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Molecular phylogeny and new classification of the genera *Eulophias* and *Zoarchias* (PISCES, Zoarcoidei)

Hyuck Joon Kwun, Jin-Koo Kim *

Department of Marine Biology, Pukyong National University, 599-1 Daeyeon 3-Dong, Nam-gu, Busan 608-737, Republic of Korea

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ABSTRACT

Morphological and osteological studies of the Zoarcoidei group have previously been undertaken, but the group (especially the genera *Eulophias* and *Zoarchias*) still remains enigmatic. Therefore, we conducted molecular phylogenetic studies on the two genera *Eulophias* and *Zoarchias* using two mitochondrial (16S rRNA and COI) and two nuclear genes (RAG2 and RNF213). Our phylogenetic analysis supported the monophyly of the suborder level of the Zoarcoidei, but rejected the previous morphology- and osteology-based classification hypotheses regarding the two genera. Conflict between mtDNA and nDNA phylogenies within the genus *Eulophias* implies that the genus shows a complicated relationship such as hybridization in the process of the evolutionary history. The genetic distances between the *Eulophias* (or *Zoarchias*) and other Zoarcoidei spp. were the greatest, showing different family-level affiliations. In addition, the mtDNA topology showed the two genera were clearly separated from each other as well as from the families Stichaeidae and Zoarcidae. Considering the new molecular phylogeny, we suggest a new classification for the two genera: (1) *Eulophias* belongs to a new family named as the Eulophiidae; (2) *Zoarchias* belongs to the family Neozoarcidae (sensu Radchenko et al., 2012b) rather than to Stichaeidae and Zoarcidae.

1. Introduction

The suborder Zoarcoidei is one of the largest groups within the Perciformes order of fishes, comprising approximately 9 families, 95 genera, and 340 species worldwide; the only common morphological characteristic known that unites this group is the presence of a single nostril (Nelson, 2006). Although morphological and osteological studies of the Zoarcoidei have been undertaken (Anderson, 1994; Makushok, 1958; Yatsu, 1985), the group remains enigmatic (Kartavtsev et al., 2009; Kim and Kang, 1991; Mecklenburg and Sheiko, 2004; Nelson, 2006). Two species of the genus *Eulophias* and 8 species of the genus *Zoarchias*, which belong to the subfamily Neozoarcinae within the family Stichaeidae (sensu Anderson, 1994), are representative of taxonomic groups of uncertain status (Kimura and Sato, 2007; Kwun and Kim, 2012; Mecklenburg and Sheiko, 2004; Yamanaka et al., 2012).

Smith (1902) and Jordan and Snyder (1902) originally distinguished these two genera as belonging to separate subfamilies within the Blenniidae (Table 1). The genus *Eulophias* was placed within the Eulophiinae, based on its members having a very slender elongate body, with many spines and few soft rays in the dorsal fin, and *Zoarchias* was placed within the Neozoarcinae, based on its members having a moderately elongate body and more soft rays than spines in the dorsal fin. Subsequently, Makushok (1958, 1961) moved *Eulophias* to the Stichaeidae based on external features, but moved the genus *Zoarchias* to the Zoarcidae, based on the caudal skeletal structure, sensory system, and mouth apparatus, and maintained the previous subfamilies (Table 1). However, Anderson (1994) suggested that the two genera be placed together in the subfamily Neozoarcinae within the Stichaeidae, based on the anterior part of the dorsal fin and the presence of a spinous first anal fin ray, gill membranes free from the isthmus, the parhypural fused to 1 + 2 hypurals, and the fifth hypural being absent (Table 1). The taxonomic position of the two genera has remained unclear (Hatooka, 2002; Kimura and Sato, 2007; Lindberg and Krasyukova, 1975; Mecklenburg and Sheiko, 2004), suggesting the need for a molecular phylogenetic study of the two genera to resolve their relationships and classification.

Many studies have considered the morphology and osteology of the Zoarcoidei group (Hilton and Kley, 2005; Voskoboinikova et al., 2010; Zemnukhov, 2012), and their phylogeny (Chereshnev et al., 2011; Kartavtsev et al., 2009; Radchenko et al., 2008, 2009, 2010a, 2010b, 2011, 2012a, 2012b). Radchenko et al. (2012b) treated *Neozoarces* (one of four genera within the Neozoarcinae in the Stichaeidae, sensu Anderson, 1994) as a member of a new family (Neozoarcidae). In addition, Zemnukhov (2012) regarded the genus

* Corresponding author. Fax: +82 51 629 5931.
E-mail addresses: kwunhj@hotmail.com (H.J. Kwun), taengko@hanmail.net (J.-K. Kim).

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Table 1
A morphology-based classification hypothesis for four genera of Neozoarcinae.

	Suborder	Family	Subfamily	Genus
Jordan and Snyder (1902)	Blennioidei	Blenniidae	Eulophiinae	*Eulophias*
			Neozoarcinae	*Neozoarces*
				Zoarchias
Makushok (1958, 1961)	Blennioidei	Stichaeidae	Azygopterinae	*Azygopterus*
			Eulophiinae	*Eulophias*
		Zoarcidae	Neozoarcinae	*Neozoarces*
				Zoarchias
Anderson (1994)	Zoarcoidei	Stichaeidae	Neozoarcinae	*Azygopterus*
				Eulophias
				Neozoarces
				Zoarchias

Azygopterus as a member of the Azygopterinae in the Stichaeidae (sensu Makushok, 1958). However, there have been no studies of the phylogenetic relationships of the genera *Eulophias* and *Zoarchias*, except for new species (Kimura and Sato, 2007; Kwun and Kim, 2012) and re-descriptions (Yamanaka et al., 2012). Therefore, the aim of the present study was to clarify the molecular phylogenetic relationships of the genera *Eulophias* and *Zoarchias*, based on mitochondrial and nuclear DNA.

2. Materials and methods

2.1. Specimen collection

Three species of the genus *Eulophias* (*E. koreanus*) and *Zoarchias* (*Z. major*, *Z. uchidai*) were collected from the southern waters of Korea by bottom-trawl and hand net in 2008–2012, respectively. Comparative species, 8 species of Zoarcidae (*Bothrocara hollandi*, *Davidijordania poecilimon*, *Lycodes japonicus*, *L. nakamurae*, *L. sadoensis*, *L. tanakae*, *L. toyamensis*, and *Zoarces gillii*), 14 species of Stichaeidae (*Anisarchus macrops*, *Askoldia variegata*, *Bryozoichthys lysimus*, *Chirolophis japonicus*, *C. wui*, *Dictyosoma burger*, *Dictyosoma tongyeongensis*, *Ernogrammus hexagrammus*, *Lumpenella longirostris*, *Lumpenus sagitta*, *Opisthocentrus tenius*, *Stichaeopsis epallax*, *Stichaeus grigorjewi*, and *S. nozawai*), and 3 species of Pholidae (*Pholis crassispina*, *P. fangi*, *P. nebulosa*) were collected from the southern and eastern coastal waters of Korea in 2008–2012. *Sillago japonica* of the Sillaginidae under Perciformes, was selected as an outgroup species. Species identification followed Hatooka (2002) and Kim et al. (2005), and one specimen of each species were used for the molecular analysis. Voucher specimens used in present study were deposited at the Pukyong National University (PKU) (Table 2).

2.2. DNA extraction, PCR, and sequencing

Total DNA was extracted from muscle tissue using Chelex 100 Resin following the manufacturer's protocol. The 16S ribosomal RNA (16S rRNA) and Cytochrome c Oxidase subunit I (COI) of mitochondrial DNA and Recombination Activating Gene subunit 2 (RAG2) of nuclear DNA were amplified by PCR with universal primers, and the Ring Finger protein 213 (RNF213) was amplified with new specific primers designed from data downloaded from the National Center for Biotechnology Information GenBank database (NCBI). Primers used for amplification are listed in Table 3. Each PCR was carried out in a MJ Mini Thermal Cycler PTC-1148 (Bio-Rad) with a total volume of 50 μl, 10 μl of total DNA, 5 μl of 10× reaction buffer, 4 μl of 2.5 mM dNTP, 1 μl of each primers set and 5 μl of FR-Taq polymerase (Biomedic). PCR cycling parameters were performed using the following protocol for each gene: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The products of PCR amplification were purified using ExoSAP-IT (United States Biochemical Corporation). PCR products were cycle sequenced using the ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems Inc.) and run on a ABI 3730XL Sequencer (Applied Biosystems Inc.).

2.3. Sequence alignment and phylogenetic analyses

Sequences of each region were modified and independently aligned using ClustalW (Thompson et al., 1994) in BioEdit ver. 7 (Hall, 1999). No indels were detected except within 16S rRNA region. The aligned sequences were compiled into three data matrices, mitochondrial, nuclear, and combined datasets. Only the mitochondrial DNA data matrix included data from the six species of other families within the Zoarcoidei that were downloaded from the NCBI GenBank database for comparison (Anarhichadidae, Bathymasteridae, Cryptacanthodidae, Neozoarcidae, Ptilichthyidae, and Zaproridae) (Table 2). Genetic distances of each region were calculated using Kimura-2 parameter (Kimura, 1980) and *P*-distance in MEGA5 (Tamura et al., 2011). Tests for substitution saturation were investigated by plotting numbers of transitions and transversions of each region against pairwise genetic distances.

Bayesian inference (BI) was performed on the mitochondrial, nuclear, and combined DNA sequences. The best-fit models of each sequences evolution were selected using MrModeltest 2.3 (Nylander, 2004). The selected models were GTR + I + G (Tavaré, 1986) with a proportion of invariable sites of 0.5672 and a gamma distribution with a shape parameter of 0.4927 for mitochondrial DNA, K80 + I + G (Kimura, 1980) with a proportion of invariable sites of 0.3552 and a gamma distribution with a shape parameter of 0.8520 for nuclear DNA, and GTR + I + G (Tavaré, 1986) with a proportion of invariable sites of 0.5379 and a gamma distribution with a shape parameter of 0.3540 for combined data. Phylogenetic trees were constructed using BEAST 1.7.2 (Drummond and Rambaut, 2007), and importing taxa data and specifying the evolutionary models were done using BEAUti (Drummond et al., 2012). The Markov chain Monte Carlo (MCMC) analyses of each region were run for 10 million generations, sampling trees every 1000 generations. The consensus trees along with posterior probabilities were visualized using FigTree Ver. 1.3.1. (Rambaut, 2010).

Maximum likelihood (ML) and Bayesian Estimation of Species Trees (BEST) analyses (Liu, 2008) were performed on the mitochondrial DNA sequences. The ML tree was constructed with HKY + I + G (Hasegawa et al., 1985) model and 1000 bootstrap replications using MEGA 5 (Tamura et al., 2011). The BEST analysis was run for 1 million generations with GTR + I + G (Tavaré, 1986) model.

3. Results

3.1. Sequence data and divergences

Mitochondrial and nuclear DNA sequences for 18 genera (including the genera *Eulophias* and *Zoarchias*) and 28 species of the Zoarcoidei were obtained. For *Z. uchidai* only mitochondrial DNA sequence data were available. The combined mitochondrial DNA sequences consisted of 1187 base pairs (bp), corresponding to the combination of 591 bp from 16S rRNA and 596 bp of the COI gene. The nuclear DNA sequences consisted of 1343 bp, corresponding to 744 bp of the RAG2 gene and 599 bp of the RNF213 gene. Overall (with the exception of the outgroup), the mitochondrial DNA sequences included 259 parsimony informative sites (Pi), a transition to transversion ratio (Ti/Tv) of 2.84, and base