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SHORT REPORT

Morphological and genetic identification of formalin-fixed gobioid larvae and description of postflexion larvae of *Paragunnellichthys* sp. and *Ctenogobiops feroculus*

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Abstract
Gobioid larvae collected from the coast of Okinawa Island, southern Japan, were identified on the basis of a combination of morphological characters and a sequence of a hypervariable region of mitochondrial 12S ribosomal RNA (12S rRNA) gene (138–145 base pairs). A short-term formalin fixation technique enabled identification using both morphological and genetic methods. Thirteen of the 21 types of gobioid larvae assessed were identified to the species level. Additionally, we described the morphology of the postflexion larvae of wormfish *Paragunnellichthys* sp. and shrimp-associated goby *Ctenogobiops feroculus*, identified for the first time in their respective genera.

Keywords 12S rRNA · DNA barcoding · Molecular identification · Fish larvae · Goby

Introduction

Gobioid fish, suborder Gobioidei (Teleostei), is an important constituent of larval fish assemblages in tropical and subtropical coastal waters (e.g. Clarke 1991; Maeda and Tachihara 2014; Isari et al. 2017). Nevertheless, the understanding of early life history of gobies is poorly known (Borges et al. 2011). This lack of current knowledge can largely be attributed to the fact that gobioid larvae are difficult to identify.

Identification of fish larvae has been traditionally based on morphological characters, which are often not sufficient to identify larval fish to the species level (Ko et al. 2013). Recently, genetic methods called DNA barcoding have been used to identify fish eggs and larvae (e.g. Victor et al. 2009; Kawakami et al. 2010; Marancik et al. 2010; Kimmerling et al. 2018). This technique can distinguish even closely related species (e.g. Baldwin et al. 2009; Tawa et al. 2014). These genetic methods enable any researcher with expertise in molecular biology to achieve results of similar precision. However, there are certain limitations in identifying fish eggs and larvae by genetic methods such as mitochondrial introgression, the lack of reference sequences in the database, and misidentification of reference data (Kawakami et al. 2010; Leis 2015). Therefore, it is preferable to identify fish eggs and larvae using a combination of morphological and genetic methods.

The larval fixation technique substantially influences the accuracy and reliability of the identification results. Previous studies successfully applied both morphological and genetic methods using larvae fixed in 70–99% ethanol (e.g. Tawa et al. 2014; Leis et al. 2015) because formalin damages DNA (Hykin et al. 2015). However, larval fixation in highly concentrated ethanol may cause more severe sample tissue shrinkage than 5–10% buffered formalin (Cunningham et al. 2000), resulting in specimens unsuitable for morphological observation. Previous studies demonstrated that by appropriately shortening the formalin fixation step, mitochondrial DNA (mtDNA) of sufficient quality can be obtained for genetic analysis (Díaz-Viloria et al. 2005; Chakraborty

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et al. 2006). However, short-term formalin fixation method has not been applied to identify fish larvae using a combination of morphological and genetic approaches.

For genetic identification of fish species, partial sequences of mtDNA, such as cytochrome *c* oxidase subunit 1 (COI), 12S ribosomal RNA gene (12S rRNA), 16S ribosomal RNA gene (16S rRNA), and cytochrome *b* gene (cyt *b*), have been used (Teletchea 2009). Among these DNA markers, a hypervariable region of 12S rRNA is a short fragment (less than 200 bp) containing sufficient interspecific differences to identify fishes at the species level except for some closely related congeners (Miya et al. 2015), and has been used for metabarcoding environmental DNA (e.g. Yamamoto et al. 2017). The region capable of species identification with a short fragment would be suitable for genetic identification of specimens in which the DNA may have been damaged due to formalin fixation. Moreover, a large amount of 12S rRNA sequence data for Japanese gobioid species is available through the DDBJ/EMBL/GenBank (12S rRNA: 293 species; COI: 270 species; cyt *b*: 177 species; 16S rRNA: 144 species, https://www.ncbi.nlm.nih.gov/nuccore).

The aim of the present study was to identify gobioid larvae based on a combination of morphological characters and a sequence of a hypervariable region of 12S rRNA. To enable identification using morphological and genetic methods, we applied short-term formalin fixation to the larvae. We also describe in detail the morphologies of larvae from two gobioid genera identified for the first time.

Materials and methods

Specimens. Larval fish samples were collected with a light trap at Shinzato Fishing Port (26°42′08″N, 127°54′00″E) on the northern coast of Okinawa Island, southern Japan (see Oka and Miyamoto 2014). Sampling was conducted once or twice a month between April 2011 and October 2012. All samples were fixed in 10% seawater formalin immediately after collection. They were sorted and transferred into 70% ethanol within 24 hours. The larval specimens were preserved for 3–7 years without replacing the storage solution. Immature gobioid fish with scales and various cryptic *Schindleria* fish species (see Kon et al. 2007) were excluded from the present study.

Morphological identification. Gobioid larvae were distinguished on the basis of their morphological characters and identified to the lowest possible taxon according to the descriptions of Neira et al. (1998), Smith and Thacker 2000, Harada and Suharti (2000), Leis and Carson-Ewart (2004), Maeda and Tachihara (2005), Maeda (2008), Okiyama (2014), and Tran et al. (2018). Taxonomic nomenclature was according to Akihito et al. (2013). When a larva was identified only to the genus or family level, we used "sp." after the genus or family name. In case of more than one species, they were distinguished with different letters (e.g. sp. A and sp. B). Terminology for morphological characters and measurements followed that of Leis and Carson-Ewart (2004). Standard lengths (SL) were measured using a digital microscope (Keyence, VHX-1000) or a vernier caliper under a stereomicroscope (Nikon, SMZ645). The following characters of the specimens described were measured based on photographs taken using digital camera (Olympus, E-M1): body depth at the pectoral-fin base (BD), head length (HL), snout length (SnL), eye diameter (ED), pre-dorsal-fin length, preanal length, caudal-peduncle length (horizontal distance from posterior end of the dorsal-fin base to caudal-fin base) and caudal-peduncle depth (least vertical distance of caudal peduncle). Dorsal-, anal-, pectoral-, and pelvic-fin rays were counted under the stereomicroscope. Measurements were made the distances along the body axis on the two dimensional images.

Genetic identification. DNA sequencing was performed on at least one individual per type distinguished by its morphological characters. Total DNA was extracted from the right eyeball of each specimen by the HotSHOT method (Truett et al. 2000). A partial sequence of mtDNA 12S rRNA region was amplified by polymerase chain reaction (PCR) in a thermal cycler (Gene Atlas 322, Astec, Fukuoka, Japan) with the primer pairs MiFishUF (5'-GTCGGTAAA ACTCGTGCCAGC-3') and MiFishUR (5'-CATAGTGGG GTATCTAATCCCAGTTTG-3') (Miya et al., 2015). PCR was conducted with a KOD FX Neo DNA polymerase Kit (Toyobo, Osaka, Japan) in a 12.5-µl total volume: 6.25 µl of 2× PCR buffer, 0.5 µl of 10 µM of each primer, 2.5 µl of 2 mM dNTPs, 0.25 µl of KOD FX Neo, 0.25 µl of template DNA, and 2.25 µl dH_2O. The PCR cycling conditions were as follows: 94 °C for 2 min, 10 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 15 s, extension at 68 °C for 20 s, 25 cycles of denaturation at 98 °C for 10 s, annealing at 53 °C for 15 s, extension at 68 °C for 20 s, and a final extension at 68 °C for 2 min. Sequences were determined by Sanger Sequencing Service (FASMAC, Kanagawa, Japan). All sequences were deposited in DDBJ/EMBL/GenBank (Accession numbers LC515643–LC515665). Voucher specimens were deposited in the collection of the Okinawa Churashima Foundation Research Center (OCF).

The DNA sequences obtained for all representative larvae were compared with sequences deposited in DDBJ/EMBL/GenBank using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using the default parameters. Identification to the species level was considered as valid if more than 98.0% of the sequence corresponded to the sequence(s) of a single species. If more than 98.0% of sequence corresponded to those of more than two species, the larva was identified as belonging to a taxon that included all of these species.

Final identification. The final identification results for each larva were determined as follows. Case I: When the larva was identified to the same species or genus by the morphological and genetic methods, it was considered that there was no discrepancy, and the taxon at the lowest taxonomic level was used as the final identification; Case II: When no highly matched sequences (> 98.0%) were detected by BLAST, the taxon identified by the morphological method was used as the final identification; Case III: When the larva was identified to the family level using the morphological method and to the species level using the genetic method, the species identified using the genetic method was used as the final identification; Case IV: When there was any inconsistency between the morphological and genetic identifications, both candidates were inspected based on the larval morphology, sequencing data, and other available information. The most likely taxon was used as the final identification.

Results

Morphological identification. A total of 133 specimens of the postflexion gobioid larvae were distinguished into 21 types based on the morphological characters. Five types (*Eleotris melanosoma*, *Eviota japonica*, *Pleurosicya bilobata*, *Redigobius bikolanus*, and *Ptereleotris microlepis*) were identified to the species level, 14 to the genus level, and two to the family level (Table 1).

Genetic identification. DNA segments of 138–145 base pairs (bp) of the 12S rRNA gene were sequenced using DNA extracted from the larvae fixed in formalin for 1 day and preserved in ethanol for 3–7 years thereafter. The sequences of 13 types of the larvae were matched > 98.0% with single species (Table 1). The sequences of larvae OCF-P4150 and OCF-P4151 matched those of *Oxyurichthys visayanus*, but also presented with high similarity (> 98.0%) to that of *Oxyurichthys* sp. and *Oxyurichthys cornutus* (Table 1). Therefore, we genetically identified these larvae as *Oxyurichthys* sp. The sequence of larva OCF-P4145 showed high similarity with those of *Trypauchenopsis intermedia* (100%) and *Trypauchenopsis* sp. (99.3%) (Table 1). Thus, we genetically identified this larva as *Trypauchenopsis* sp. The sequences of larva OCF-P4144 matched with those of *Bunaka gyrinoides* (100%) and *E. melanosoma* (99.3%) (Table 1). Therefore, we genetically identified it as Eleotridae sp. No sequences were matched for the remaining five larval types (< 95.0% accuracy) (Table 1). The sequences of OCF-P4150 and OCF-P4159 were the same as those of OCF-P4151 and OCF-P4160, respectively, and were identified as the same respective types based on their morphological characters.

Final identification. Morphological and genetic methods identified 13 types to the species level and seven types to the genus level (Table 1; Figs. 1–3). Only one larval type (OCF-P4144) was inconsistently matched by both methods (Case IV). *Bunaka gyrinoides* and *E. melanosoma* were initially considered candidate species. The sequence of OCF-P4144 matched > 98.0% with those of *B. gyrinoides* (accession No. LC499417) and *E. melanosoma* (accession No. LC506283). Moreover, it also matched 97.2% with the sequence of *E. melanosoma* (accession No. AF265372). However, no other sequences for *B. gyrinoides* were found. The morphology of the larva OCF-P4144 was closely similar to that of *E. melanosoma* of Maeda and Tachihara (2005) but differed from that of *B. gyrinoides* of Okiyama (2014). Thus, we identified larva OCF-P4144 as *E. melanosoma*.

Descriptions of larvae. The present study is the first to clarify the larval morphologies of two gobioid taxa. We describe the morphologies of *Paragunnellichthys* sp. and *Ctenogobiops feroculus* larvae in some detail, as these were the first described species in their respective genera. Range and mean of SL and sampling month for each larval type are listed in Electronic Supplementary Materials (ESM) Table S1.

Paragunnellichthys sp.

(Table 2; Fig. 2)

Material examined. OCF-P4164, postflexion stage, 14.6 mm SL, 20 October 2012.

Description. Body very elongate, depth 7.1% of SL. Head small, length 13.8% of SL. Eye small, diameter 20.2% of HL. Myomeres 22 + 26 = 48. Mouth large; posterior margin of the maxilla reaching vertically through middle of pupil; lower jaw protruding anteriorly. Dorsal-fin base single and long, length 80.5% of SL. Anal-fin base long, length 42.1% of SL. Pectoral fin not reaching to anus. Pelvic fin small, 15.2% of HL.

Body less pigmented; four melanophores along lateral midline on caudal peduncle; two melanophores along each dorsal and ventral midline on caudal peduncle; internal melanophores on dorsal part of gas bladder.

Remarks. Larva OCF-P4164 had a very elongate body and long dorsal- and anal-fin bases, consistent with the characteristics of the family Microdesmidae (Smith and Thacker 2000; Leis and Carson-Ewart 2004). Of Microdesmidae, *Gunnellichthys* and *Paragunnellichthys* are known from the Indo-Pacific (Leis and Carson-Ewart 2004). Although the number of fin rays of OCF-P4164 closely matched that of *Paragunnellichthys seychellensis* (see Dawson 1967), this species is not known from Okinawa Island. *Paragunnellichthys* sp. was recorded with a voucher specimen (HMNH-P6154) from Irabu Island (Yoshigou 2014) and with photographs from Okinawa and Iriomote Islands in southern Japan (http://fishpix.kahaku.go.jp/fishimage-e/index.html).

Table 1 Morphological and genetic identification of gobioid larvae collected on the northern coast of Okinawa Island

Catalog number of voucher	Morphological identification		Genetic identification			Final determined identification	
	Taxon name	Reference(s)	Taxon name	% Identity	GeneBank Accession no.	Taxon name	Case
OCF-P4144	*Eleotris melanosoma*	1	*Bunaka gyrinoides*	100	LC499417	*Eleotris melanosoma*	Case IV
			Eleotris melanosoma	99.3	LC506283		
OCF-P4146	*Eviota japonica*	3	*Eviota japonica*	98.6	LC385197[a]	*Eviota japonica*	Case I
OCF-P4147	*Pleurosicya bilobata*	3	*Pleurosicya bilobata*	99.3	LC340269	*Pleurosicya bilobata*	Case I
OCF-P4148	*Redigobius bikolanus*	2, 4	*Redigobius bikolanus*	98.6	LC500737[a]	*Redigobius bikolanus*	Case I
OCF-P4149	*Ptereleotris microlepis*	5	*Ptereleotris microlepis*	100	LC104730	*Ptereleotris microlepis*	Case I
OCF-P4145	*Trypauchenopsis* sp.	2	*Trypauchenopsis intermedia*	100	LC519445[a]	*Trypauchenopsis* sp.	Case I
			Trypauchenopsis sp.	99.3	AP019362		
OCF-P4150	*Oxyurichthys* sp.	2, 3, 6	*Oxyurichthys visayanus*	100	LC092052[a]	*Oxyurichthys* sp.	Case I
			Oxyurichthys sp.	99.3	LC340245		
			Oxyurichthys cornutus	98.6	LC499571[a]		
OCF-P4152	*Amblygobius* sp. A	3	*Amblygobius phalaena*	100	LC458314[a]	*Amblygobius phalaena*	Case I
			Amblygobius phalaena	99.3	LC049765		
OCF-P4153	*Amblygobius* sp. B	3	*Amblygobius nocturnus*	100	LC049764	*Amblygobius nocturnus*	Case I
OCF-P4154	*Favonigobius* sp. A	3, 6, 7	*Favonigobius gymnauchen*	100	LC506699[a]	*Favonigobius gymnauchen*	Case I
			Favonigobius gymnauchen	99.3	LC499500[a]		
OCF-P4155	*Favonigobius* sp. B	3, 6, 7	*Favonigobius* sp.	99.3	LC506281	*Favonigobius* sp. B	Case I
OCF-P4156	*Valenciennea* sp.	3	*Valenciennea longipinnis*	100	LC049780[a]	*Valenciennea longipinnis*	Case I
OCF-P4157	*Eviota* sp. A	3	*Eviota abax*	100	LC458284[a]	*Eviota abax*	Case I
OCF-P4158	*Eviota* sp. B	3	*Eviota distigma*	100	LC146314	*Eviota distigma*	Case I
OCF-P4159	*Eviota* sp. C	3	No matched sequence	-	-	*Eviota* sp. C	Case II
OCF-P4161	*Eviota* sp. D	3	No matched sequence	-	-	*Eviota* sp. D	Case II
OCF-P4162	*Eviota* sp. E	3	No matched sequence	-	-	*Eviota* sp. E	Case II
OCF-P4163	*Gunnellichthys* sp.	3	*Gunnellichthys pleurotaenia*	100	LC499516	*Gunnellichthys pleurotaenia*	Case I
OCF-P4164	*Paragunnellichthys* sp.	5, 8, 9	No matched sequence	-	-	*Paragunnellichthys* sp.	Case II
OCF-P4165	Gobiidae sp. A	-	*Ctenogobiops feroculus*	100	LC499457	*Ctenogobiops feroculus*	Case III
OCF-P4166	Gobiidae sp. B	-	No matched sequence	-	-	Gobiidae sp. B	Case II

Reference codes: 1 Maeda and Tachihara (2005); 2 Maeda (2008); 3 Okiyama (2014); 4 Tran et al. (2018); 5 Leis and Carson-Ewart (2004); 6 Harada and Suharti (2000); 7 Neira et al. (1998); 8 Smith and Thacker (2000); 9 Dawson (1967)

No matched sequence no matched sequences with high rate of over 95%

a the presence of the sequence showing the same match rate in the same species

Thus, OCF-P4164 was identified as a member of the genus *Paragunnellichthys*, but further study is needed to determine whether it is the same species as HMNH-P6154 and the other photographed specimens.

Among the five genera of the family Microdesmidae (*Clarkichthys*, *Cerdale*, *Microdesmus*, *Gunnellichthys*, and *Paragunnellichthys*), the postflexion larvae of four genera, excluding *Clarkichthys*, have been described in previous studies (Smith

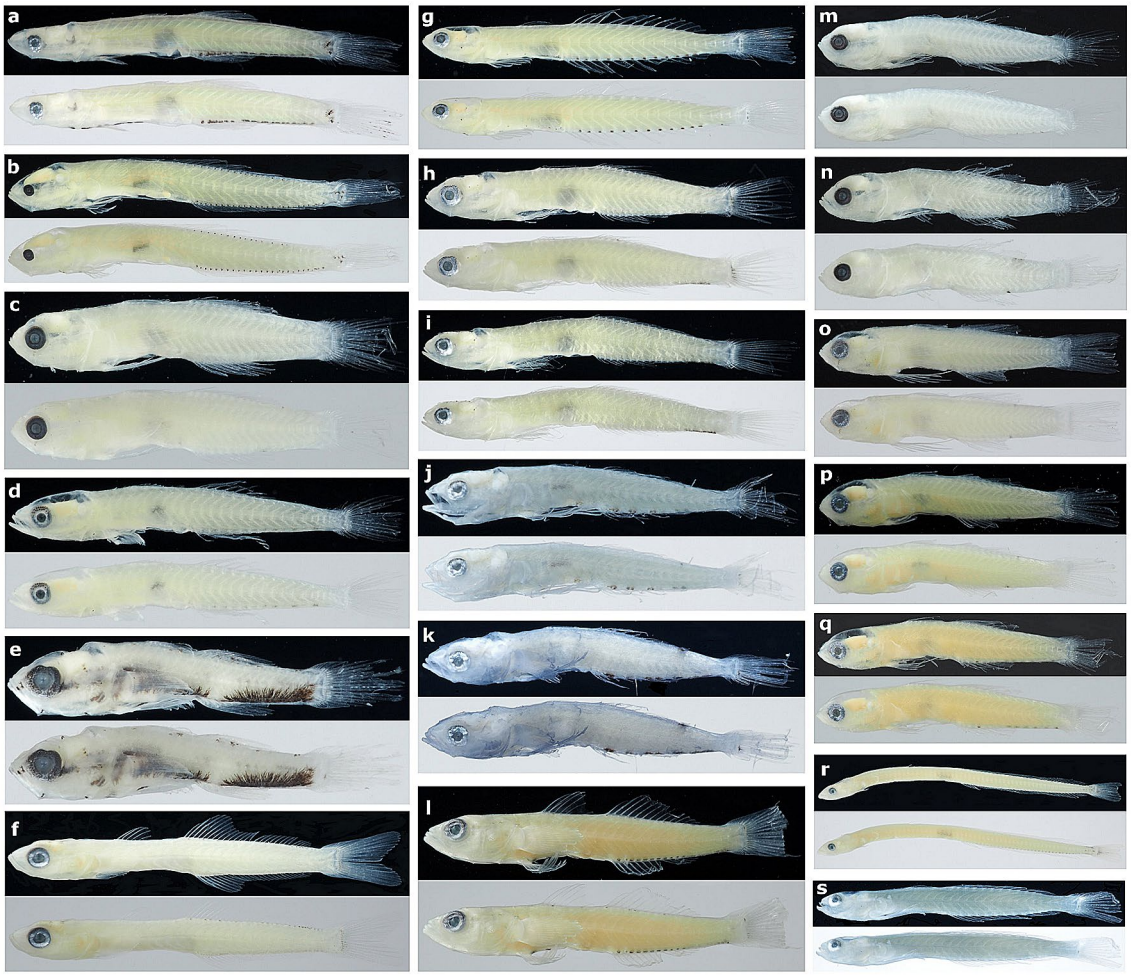

Fig. 1 Photograph of preserved gobioid larval specimens collected on the northern coast of Okinawa Island. **a** *Eleotris melanosoma* (OCF-P4144; 10.8 mm SL), **b** *Trypauchenopsis* sp. (OCF-P4145; 8.6 mm SL), **c** *Eviota japonica* (OCF-P4146; 8.0 mm SL), **d** *Pleurosicya bilobata* (OCF-P4147; 7.4 mm SL), **e** *Redigobius bikolanus* (OCF-P4148; 5.1 mm SL), **f** *Ptereleotris microlepis* (OCF-P4149; 17.7 mm SL), **g** *Oxyurichthys* sp. (OCF-P4150; 13.0 mm SL), **h** *Amblygobius phalaena* (OCF-P4152; 9.6 mm SL), **i** *Amblygobius nocturnus* (OCF-P4153; 10.6 mm SL), **j** *Favonigobius gymnauchen* (OCF-P4154; 7.6 mm SL), **k** *Favonigobius* sp. B (OCF-P4155; 6.3 mm SL), **l** *Valenciennea longipinnis* (OCF-P4156; 16.6 mm SL), **m** *Eviota abax* (OCF-P4157; 8.5 mm SL), **n** *Eviota distigma* (OCF-P4158; 6.6 mm SL), **o** *Eviota* sp. C (OCF-P4159; 7.7 mm SL), **p** *Eviota* sp. D (OCF-P4161; 7.0 mm SL), **q** *Eviota* sp. E (OCF-P4162; 8.4 mm SL), **r** *Gunnellichthys pleurotaenia* (OCF-P4163; 22.6 mm SL), **s** Gobiidae sp. B (OCF-P4166; 12.0 mm SL)

and Thacker 2000; Leis and Carson-Ewart 2004; Okiyama 2014). All were characterized by very elongate body, small pelvic fins, and long dorsal- and anal-fin bases. The postflexion larvae of *Gunnellichthys* and *Paragunnellichthys* were distinguished from those of *Cerdale* and *Microdesmus* by the lack of melanophores around pelvic-fin base and gut. The postflexion larvae of *Paragunnellichthys* were distinguished from those of *Gunnellichthys* by the lack of paired melanophores along each side of anal-fin base.

Ctenogobiops feroculus

(Table 2; Fig. 3)

Material examined. OCF-P4165, postflexion stage, 8.0 mm SL, 20 May 2011.

Description. Body elongate, depth 18.1% of SL. Head moderate, length 26.5% of SL. Eye small, diameter 23.5% of HL. Myomeres 7 + 17 = 24. Second dorsal- and anal-fin

Table 2 Fin counts and morphological measurements of postflexion larvae of *Paragunnellichthys* sp. and *Ctenogobiops feroculus* collected on the northern coast of Okinawa Island

Species	Voucher number	Fin counts				Morphological measurements								
		D	A	P_1	P_2	SL (mm)	BD (% SL)	HL (% SL)	SnL (% SL)	ED (% SL)	PAL (% SL)	PDL (% SL)	CPL (% SL)	CPD (% SL)
Paragunnellichthys sp.	OCF-P4164	48	29	10	I, 2	14.6	7.1	13.8	2.4	2.8	54.6	18.5	4.1	3.1
Ctenogobiops feroculus	OCF-P4165	VI-I, 11	I, 11	19	I, 5	8.0	18.1	26.5	7.3	6.2	51.8	35.8	18.0	7.9

D dorsal fin, *A* anal fin, P_1 pectoral fin, P_2 pelvic fin, *SL* standard length, *BD* body depth at pectoral-fin base, *HL* head length, *SnL* snout length, *ED* eye diameter, *PAL* preanal length, *PDL* predorsal length, *CPL* caudal peduncle length, *CPD* caudal peduncle depth

Asterisks indicate showing total numbers of elements, because spines and soft rays were difficult to distinguish

base moderate, length 27.7% and 28.5% of SL. Pelvic fins connected by membrane between last rays; lacking frenum (possibly undeveloped). Pectoral and pelvic fins not reaching to anus.

Body lightly pigmented; melanophores at angle of lower jaw, along isthmus, and on pelvic-fin base; unpaired melanophores along each side of anal-fin base; single row of melanophores along ventral midline of caudal peduncle; single row of faint melanophores along lower caudal-fin ray; internal melanophores around otic capsule, dorsal part of gas bladder, and above anus.

Remarks. The postflexion larvae of *C. feroculus* could be distinguished from previously reported gobioid larvae by the combination of fin-ray count, body size, and pigment pattern. The fin-ray count and body size of the postflexion larvae of *C. feroculus* were similar to those of the larvae of *Oligolepis* sp. of Okiyama (2014). However, *C. feroculus* differ from *Oligolepis* sp. in lacking internal pigmentation above posterior end of anal-fin base (vs. having conspicuous internal pigmentation above posterior end of anal-fin base), having single row of melanophores along ventral midline of caudal peduncle (vs. no melanophores along ventral midline of caudal peduncle), and having unpaired melanophores along anal-fin base (vs. paired melanophores along anal-fin base).

Discussion

We revealed the morphology of the postflexion larvae of *Paragunnellichthys* sp. (Microdesmidae) and *C. feroculus* (Gobiidae) for the first time in their respective genera. These postflexion larvae were in the developmental stage with poor pigmentation and almost complete fins, and they would be at the stage just before settlement. The microdesmid postflexion larvae at the settlement stage have been reported from both Indo-Pacific and Atlantic Oceans. The settlement size of Indo-Pacific *Gunnellichthys* larvae is 22.6–28.0 mm SL (Leis and Carson-Ewart 2004; present study), and the size of the larvae of the Atlantic microdesmid *Cerdale* and *Microdesmus* is 15–36 mm SL (Smith and Thacker 2000). The size of the postflexion larva of *Paragunnellichthys* sp. is 14.6 mm SL; thus this is similar to the smallest settlement size of Atlantic microdesmid species. The larval morphology of *C. feroculus* was the first to be described among the 12 genera (Karplus and Thompson 2011) of the shrimp-associated gobies. The size of the postflexion larvae of *C. feroculus* (8.0–8.4 mm SL) was included in the typical size at settlement of Gobiidae members, 6–9 mm SL (Victor et al. 2010).

Using a combination of morphological and genetic methods, we identified 13 of the 21 types (61.9%) of gobioid larvae to the species level. We were unable to fulfil our

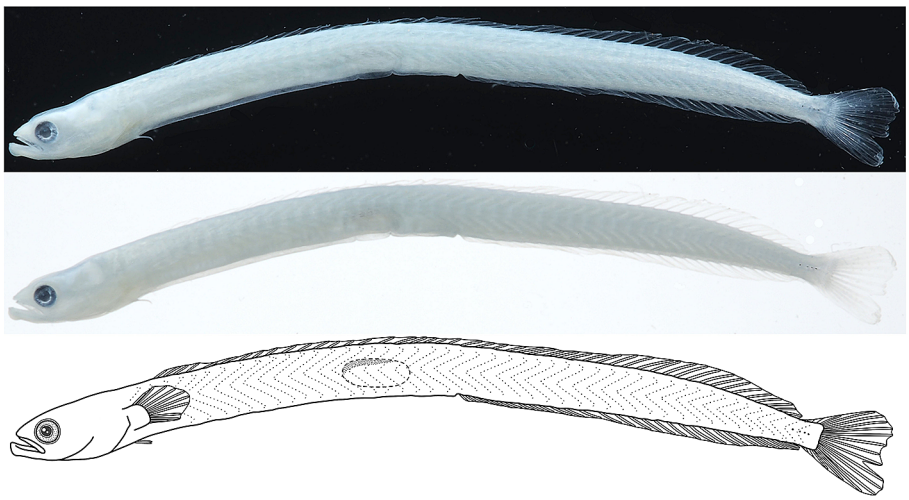

Fig. 2 Postflexion larva of *Paragunnellichthys* sp. (OCF-P4164; 14.6 mm SL) collected on the northern coast of Okinawa Island

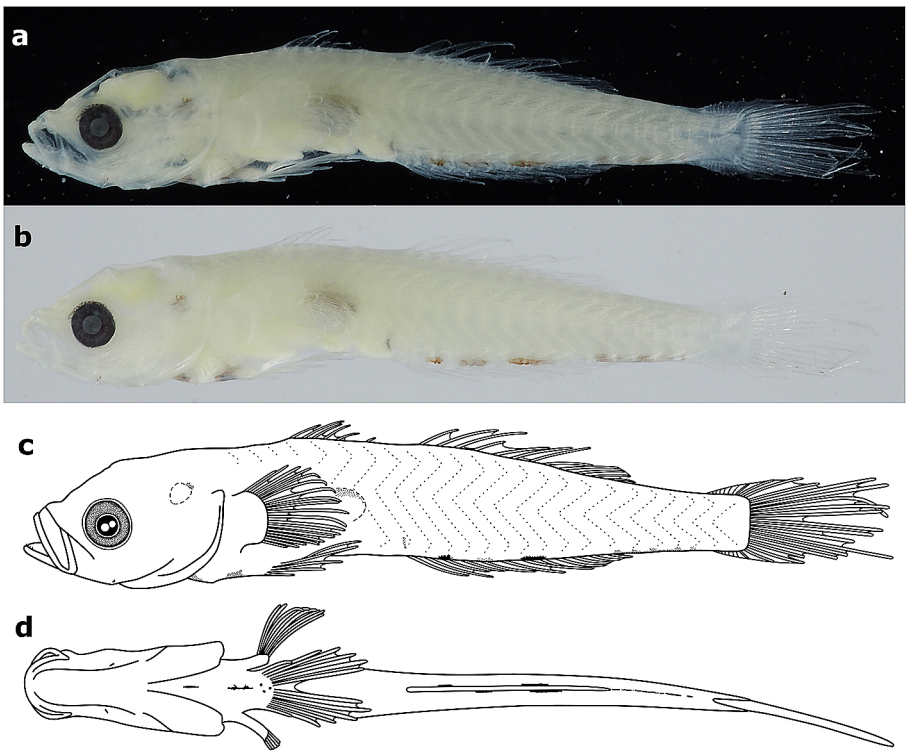

Fig. 3 Postflexion larva of *Ctenogobiops feroculus* (OCF-P4165; 8.0 mm SL) collected on the northern coast of Okinawa Island. **a**–**c** lateral views, **d** ventral view

objective of identifying all larvae to the species level, which indicates that there are still certain limitations in identifying gobioid larvae. To enable the identification of all larvae to species level, more comprehensive larval descriptions and a large number of databases containing DNA reference sequences, which constitute the basis of species identification, are required. To date, many identification guidebooks of fish larvae have been published (Leis 2015) and some large-scale sequencing projects are being undertaken to identify fish (e.g. Ward et al. 2005; Miya et al. 2015). However, neither larval descriptions nor reference sequences suffice (Leis 2015; Steinke et al. 2017). Among the 518 species in 127 gobioid fish genera found in Japanese waters (Akihito et al. 2013), the early stages of 142 taxa in 71 genera (including fishes identified to the genus or family level) are described in an atlas of early stage fishes in Japan (Okiyama 2014). The number of reference sequences of 12S rRNA regions used in the present study was 293 gobioid species in 96 genera (https://www.ncbi.nlm.nih.gov/nuccore). The identification of gobioid larvae should dramatically improve as the numbers of larval descriptions and reference sequences increase. The information and data derived therefrom should help to

elucidate larval ecology, which has been heretofore obscure as it has been difficult to identify fish species based solely on their morphological characters.

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