaluation of Parabrotulidae as an ophidiiform or zoarcoid family by Anderson (1994) concluded that parabrotulids are closest to the ophidiiforms since the caudal structure, the paired nostrils and the bilobed ovary of parabrotulids differ from that of the zoarcids. While Nielsen et al. (1999) did not consider the Parabrotulidae to be an ophidiiform taxon, Nelson (1994, 2006) placed Parabrotulidae in the Ophidiiformes. Recently the question of parabrotulid evolutionary relationships was summarized as "The issue of their correct phylogenetic position remains unresolved because of a lack of recent detailed studies and because broad-scale molecular phylogenies such as that of Betancur-R et al. (2013) have not sampled them" (Nelson et al., 2016).

In this paper, we examine molecular sequence data generated from *Parabrotula tanseimaru* Miya and Nielsen, 1991 and place that in the context of a molecular phylogeny utilizing data from Betancur-R et al. (2013) to determine the phylogenetic placement of this mysterious family.

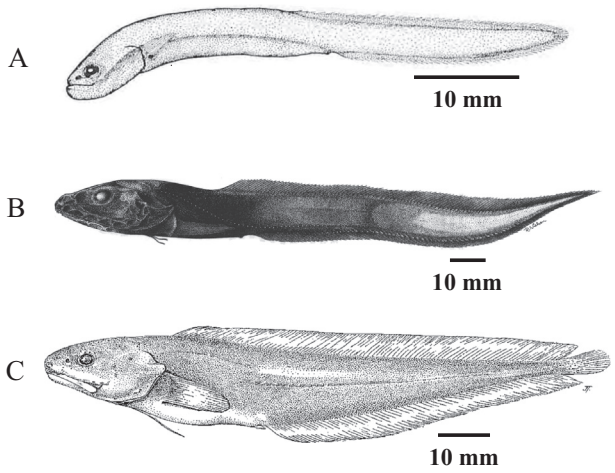

Fig. 1. Representative illustrations of *Parabrotula tanseimaru* and potential near relatives with 10 mm scale bars included. (A) Illustration of *Parabrotula tanseimaru* adapted from Miya and Nielsen (1991). (B) Illustration of a representative zoarcoid from the Zoarcidae, *Lycodes adolfi* (Nielsen and Fosså, 1993). (C) Illustration of a representative ophidiiform from the Bythitidae, *Brosmophysis marginata* (Ayres, 1854).

2. Methods

2.1. Sample collection, DNA extraction and sequencing

A single individual of *Parabrotula tanseimaru* was examined for this paper. The specimen was collected during a cruise of R/V Tansei-maru (KT-01-1) from a fixed station at the center of Sagami Bay (35°00′N, 139°20′E, ca. 1500 m depth) on March 6, 2001. An Isaacs-Kidd Midwater Trawl with a mesh size of 0.67 mm was towed obliquely with sampling depths from 0 to 890 m and the specimen placed in 100% ethanol immediately after collection. The specimen was 41 mm SL and assigned the Chiba Natural History Museum and Institute catalog number CBM-ZF 10557.

Total genomic DNA from preserved tissue was extracted with a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Inc., www.qiagen.com) following the manufacturer's protocol. A Sanger derived partial Cytochrome Oxidase I (COI) sequence was generated with the primer pair L6199-COI and H6855-COI (Miya and Nishida, 1999). Amplification from total genomic DNA with short PCR followed the reaction conditions and PCR profile of Miya and Nishida (1999) with the exception that TaKaRa EX Taq polymerase (TaKaRa Bio Inc., www.takara-bio.com) was used. High-throughput sequencing of the extracted DNA was conducted by preparing 200 ng of DNA for Illumina sequencing with a KAPA HyperPlus Kit (KAPA Biosystems Inc., www.kapabiosystems.com). The library was sequenced as one-third of a run on an Illumina MiSeq with 300 base pair paired-end sequencing and version 3 chemistry.

2.2. Raw high-throughput sequence data quality control

Raw sequence data were processed for quality with Trimmomatic version 0.32 (Bolger et al., 2014) providing Nextera adapters for trimming and with the following other specifications LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 and MINLEN:36. Examination of the data with FastQC version 0.11.2 verified the data were cleaned sufficiently. To check for overlapping paired end reads, FLASH version 1.2.11 (Magoč and Salzberg, 2011) was used with maximum overlap (-M) set to 250 base pairs. The merged reads and unpaired reads were then retained for a mapping based assembly.

2.3. High-throughput sequence data assembly

A mapping based assembly was chosen to focus on specific phylogenetic loci. To find potential loci for mapping, a near relative of *Parabrotula* was first identified. The newly determined COI sequence (GenBank Accession KY509536) was searched against the NCBI nucleotide reference database with BLASTN megablast (Altschul et al., 1997; Morgulis et al., 2008). Affinities were indicated to ophidiiform taxa from the Bythitidae (best match

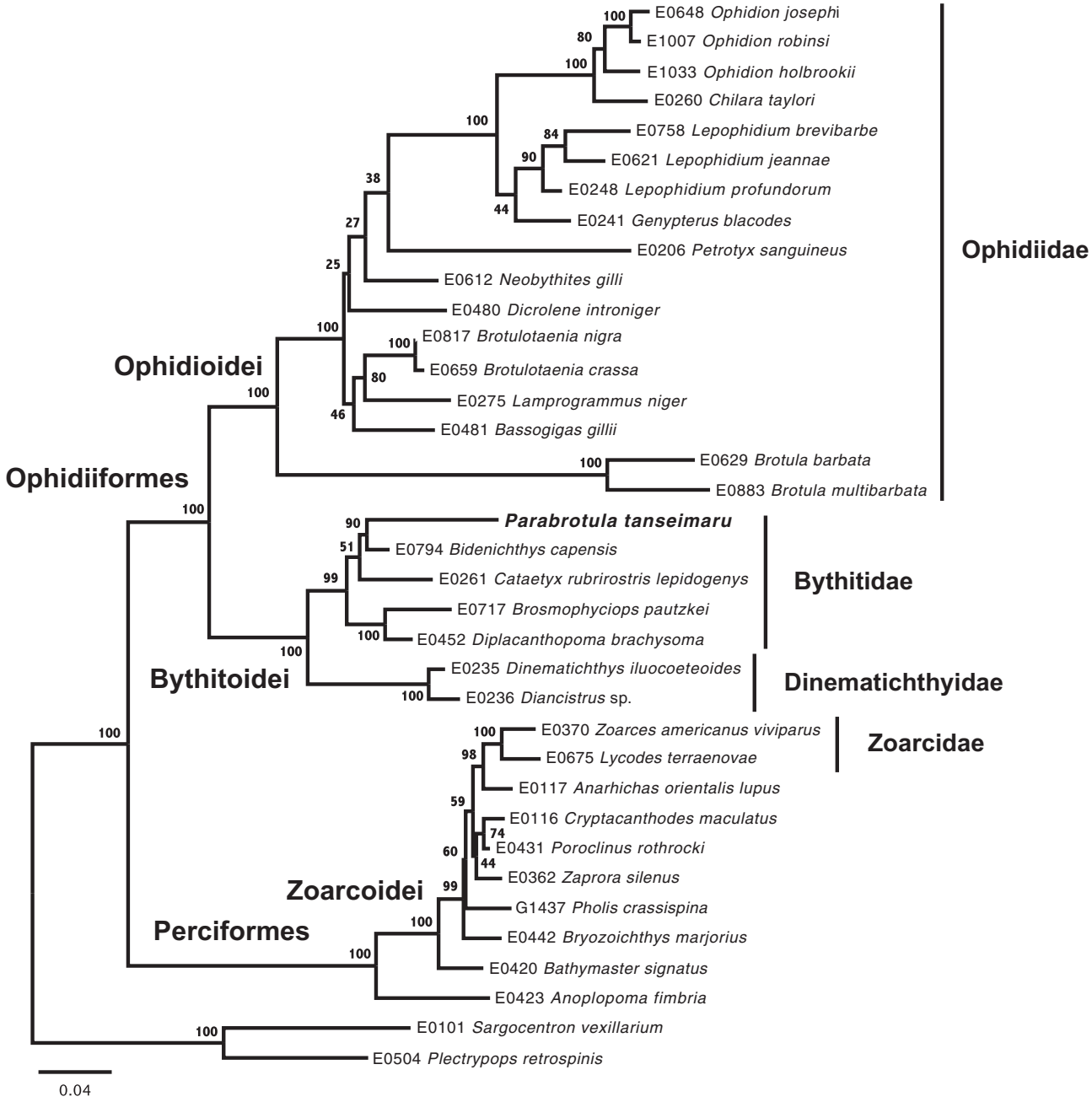

Fig. 2. A Maximum-Likelihood (ML) phylogenetic hypothesis of the evolutionary relationships of *Parabrotula tanseimaru*. The phylogenetic tree was generated from the alignment of ten genetic loci modeled under five separate partitions as determined by Partition Finder (described in Table 2). All three codon positions of the protein coding genes ($1_N2_N3_N$) and the ribosomal 16S unit were included in the alignment. Nucleotide evolution was modeled under a General Time Reversible (GTR) model of sequence evolution with Gamma distributed rate variation (Γ). Values at nodes indicate boostrap support values. The tree is rooted with holocentriform outgroup taxa. *Parabrotula tanseimaru* is most closely related to *Bidenichtys* (Ophidiiformes, Bythitidae, bootstrap support = 90%) not any zoarcoid. Taxon names are prepended with Euteleost Tree of Life identifiers, except the newly determined sequence data from *Parabrotula tanseimaru*. Two species epithets indicates that sequences derived from the two species were combined to generate genus-level composite taxa for analysis (Betancur-R et al., 2013).

Diplacanthopoma brachysoma, e-value 0.0, percent similarity 82%). The most complete set of bythitid genetic loci from Betancur-R et al. (2013) with the Euteleost Tree of Life Identifier E0261, *Cataetyx rubrirostris/lepidogenys,* was selected to provide seeds for mapping assembly. Sequences for E0261 were either generated from *C. rubrirostris* or *C. lepidogenys* and were previously merged to form a more complete dataset by Betancur-R et al. (2013). If a gene was not available from either of these taxa, an alternative taxon from the same order was selected to provide a gene sequence for a seed.

An initial mapping step was performed with Mira version 4.0.2 (Chevreux et al., 1999), then a baiting and iterative mapping approach was conducted with MITObim version 1.7 (Hahn et al., 2013).

2.4. Sequence alignment

Resulting assemblies for each seed were combined with an aligned data matrix consisting of potential relatives including zoarcoids and ophidiiforms available at http://dx.doi.org/10.5061/dryad.c4d3j (Betancur-R et al., 2013). The unaligned data from Betancur-R et al. (2013) is available separately from GenBank. Sequence data from relatives of Zoarcidae is available from the

following families: Anarhichadidae, Anoplopomatidae, Bathymasteridae, Cryptacanthodidae, Pholidae, Stichaeidae (not monophyletic) and Zaproridae (Betancur-R et al., 2013). The most representative sequence in terms of completeness of gene sequences was chosen from each family including the two independent lineages of stichaeids and the most divergent lineages of Zoarcidae. To represent ophidiiforms, all individuals from Betancur-R et al. (2013) were included in the data matrix. Outgroup sequences from holocentriform taxa were chosen based on overall completeness and representing the widest divergence within that lineage. New sequences from *Parabrotula* were aligned to the existing alignments with MAFFT version 7.130b (Katoh et al., 2002; Katoh and Toh, 2008). Alignments were subsequently evaluated for quality, translation into acids for protein coding sequences, and long ends from *Parabrotula* trimmed in Mesquite version 3.04 (Maddison and Maddison, 2011). Three data sets were generated through different treatments of the third codon positions of protein coding genes. All codon positions were included ($1_N2_N3_N$), third codon positions were recoded as purines or pyrimidines (RY-coding, $1_N2_N3_{RY}$) or third codon positions were excluded (1_N2_N).

2.5. Tree construction

Partitioning schemes for each data set ($1_N2_N3_N$, $1_N2_N3_{RY}$, 1_N2_N) were generated with PartitionFinder version 1.1.1 (Lanfear et al., 2012). Protein coding genes were divided by codon positions, while 16S RNA was treated as a single possible partition. Branches were linked and a (GTR) model with a Gamma distributed rate variation (Γ) was specified. The best partitioning scheme was chosen by the Bayesian information criterion (BIC) with a greedy searching scheme. For each data set, the best partition was specified for a tree search with partitioned Maximum Likelihood (ML) analysis within the Randomized A(x)ccelerated Maximum Likelihood (RAxML) program version 8.2.9 (Stamatakis, 2006; Stamatakis and Ott, 2008). Confidence of inferred relationships was established by conducting bootstrap replicates with the rapid bootstrap algorithm (-f a) and automatic stopping specificied (-N autoMRE).

2.6. Hypothesis testing

With the objective partitioning scheme defined by ParitionFinder above, an unconstrained best tree was generated with RAxML version 8.2.9 with the $1_N2_N3_N$, $1_N2_N3_{RY}$ and 1_N2_N data sets. For each data set a constrained tree with the alternative placement of *Parabrotula* was then made by requiring *Parabrotula* to be most closely related to zoarcids in the dataset (-g option in RAxML). Per site likelihoods were calculated by supplying both the best tree and constrained tree to RAxML (–f g and –z treefile). The per site likelihoods were supplied to the subprograms of the CONSEL package version 0.20 (makermt, consel, catpv) (Shimodaira and Hasegawa, 2001) to test the two hypotheses of *Parabrotula* as an ophidiiform or zoarcoid.

3. Results and discussion

3.1. Raw sequence quality control

Of the 7,056,558 initial read pairs, 58.9% remained after trimming as paired reads. Forward only reads accounted for 39.6% of read pairs and reverse only for 0.2%. Completely removed read pairs were 1.2% of the total. FLASH combined 95% of the paired reads into a single read resulting in approximately 7 Million unpaired reads applied to the mapping based assembly.

Table 1
Gene abbreviation, full name, and species of origin for nucleotide sequences utilized as seeds for mapping based assembly. The GenBank accession for each seed is given as well the accession assigned to the *Parabrotula tanseimaru* sequences.

Gene abbreviation	Gene full name	Seed species	Seed accession	*Parabrotula* accession
16S	16S ribosomal RNA gene	*Cataetyx rubrirostris*	KJ010623	KY509530
ENC1	Ectodermal-neural cortex 1-like protein	*Cataetyx lepidogenys*	KF139419	KY509531
FICD	Fic domain protein	*Cataetyx rubrirostris*	KC825526	KY509532
KIAA1239	Leucine-rich repeat and WD repeat-containing protein	*Cataetyx rubrirostris*	KC826351	KY509533
MYH6	ClepA cardiac muscle myosin heavy chain 6	*Cataetyx lepidogenys*	JX190481	KY509534
PANX2	Pannexin 2	*Ophidion robinsi*	KC827781	KY509535
PTCHD4	Patched domain-containing 4	*Cataetyx rubrirostris*	KC828567	KY509540
RAG1	Recombinase activating protein 1	*Cataetyx lepidogenys*	JX190868	KY509537
RHOD	Rhodopsin	*Cataetyx laticeps*	EU637947	KY509538
RIPK4	Receptor-interacting serine-theronine kinase 4	*Ophidion robinsi*	KC829277	KY509539

Table 2
For each data set in the study as named by the treatment of third codon positions in protein coding genes, the total number of characters, distinct alignment patterns, and number of partitions as defined by PartitionFinder are given. The composition of each partition is presented with abbreviations matching Table 1, the codon position is appended to the gene name (*i.e.* RAG11 is the first codon position of RAG1).

Data set	Number of characters	Distinct alignment patterns	Partitions	Preferred partitioning scheme composition
$1_N2_N3_N$	9259	3255	1	16S
			2	ENC11, KIAA1, MYH62, PTCHD11, RAG12, RH2
			3	ENC12, FICD2, KIAA2, PANX22, PTCHD12, RIPK42
			4	ENC13, FICD3, KIAA3, MYH63, PANX23, PTCHD13, RAG13, RH3, RIPK43
			5	FICD1, MYH61, PANX21, RAG11, RH1, RIPK41
$1_N2_N3_{RY}$	9259	2325	1	16S
			2	ENC11, FICD1, KIAA1, MYH61, PANX21, RAG11, RH1, RIPK41
			3	ENC12, FICD2, KIAA2, PANX22, PTCHD11, PTCHD12, RAG12, RH2, RIPK42
			4	ENC13, FICD3, KIAA3, MYH62, MYH63, PANX23, PTCHD13, RAG13, RH3, RIPK43
1_N2_N	6746	1662	1	16S
			2	ENC11, KIAA1, MYH62, PTCHD11, RAG12, RH2
			3	ENC12, FICD2, KIAA2, PANX22, PTCHD12, RIPK42
			4	FICD1, MYH61, PANX21, RAG11, RH1, RIPK41

3.2. Sequence assembly, alignment, and analysis

MITObim reached stationarity after eight iterations, from which we identified ten well-assembled loci accessioned into GenBank (Table 1, Supplemental Table S1). The sequence data from *Parabrotula* is combined with thirty-three potential relatives and two outgroup taxa (Supplemental Table S1) with the characteristics and optimal partitioning scheme of each data set presented in Table 2. The molecular phylogenetic analyses resolve the two suborders of Ophidiiformes Bythitoidei and Ophidioidei with high support (bootstrap support BS $\geqslant$ 99%) across all three data sets. The monophyly and placement of Ophidiidae, Dinematichthyidae, and Bythitidae are consistent and highly supported (BS $\geqslant$ 99%) across all three data sets.

The molecular phylogenetic analyses also place *Parabrotula* within the Bythitidae for all data sets, the $1_N2_N3_N$ data set results are presented in Fig. 2. The placement of *Parabrotula* as a zoarcoid is rejected in favor of a placement as an ophidiiform with the approximately unbiased test, $1_N2_N3_N$ p – value 4×10^{-90}, $1_N2_N3_{RY}$ p – value 2×10^{-45}, 1_N2_N p – value 4×10^{-49} (Shimodaira, 2004).

3.3. Molecular evidence that parabrotulidae is an ophidiiform taxon

Two different approaches, BLASTN megablast of a Sanger sequenced mitochondrial DNA fragment, and a mapping based assembly and phylogenetic analysis from ten independent loci, indicate that Parabrotulidae is an ophidiiform taxon. The alternative hypothesis, Parabrotulidae as a zoarcoid, is strongly rejected through statistical testing. *Parabrotula* in the phylogenetic analysis is a member of Bythitidae within the Ophidiiformes.

4. Conclusions

While the Parabrotulidae demonstrates some similarity to zoarcoids by the presence of only one fin ray for each vertebra, it is possible that this convergence arose due to the large reduction in body size that has occurred in this family (maximum size of 60 mm SL). Other morphological characteristics such as caudal structure, paired nostrils and a bilobed ovary (Anderson, 1994) as well as the phylogenetic analysis presented here indicate that parabrotulids are ophidiiforms. The phylogenetic analyses place Parabrotulidae within the suborder Bythitoidei, which is known to be viviparous as is *Parabrotula tanseimaru* (Miya and Nielsen, 1991; Møller et al., 2016). The other suborder of Ophidiiformes, Ophidioidei is oviparous. Recently, the morphological distinct Aphyonidae (Ophidiiformes) were demonstrated to form a monophyletic clade within Bythitidae, and the family was not maintained (Møller et al., 2016). Our results indicate that another family level taxon, Parabrotulidae (including the genera *Leucobrotula* and *Parabrotula*), should be considered a monophyletic clade within Bythitidae.

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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.2017.01.016.

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