Hormone injection-induced maturity and insemination of the bronze puffer *Auriglobus modestus*

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Abstract : To improve breeding techniques for aquarium freshwater pufferfishes, hormone injection (hCG, 10 IU per g)-induced maturity and insemination of *Auriglobus modestus* from Thailand were undertaken, and morphological development of eggs and early stage larvae observed. Artificial insemination was performed two to five days after hormone injection. Although no fertilized eggs resulted from artificial insemination utilizing a wet (freshwater) method, an isotonic (sodium lactate ringer solution) method produced 458 fertilized eggs out of 787 eggs (fertilization rate 61.7%) spawned by one female, although only 8 larvae (1.7%) eventually hatched. Eggs were oval [long axis 2.05 ± 0.06 mm, short axis 1.68 ± 0.06 mm (n = 5)], translucent, demersal, and adhesive, and contained a number of small yellow oil globules. Oval eggs are unusual among pufferfishes, although common in the genus *Chonerinos*, an indication of the genetic closeness of the latter and *Auriglobus*, but also suggesting ecological similarity. The eggs hatched after 2 days, larvae one day after hatching [4.54 ± 0.34 mm NL (n = 9)] having a large yolk sac. However, the mouth remained unopened, the optic vesicle uncolored, and the pectoral fin membrane undeveloped, except for small knob-like rudiments. Seven days after hatching, larvae [5.22 ± 0.10 mm NL (n = 3)] had lost the yolk sac and acquired rudimentary soft rays in the pectoral, dorsal and anal fins. All larvae died after eight days. DNA barcoding comparisons (COI gene, 652 bp) of several pufferfish genera indicated a close genetic relationship of *Auriglobus* and *Chonerhinos*, being nested in the same clade.

Key words : *Auriglobus modestus*, induced breeding, hormone injection, artificial insemination, DNA barcoding

Introduction

An understanding of their reproductive biology is important for sustaining exhibitions of aquarium fishes. Fresh and brackish water pufferfishes include about 8 genera (41species) out of some 30 genera (200 species) of tetraodontids overall[1, 2]. Because most of the former are included in the aquarium trade[3], it is necessary to improve artificial breeding techniques, not only for aquarium exhibition but also for protection of natural ecosystems[4].

Fresh and brackish water pufferfishes have variable reproductive features. The African species *Tetraodon schoutedeni* has been reported as scattering demersal adhesive eggs during tandem swimming, males biting and clinging to the females[5]. The South American genus *Colomesus* is thought to spawn on flood plains during the wet season[6]. Among six Asian genera, three species of *Carinotetraodon* perform pair spawning, scattering small demersal adhesive eggs[7], and species of *Pao* and *Leiodon* are characterized by pair spawning of relatively large eggs in a single layered batch, subsequently protected by the male[4, 7]. *Dichotomyctere ocellatus* and *D. nigroviridis* have been reported to scatter many small demersal adhesive eggs[7, 8]. Limited reports have indicated that *Chonerhinos naritus* migrates from coastal to inland waters, spawning oval eggs[9, 10]. However, nothing is known of spawning in *Auriglobus* species, also inhabiting fresh to brackish water.

Southeast Asian *Auriglobus modestus* is often traded as an aquarium fish[3, 11]. From a taxonomic viewpoint, the genus *Auriglobus* shares many morphological features (i.e.,

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numbers of dorsal and anal fin rays, and vertebrae) with the genus *Chonerhinos*[12, 13], and had been included in the latter following its establishment by Bleeker[14], until Fraser-Brunner[15] separated them on the basis of adult size difference[16].

Because the reproductive features of fresh and brackish water pufferfish species of the same genus are often similar, the close genetic relationship of *Auriglobus* and *Chonerinos* might also be reflected by such similarity.

This paper reports some reproductive information for *A. modestus*, resulting from hormone injection-induced maturity and subsequent insemination undertaken at Osaka aquarium NIFREL in 2021.

Materials and Methods

Parental fish

Sixteen wild pufferfishes collected from Bangpakong River, Chachoeng Sao, Thailand, were purchased from a Japanese fish trader (Rio Co. Ltd.), ten in June 2019, and six in October 2020, although three of the formers died before reproductive experiments began in December 2019. The fish were separated into five closed circulating/filtering freshwater tanks at Osaka Aquarium NIFREL, each accommodating two or three individuals, to avoid any conflict or biting behavior [2 tanks: 90 cm (L) × 36 cm (D) × 45 cm (H); 2 tanks: 60 cm (L) × 36 cm (D) × 45 cm (H); 1 tank: 70 cm (L) × 70 cm (D) × 60 cm (H)]. Water temperature was maintained at 26 ℃, and pH between 7.59-7.69, the lighting regime being 1200 Lux 12 h per day (7:00-19:00). Half of the water was replaced once a week. Commercially prepared chironomid larvae and dried meal worms (Natural Pet Foods Co., Ltd.) were provided to fish satiation level once a day, with remaining food and excrement being removed daily.

Morphological identification

Parental fish were identified based on caudal peduncle height and the direction of skin spines below the pectoral fin, according to the identification key for five species of *Auriglobus* given by Roberts[13]. Total length, standard length and caudal peduncle height were measured to 0.1mm. Spines on the body surface of dead specimens were stained with Alizarin Red and observed under a stereoscopic microscope.

DNA extraction, PCR, and DNA sequencing

Partial nucleotide sequences of the mitochondrial cytochrome oxidase subunit I (COI) gene (652 bp) in the 16 parental fish (Amod01-Amod16) were sequenced and used for species identification by DNA barcoding. In addition, sequences of 4 additional pufferfish species (*Pao cochinchinensis, Tetraodon miurus, Arothron firmamentum, and Sphoeroides pachygaster*) were also determined in order to include them in the dataset for phylogenetic analysis. In each individual, genomic DNA was extracted from the right pectoral fin using the QuickGene DNA tissue kit S (KURABO). COI partial sequences were amplified by PCR reaction with a primer set slightly modified from those designed by Ivanova et al.[17] (COI-FishF2-t1: 5'-TCG ACTAATCATAAAGATATCGGCAC-3, COI-FishR2-t1-Amod: 5'-ACCTCTGGGTGGCCAAAGAATCAAAA-3). The PCR amplification method involved initial denaturation at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 52 °C for 40 sec, and extension at 72 °C for 1 min, using Ex-Taq DNA Polymerase (Takara Bio). PCR products were purified using Agencourt AMPure XP (Beckman Coulter) and sequenced on an automated DNA sequencer SeqStudio™ Genetic Analyzer (Thermo Fisher Scientific) using amplification primers and the BigDye Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific). The nucleotide sequences obtained in this study were submitted to the DNA Data Bank of Japan (DDBJ, Accession Nos. LC704694– LC704713).

Species identification by BLAST search and phylogenetic analysis

To confirm the species of the parental fish used in this study, a BLAST search was carried out against the NCBI nucleotide database (i.e., DDBJ/EMBL/GenBank), using COI partial sequences of each individual as a query. The species and sequences analyzed are summarized in Table 1.

Table 1. Species and accession numbers from NCBI nucleotide database (i.e., DDBJ/EMBL/GenBank) used for phylogenetic analysis based on COI partial sequences

Accession No.	Species	Reference
LC704694-703	Amod01-Amod10	Present study (2019)
LC704704-709	Amod11-Amod16	Present study (2020)
LC704710	*Pao cochinchinensis*	Present study
LC704711	*Tetraodon miurus*	Present study
LC704712	*Arothron firmamentum*	Present study
LC704713	*Sphoeroides pachygaster*	Present study
LC586271	*Pao suvattii*	Yamada et al.[26]
AP011917	*Auriglobus modestus*	Yamanoue et a1.[27]
JQ681769	*Auriglobus modestus*	Santini et al.[28]
AP011917	*Auriglobus modestus*	Yamanoue et a1.[27]
JQ681770	*Auriglobus nefastus*	Santini et al.[28]
JQ681848	*Chonerhinos naritus*	Santini et al.[28]
KF027531	*Chonerhinos naritus*	Santini et al.[29]
LC586270	*Pao abei*	Yamada et al.[26]
AP011938	*Pelagocephalus marki*	Yamanoue et a1.[27]
AP011936	*Omegophora armilla*	Yamanoue et a1.[27]
AP006743	*Canthigaster coronata*	Yamanoue et a1.[30]
AP011924	*Leiodon cutcutia*	Yamanoue et a1.[27]
AP011928	*Chelonodontops pleurospilus*	Yamanoue et a1.[27]
AP011923	*Tetraodon mbu*	Yamanoue et a1.[27]
JQ681839	*Pao palembangensis*	Santini et al.[28]
AP011925	*Pao cochinchinensis*	Yamanoue et a1.[27]
JQ681838	*Dichotomyctere nigroviridis*	Santini et al.[28]
AP011913	*Polyspina piosae*	Yamanoue et a1.[27]
AP009537	*Torquigener brevipennis*	Yamanoue et a1.[30]
AP006045	*Takifugu rubripes*	Yamanoue et a1.[31]
AP011919	*Carinotetraodon salivator*	Yamanoue et a1.[27]
AP009538	*Lagocephalus wheeleri*	Yamanoue et a1.[30]
AP011909	*Colomesus asellus*	Yamanoue et a1.[27]
AP006238	*Mola mola*	Yamanoue et a1.[32]

The phylogenetic analysis compared sequences of the 16 parental *Auriglobus* specimens (Amod01-Amod16), four newly sequenced pufferfish species (*Pao conchinchinensis, Tetraodon miurus, Arothron firmamentum,* and *Sphoeroides pachygaster*), and published data for 18 additional pufferfish species (21 individuals). The ocean sunfish (*Mola mola*) was used as an outgroup. Nucleotide sequences were aligned by MAFFT v7.392[18], and the best substitution model selected based on the Bayesian Information Criterion (BIC). A phylogenetic tree was then constructed by the maximum-likelihood (ML) method, the model selection and ML tree construction being carried out using the MEGA X program package[19]. The reliability of each tree node was assessed by the bootstrap method with 1,000 replicates.

Haplotype network

To examine the genetic relationship among COI haplotypes of the 16 parental individuals and those of closely related species (*Auriglobus modestus, A. nefastus,* and *Chonerhinos naritus*), a minimum spanning network[20] was constructed using the software package PopArt (http://popart.otago.ac.nz).

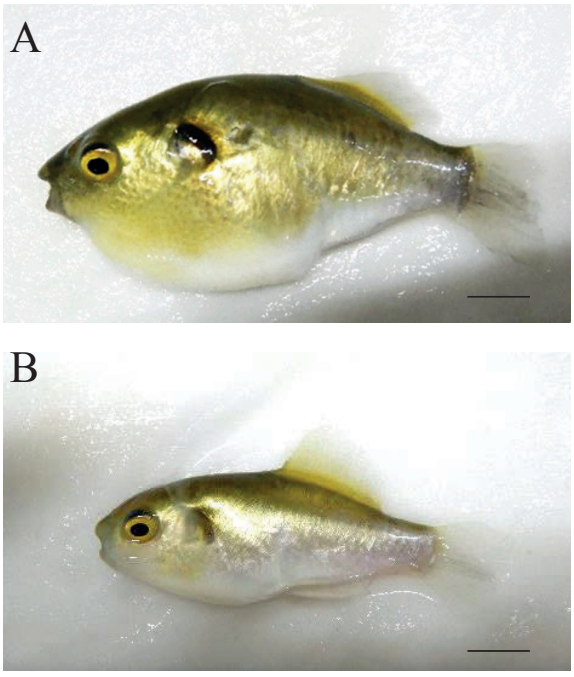

Fig. 1. Mature parental *Auriglobus modestus*: A, adult female; B, adult male. Each scale bar shows 1.0 cm

Hormone injection and artificial insemination

Mature females (79.6-91.9mm SL, 25.4-35.3g BW) were larger than mature males (54.9-64.5mm SL, 7.4-10.7g BW) (Fig. 1) and had the edge of the genital pore expanded. The nasal pores became black in both sexes. Human chorionic gonadotrophin (hCG, ASKA Pharmaceutical Co., Ltd.) was injected (10 IU per g)[4] on the left side of the caudal peduncle of specimens on four occasions, January 4th, and February 1st, 13th and 26th in 2021. Two to five days following such injection, artificial insemination (one female with one to four males) was carried out five times, January 7th, February 4th, 6th, 17th and March 1st, the first four utilizing a wet method (eggs and sperm mixed slowly in 5ml freshwater, with a quantity of additional freshwater added subsequently), and on the final occasion, an isotonic method [eggs and sperm mixed slowly in 5ml L-sodium lactate ringer solution (TERUMO SOLULACT), with a quantity of additional freshwater added subsequently].

Incubation, rearing and observation of eggs and larvae

The fertilized eggs were transferred into a 1.5 *l* bottle or 5 *l* plastic tank containing freshwater with 1ppm methylene blue. The water temperature was maintained at 26 ℃ throughout incubation and rearing. Half of the water was replaced on three occasions each day. The hatched larvae were reared in a 5 *l* plastic tank containing 1 ‰ brackish water, since the relatively low hatching rate of eggs in freshwater (described below) suggested that the latter was unsuitable for incubation and rearing. Half of the water was replaced twice a day. Larvae were fed with *Brachionus plicatilis* from two days after hatching.

Live eggs and larvae (unanesthetized) and fixed specimens were observed under a stereoscopic microscope equipped with a 3R Anyty WiFi Microscope ver.6.9.3 and photographed digitally. Total lengths (TL) and notochord lengths (NL) of live fishes were measured to 0.01mm.

Results

Identification of parental fish

Morphology: In preserved specimens, spines on the body below the pectoral fin were close-set and directed dorsally. In the living specimens, the caudal peduncle height was 14.4-17.1 % of the standard length.

DNA barcoding: Mitochondrial COI sequences in the 16 parental fish were identical or nearly so (pairwise nucleotide sequence identity among individuals: 99-100 %). The BLASTN search against the NCBI nr database revealed that the sequences corresponded very closely to those of *A. modestus, A. nefastus*, and *C. naritus*, the phylogenetic analysis indicating that the parental fish and latter species formed a monophyletic clade with 100 % bootstrap probability (Fig. 2). The phylogenetic and haplotype network analyses also showed that some of the COI sequences in the parental fish were wholly

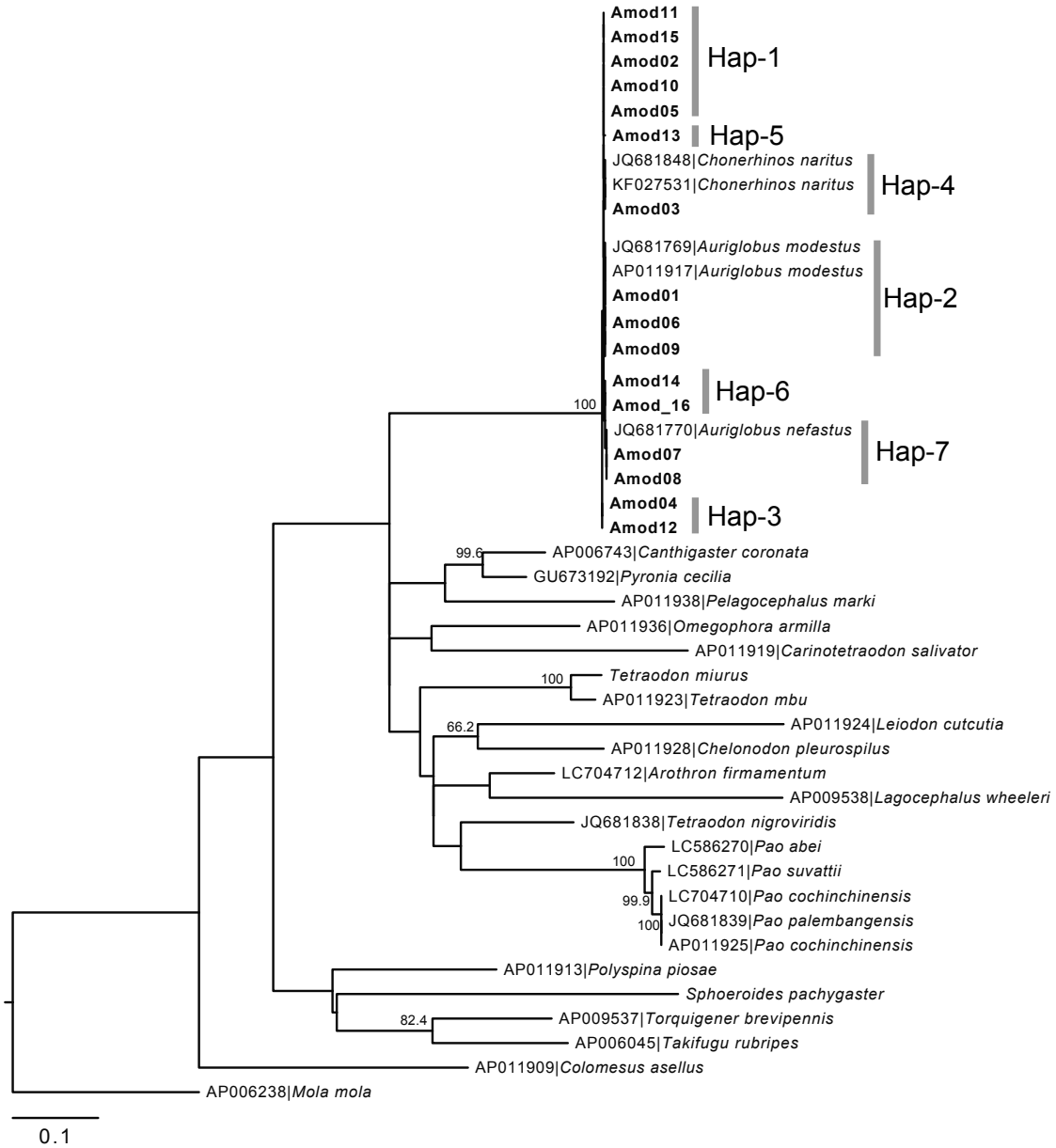

Fig. 2. Maximum likelihood tree of the COI partial sequences in the 16 parental fish (Amod01-Amod16, Hap-1-Hap-7) and 21 representative pufferfishes (3 sequenced in this study and 18 obtained from NCBI database). Numbers on tree nodes indicate bootstrap probabilities with 1,000 replicates. For simplicity, values only > 50 % and major clades are shown.

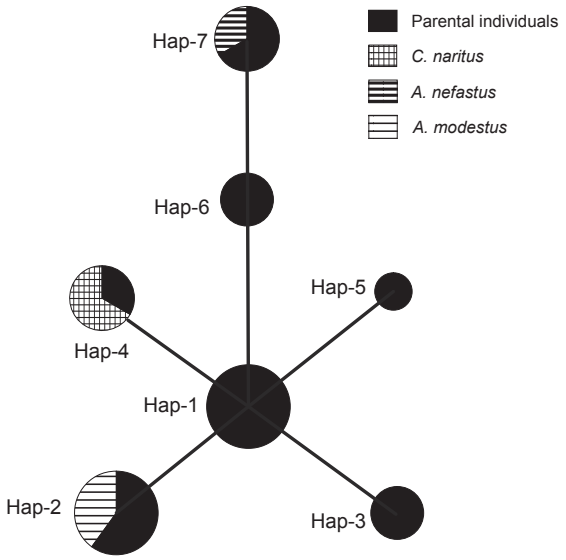

Fig. 3. Minimum spanning network for COI haplotypes (Hap-1-Hap-7) of the 16 parental fish and 3 closely related species.

identical to those in one of the other 3 pufferfish species, and the sequences were eventually separated into 7 haplotypes (Hap-1-Hap-7) that differed by 1-3 bps (Figs. 2 and 3). The sequences of *A. modestus* obtained from NCBI nucleotide database (AP011917 and JQ681769) corresponded to Hap-2, those of *C. naritus* (KF027531 and JQ681848) corresponded to Hap-4, and that of *A. nefastus* (JQ681740) corresponded to Hap-7 (Fig. 3).

Artificial fertilization and hatching

The mean number of spawned eggs was 625.6 (range 272-928, standard deviation SD 281.7, n = 5). No eggs were fertilized in the trials utilizing the wet method, whereas the isotonic method yielded 458 fertilized eggs out of 747 eggs spawned (61.3 %). Three days later, eight larvae hatched successfully (1.7 %).

Eggs and Larvae

Fertilized eggs: The oval eggs [mean long and short axes 2.05 (0.06 SD) and 1.68 (0.06 SD) mm, respectively, n = 5] were demersal, adhesive and transparent, with a mass of many small yellow oil globules (Fig. 4A; 3 hours after fertilization). Two days after fertilization, the embryo had already appeared with transparent optic vesicles and a few melanophores dorsally on the head,

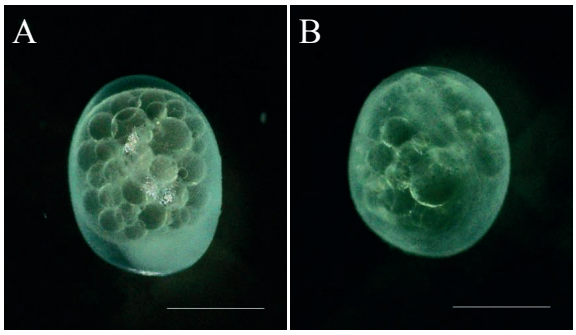

Fig. 4. Fertilized eggs: A, 3 hours after fertilization; B, 2 days after fertilization. Each scale bar shows 1.0 mm

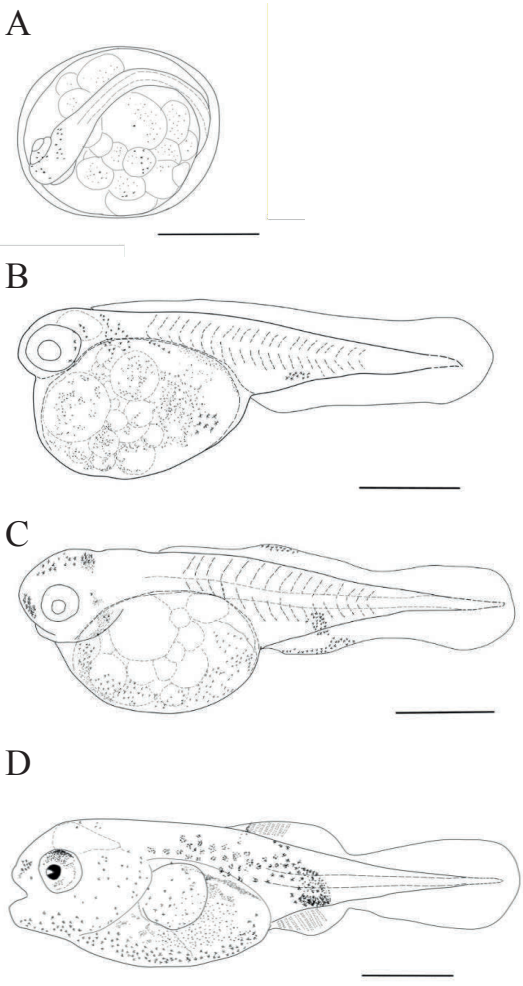

Fig. 5. Developmental series of *Auriglobus modestus*: A, fertilized egg 2 days after fertilization; B, 4.58 mm larva 1 day after hatching; C, 5.10 mm larva 2 days after hatching; D, 5.50 mm larva 7 days after hatching. Each scale bar shows 1.0 mm

eyes and gut (Figs. 4B, 5A). The eggs hatched 3 days after fertilization.

One day after hatching [Fig. 5B, 4.58mm TL; mean TL and NL 4.85 and 4.54 mm (0.35 and 0.34 mm SD), respectively (n = 3)], the yolk sac was large, with many oil globules remaining. The mouth remained closed, and optic vesicles were translucent. Small pectoral fin fold rudiments were observed in a fixed specimen. Fin folds were apparent along the dorsal aspect from the central nuchal region to the anus. Melanophores were present on the posterior surface of eyes, posterior-lateral sides of head, nuchal region, posterior-lower sides of yolk, lateral gut surface and ventral-middle edge part of the tail. Myotomes numbered 11 + 12 =23.

Two days after hatching [Fig. 5C, 5.10 mm TL; mean TL and NL 5.06 and 4.77 mm (0.03 and 0.04 mm SD), respectively (n = 3)], the yolk sac had diminished and the mouth opened, although feeding was not observed. The optic vesicles remained translucent. The dorsal and ventral fin folds had become slightly notched at the caudal peduncle. Melanophores were apparent on the snout, dorsal surface of the head, dorsal surface of the gut, ventral surface of the belly, and on the edges of the dorsal and anal fin folds.

Five days after hatching (4.51mm TL), some oil globules remained. The optic vesicles were black, and pectoral fin folds larger. Melanophores covered the lateral gut surface and the entire body, except the caudal peduncle. Feeding on *Brachionus* larvae was observed.

Seven days after hatching [Fig. 5D, 5.50 mm TL; mean TL and NL 5.47 and 5.22 mm (0.05 and 0.10 mm SD), respectively (n = 3)], the body was spherical, and the yolk sac completely absorbed. Rudimentary soft rays in the pectoral, dorsal and anal fins were apparent.

All larvae had died by eight days after hatching, the reason unknown.

Discussion

On the bases of morphological features, such as spine condition below the pectoral fin and caudal peduncle height, key characteristics for identifying *Auriglobus* species[13], the present parental fish were identified as *A. modestes*, the spines being close-set and directed dorsally, and the caudal peduncle height 13.5-14.9 % of standard length (other *Auriglobus* species have the spines less close-set and directed dorsoposteriorly or posteriorly, and the caudal peduncle height 10.1-13.9 % of standard length).

On the other hand, some of the seven COI haplotypes observed in the present parental fish corresponded to those in *A. modestus, A. nefastus* and *C. naritus*, obtained from the GenBank database, indicating either a close relationship of the former with the latter species, or problems of identification. However, on the basis of overall morphological and molecular data, all of the parental fish were identified as *A. modestus*.

In reality, *Auriglobus modestus* and *Chonerhinos naritus* are similar in spawning oval eggs, resulting in relatively undeveloped hatched larvae with a large yolk sac, unopened mouth, unpigmented optic vesicles, and without membranous pectoral fins (present study and previous reports[9, 10]). These features are not shared by other fresh- and brackish water pufferfish genera, such as *Tetraodon*[5], *Carinotetraodon*[7], *Dichotomyctere*[7, 8, 21], *Pao* [7], and *Leiodon*[4, 7], all of which spawn spherical eggs and have better developed hatched larvae. Their similarity in egg and larval characters apparently support both similar life-history aspects and/or genetic similarities between *A. modestas* and *C. naritus*.

Oval eggs are usually considered to be spawned by groups such as bitterlings (Cyprinidae) (spawning into bivalve shells), and gobies (Gobioidea) and damselfishes (Pomacentridae) (spawning adhesive eggs thickly onto a substrate), being relatively rare in open-water spawning fishes, except in species such as *Engraulis japonicus* and *Scarus* spp. The functional significance of the oval shape of such eggs is unknown[22-25].

On the other hand, the oval eggs of *A. modestus* and *C. naritus*[9, 10] are demersal and adhesive, and therefore of possibly differing ecological significance from those of *E. japonicus* and *Scarus* spp. However, this requires further investigation.

Dring the present study, eggs of *Auriglobus modestus*

could be fertilized only by utilizing the isotonic method. However, the hatching rate was low following freshwater incubation, probably indicating less than optimal rearing conditions for *A. modestus* eggs. On the other hand, the closely related species *Chonerhinos naritus* can be artificially fertilized and resultant larvae reared utilizing brackish water conditions (18 psu)[9, 10]. Because *A. modestus* is also distributed in rivers and estuaries[3], it is likely to spawn and develop in such conditions, like *C. naritus*. Future studies to establish an artificial breeding method for *A. modestus* should consider the salinity conditions best suited to insemination, incubation and rearing.

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Bronze puffer *Auriglobus modestus* のホルモン投与による人工催熟及び授精

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和文要旨：淡水フグ類の水族館における魚類の育成技術の向上のため、タイより入手した*Auriglobus modestus*のホルモン処理（hCG, 10 IU per g）による人工催熟および人工授精を実施し（処理後２- ５日目）、初期発育の観察を行った。親魚を含むフグ科魚類で行ったDNAバーコーディング（COI gene, 652 bp）より、これまで形態的に近縁と考えられてきた*Auriglobus*属と*Chonerhinos*属の2属は遺伝学的にも近縁であることが示唆された。5回実施した人工授精ではメス1尾あたり625.6±281.7 (*n*=5)の卵が得られ、湿導法では一切授精しなかった一方、等調法により１尾のメスの産卵した787卵中458卵が受精 (受精率61.7%), 8卵が孵化に至った (ふ化率1.7%)。卵は透明で、黄色い小油球を多数含んだ楕円形沈性付着卵で、卵形は長径2.05±0.06mm、短径1.68±0.06mmであった (*n* = 5)。なお、楕円形卵は*Chonerhinos*属と共通であり、両属の遺伝的近縁さのみならず初期生態的な類似性も示唆するが、フグ類では他に例を見ない特徴である。卵は2日で孵化に至り、1日齢の孵化仔魚は脊索長4.54 ± 0.34 mm (*n* = 9)で、未開口で眼胞は無色透明、大きな卵黄嚢を有し、瘤状の胸鰭原基が見られた。7日齢では脊索長5.22 ± 0.10 mm (*n* = 3)に達し、卵黄は消失し、胸鰭、背鰭、臀鰭に鰭条の原基を確認した。仔魚は8日齢で斃死した。