Identification of larvae of two *Gymnocanthus* (Cottidae) species based on melanophore patterns

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Abstract *Gymnocanthus herzensteini* and *G. intermedius* (Cottidae) larvae are common in northern Japan but can be difficult to distinguish. In this study, morphological observations and mitochondrial DNA analyses were conducted to determine if melanophore patterns could be used to distinguish the two species. The morphological observations identified two groups with distinct melanophore patterns. Subsequent molecular analyses determined with high bootstrap values that the group with numerous body melanophores was *G. intermedius*, and the group with fewer melanophores was *G. herzensteini*. These results indicate that melanophore distribution patterns in larvae are useful for identifying *G. intermedius* and *G. herzensteini*.

Keywords *Gymnocanthus* · sculpins · melanophores · morphology · DNA barcoding

Introduction

The family Cottidae (Teleostei) comprises about 70 genera and 275 species, which generally spawn demersal eggs on various matrixes, such as rocks, sand, seaweeds, sponges or other structures (Munehara 2011). The hatchlings are pelagic, but the flexion and postflexion larvae are benthic. *Gymnocanthus herzensteini* and *G. intermedius* occur sympatrically and concurrently over shallow sandy bottom in northern Japan, and have similar early life histories; they spawn in winter and move to the sandy bottom from April to May (Munehara et al. 2009). Adults of these two species can be distinguished based on differences in the skin flaps on the eyes, the form of the dorsal fin, the distribution of melanophores on the pectoral fins (with 3-4 dark transverse bands, but narrow than pupil in *G. intermedius*), and the number of spines and fin rays (Nakabo and Kai 2013), but these diagnostic characteristics can be similar in immature individuals.

The characteristics used for identifying the early life stages, including the numbers of spines, fin rays and myomeres (Okiyama 1988), are easily destroyed during collection. Furthermore, morphological data are available for *G. intermedius* larvae and juveniles (collected by larval net, Okiyama 1988), but for *G. herzensteini* larvae only through the preflexion stage (reared, Kyushin 1970). These obstacles have limited our understanding of ecological characteristics of individual species such as larval and juvenile distribution and abundance, and recruitment dynamics.

Another characteristic that can be used to distinguish the early life stages of several fish groups is melanophores on other body parts (e.g., the head, gut, body, finfolds and fins for Agonidae, Busby 1998; the antero-ventral postanal and gut dorsal to the pelvic bone for Lutjanidae, Clarke et al. 1997). In additional to morphological identification methods, genetic methods such as DNA barcoding are increasingly being used to identify specimens, but to develop a global DNA-based barcode identification system requires nucleotide sequence data from numerous species (Lakra et al. 2011). In the present study, morphological and molecular analyses together confirmed that melanophore distribution patterns can be used to distinguish larvae of *G. herzensteini* and *G. intermedius*.

61 **Materials and methods**

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63 Samples were collected in Funka Bay, southwest Hokkaido. For molecular analyses, 18 larvae were
64 collected with a hand net by SCUBA diving in May 2010. Specimens used for the examination of
65 morphological characters were collected by SCUBA diving in May of 2010 (n=18) and using a sledge
66 net (Munehara et al. 2009) in May of 2012 (n=1,752). *Gymnocanthus* larvae were identified based on
67 descriptions published in Kojima (1988) and Shiogaki (1988). The developmental stages described by
68 Leis and Carson-Ewart (2000) were used. All larvae were observed under a microscope (BX51,
69 OYMPUS), and the body length (BL) was measured to the nearest 0.01 mm. The body-part terms and
70 morphological characters described by Leis and Carson-Ewart (2000) were used. All specimens
71 collected in May of 2012 were deposited in the Hokkaido University Museum, Hakodate (HUMZ, *G.*
72 *intermedius*: HUMZ 223343, *G. herzensteini*: HUMZ 223344).
73 Genomic DNA was extracted from the whole body of all samples using a QuickGene DNA
74 extraction kit (KURABO, Japan) following the manufacturer's protocol. A 679 bp of the
75 mitochondrial cytochrome c oxidase subunit I (COI) region that has enough substitution rates to
76 distinguish species in *Gymnocanthus* (Yamazaki et al. 2013) was analyzed; this region is used by
77 taxonomists as a standard DNA barcode sequence. The COI region was amplified by PCR using the
78 primers FishF1 and FishR1 (Ward et al. 2005) following the experimental protocol of Yamazaki et al.
79 (2013). Sequences were aligned using MEGA5 (Tamura et al. 2011) with default settings and
80 manually corrected. The maximum likelihood (ML) tree was constructed using RAxML ver. 7.2.8
81 (Stamatakis 2006). MrModeltest2.3 (Nylander 2004) was used to determine the appropriate model of
82 sequence evolution. The best-fit model selected using the heuristic algorithm based on the Akaike
83 information criterion was the general time reversible model (GTR) of nucleotide substitution with the
84 gamma distribution shape parameter (G) and a proportion of invariable positions (I). The robustness
85 of nodes was estimated from 1,000 bootstrap replicates. Seven *G. intermedius*, eight *G. herzensteini*,
86 and one individual each of *G. detrisus*, *G. galeatus*, *G. pistilliger*, *G. tricuspis* and *Myoxocephalus*
87 *stelleri* that already detected the species specific nucleotides by Yamazaki et al. (2013) were added as
88 an out-group, and sequences of 51 species of Cottidae from GenBank were also included in the

molecular analysis (see electronic supplementary material: S1). Sequence data of *G. herzensteini* and *G. intermedius* are shown in S2, and all individual sequence data have been deposited in GenBank (Accession numbers are shown in Table 1).

Results and discussion

Based on the melanophore distribution patterns, 1,752 larvae were divided into two groups (Fig. 1): A (n=1,260) and B (n=492). Group A ranged in size from 9.01–16.84 mm BL. Melanophores occurred on the dorsal side of the head and nape in all group A specimens. An oblique series of melanophores also occurred on the tail under the second dorsal fin (melanophores on tail) in some flexion specimens at 9.22–11.97 mm BL, and in all flexion and postflexion specimens larger than 11.97 mm BL, but not in flexion larvae at 9.01–9.22 mm BL. On the first dorsal fin, melanophores were observed on spines I to VII from the base to over half the length of the spine, sometimes to the distal end. Group B ranged in size from 9.06–18.27 mm BL. Melanophores also occurred on the dorsal side of the head and nape in all group B individuals, but melanophores on the tail developed later than in group A; melanophores on the tail occurred in some flexion specimens at 11.64–14.49 mm BL, and in all flexion and postflexion specimens larger than 14.49 mm BL, but did not occur in flexion larvae at 9.06–11.64 mm BL. On the first dorsal fin, melanophores were observed on spines I to VII either only at the base or discontinuously from the base to the distal end, and formed a band horizontal to the base (Table 1).

The density of melanophores on both the dorsal surface of the head to nape and the first dorsal fin was high in group A and low in group B. Melanophores on the first dorsal fin extended from the base to between half the length of the fin and the distal end in both stages of group A, but in group B, they occurred at the base in the flexion stage, and at the base or sometimes in a narrow band horizontal to the dorsal base on the middle height in the flexion and postflexion stages. Melanophores on the tail were continuous in group A flexion and postflexion larvae, but sometimes discontinuous in group B flexion larvae.

In the molecular analysis, 18 larvae were sequenced. These morphological characteristics for each specimen are shown in Table 2 (6 flexion larvae and 1 postflexion larva in group A, and10 flexion larvae and 1 postflexion larva in group B). The length of the partial COI sequence was 679 bp, and it included 32 variable sites and 31 parsimony informative sites. All larvae in both groups A and B were divided into *G. intermedius* and *G. herzensteini* clades, respectively, with high (>95%) bootstrap values (Fig. 2).

These results indicate that melanophore distribution patterns can be used to identify *G. intermedius* and *G. herzensteini* flexion and postflexion larvae. Using this diagnosis, we can clarify the differences in the early life histories of these species.

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Fig. 1 Distribution of melanophores in *G. herzensteini* and *G. intermedius* larvae. The top individuals in each column are flexion larvae, the middle and bottom individuals are postflexion larvae. The bottom figure shows the back of the head from above

Fig. 2 Molecular phylogenetic tree of two species and other Cottidae species. The tree was estimated based on analysis of the COI region (626 bp) using the maximum likelihood (ML) tree method. The numbers show the bootstrap value. The larvae of *Gymnocanthus intermedius* are shown in A1–A7, and *G. herzensteini* are shown in B1–B11

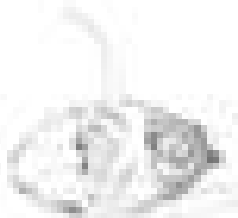
Table 1 Specimens data of *Gymnocanthus* larvae for molecular and morphological analyses (A1–A7: *G. intermedius*; B1–B11: *G. herzensteini*)

Specimen name	Accession no.	Stages	BL (mm)	Melanophore distribution patterns		
				Head & nape	1st dorsal fin	Tail
A1	JQ406330	Flexion	9.13	Higher	>Half	Absent
A2	JQ406331	Flexion	11.92	Higher	Distal end	Continue
A3	JQ406332	Flexion	11.56	Higher	Distal end	Continue
A4	JQ406333	Flexion	9.02	Higher	>Half	Absent
A5	JQ406334	Flexion	12.74	Higher	Distal end	Continue
A6	JQ406335	Flexion	13.05	Higher	Distal end	Continue
A7	JQ406336	Postflexion	13.30	Higher	Distal end	Continue
B1	JQ406315	Flexion	14.13	Lower	Base	Continue
B2	JQ406317	Flexion	12.08	Lower	Base	Discontinue
B3	JQ406318	Flexion	12.00	Lower	Base	Continue
B4	JQ406319	Flexion	12.80	Lower	Base	Discontinue
B5	JQ406320	Flexion	12.23	Lower	Base	Discontinue
B6	JQ406321	Flexion	13.89	Lower	Base	Continue
B7	JQ406322	Flexion	9.06	Lower	Base	Absent
B8	JQ406323	Flexion	14.57	Lower	Base	Continue
B9	JQ406324	Flexion	15.18	Lower	Base	Discontinue
B10	JQ406325	Postflexion	17.15	Lower	Base/bands	Continue
B11	JQ406326	Flexion	16.33	Lower	Base/band	Continue

group A
(*G. intermedius*)

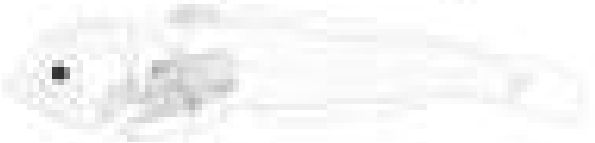
9.04 mm BL

11.52 mm BL



12.40 mm BL

group B
(*G. heterostoma*)



9.06 mm BL

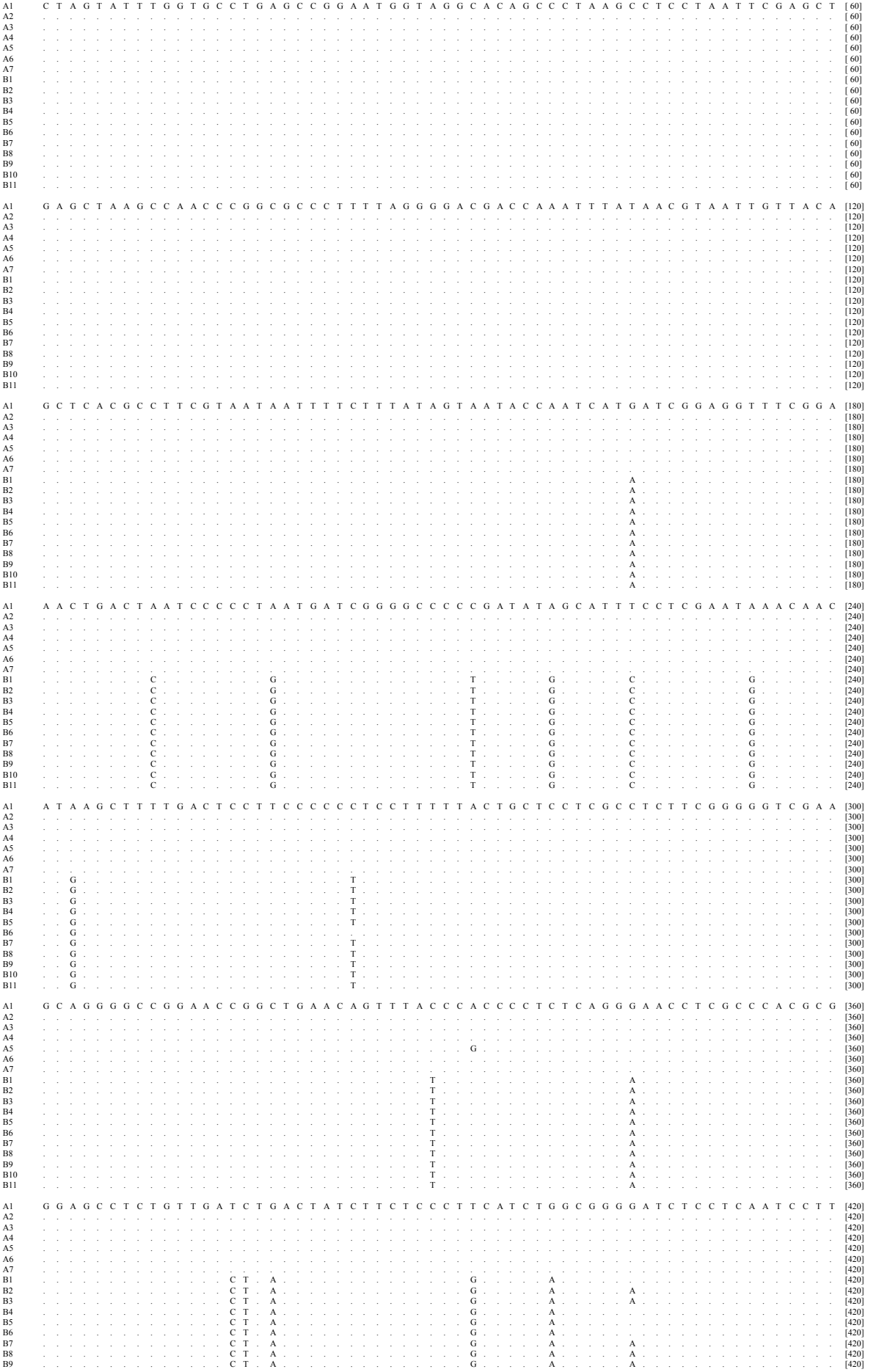
12.00 mm BL

10.79 mm BL

S1 The accession number of out-group

Genus	Species	Accession no.
Scorpaenichthys	*marmoratus*	GU440517
Hemilepidotus	*hemilepidotus*	JQ354115
	zapus	HQ712450
	spinosus	JQ354118
	jordani	JQ712443
	papilio	HQ712449
Trichocottus	*brashnikovi*	HQ712651
Artediellus	*scaber*	HQ712300
Radulinus	*asprellus*	KF918897
	boleoides	JQ354530
Icelus	*sekii*	now registering
	mororanis	now registering
	spatula	KC015498
	bicornis	KC015492
Icelinus	*borealis*	JQ354140
	burchami	FJ164690
	oculatus	GU440355
	quadriseriatus	GU440356
	cavifrons	GU440353
	filamentosus	JQ354144
	tenuis	GU440357
Leptocottus	*armatus*	JQ354167
Triglops	*pingelii*	KC016007
	macellus	JQ354527
	nybelini	KC016003
	murrayi	KC015975
Enophrys	*bison*	JQ354085
	lucasi	HQ712365
	deceraus	HQ712364
Myoxocephalus	*octodecemspinosus*	KC015729
	jaok	KC351883
	brandtii	KC351878

	polyacanthocephalus	JQ354240
	ochotensis	JF278619
	stelleri	JQ406360
Chitonotus	*pugetensis*	JW354045
Orthonopias	*triacis*	GU440439
Oligocottus	*rimensis*	GU440428
	rubellio	GU440429
	maculosus	FJ164926
	snyderi	HQ557145
Synchirus	*gilli*	GU440542
Artedius	*corallinus*	GU440235
	harringtoni	JQ353991
	notospilotus	GU440238
	fenestralis	JQ353989
	lateralis	JQ353992
Gymnocanthus	*galeatus*	JQ406292
	detrisus	JQ406279
	tricuspis	JQ406372
	pistilliger	JQ406365



B10	. . . . C T . A . . . . G . . . . . A . . . . . A	[420]
B11	. . . . C T . A . . . . G . . . . . A	[420]

A1	G G G G C A A T C A A T T T T A T C A C A A C C A T C A T T A A C A T A A A A C C C C C A G C C A T T T C A C A G T A C	[480]
A2	. . . .	[480]
A3	. . . .	[480]
A4	. . . .	[480]
A5	. . . . T	[480]
A6	. . . .	[480]
A7	. . . .	[480]
B1	. . . . . C . . T . . . . T . . . . G . . . . C . . T	[480]
B2	. . . . . C . . T . . . . T . . . . G . . . . C . . T	[480]
B3	. . . . . C . . T . . . . T . . . . G . . . . C . . T	[480]
B4	. . . . . C . . T . . . . T . . . . G . . . . C . . T	[480]
B5	. . . . . C . . T . . . . T . . . . G . . . . C . . T	[480]
B6	. . . . . C . . T . . . . T . . . . G . . . . C . . T	[480]
B7	. . . . . C . . T . . . . T . . . . G . . . . C . . T	[480]
B8	. . . . . C . . T . . . . T . . . . G . . . . C . . T	[480]
B9	. . . . . C . . T . . . . T . . . . G . . . . C . . T	[480]
B10	. . . . . C . . T . . . . T . . . . G . . . . C . . T	[480]
B11	. . . . . C . . T . . . . T . . . . G . . . . C . . T	[480]

A1	C A G A C C C C T C T T T T C G T G T G G T C T G T C C T T A T T A C G G C C G T C T T A C T G C T T C T C T C C C T C	[540]
A2	. . . .	[540]
A3	. . . .	[540]
A4	. . . .	[540]
A5	. . . .	[540]
A6	. . . .	[540]
A7	. . . .	[540]
B1	. . A . . . . C . . . . T	[540]
B2	. . A . . . . C . . . . T	[540]
B3	. . A . . . . C . . . . T	[540]
B4	. . A . . . . C . . . . T	[540]
B5	. . A . . . . C . . . . T	[540]
B6	. . A . . . . C . . . . T	[540]
B7	. . A . . . . C . . . . T	[540]
B8	. . A . . . . C . . . . T	[540]
B9	. . A . . . . C . . . . T	[540]
B10	. . A . . . . C . . . . T	[540]
B11	. . A . . . . C . . . . T	[540]

A1	C C A G T C C T T G C T G C C G G A A T T A C A A T A C T T T T A A C A G A C C G A A A C C T T A A C A C C A C C T T T	[600]
A2	. . . .	[600]
A3	. . . .	[600]
A4	. . . .	[600]
A5	. . . .	[600]
A6	. . . .	[600]
A7	. . . .	[600]
B1	. . . . C . . . . G . . . . T	[600]
B2	. . . . T . . . . G . . . . T	[600]
B3	. . . . T . . . . G . . . . T	[600]
B4	. . . . C . . . . G . . . . T	[600]
B5	. . . . C . . . . G . . . . T	[600]
B6	. . . . T . . . . G . . . . T	[600]
B7	. . . . T . . . . G . . . . T	[600]
B8	. . . . T . . . . G . . . . T	[600]
B9	. . . . T . . . . G . . . . T	[600]
B10	. . . . T . . . . G . . . . T	[600]
B11	. . . . T . . . . G . . . . T	[600]

A1	T T T G A T C C G G C C G G G G G C G G T G A C C C	[626]
A2	. . . .	[626]
A3	. . . .	[626]
A4	. . . .	[626]
A5	. . . .	[626]
A6	. . . .	[626]
A7	. . . .	[626]
B1	. . . . A . . . . . G	[626]
B2	. . . . A . . . . . G	[626]
B3	. . . . A . . . . . G	[626]
B4	. . . . A . . . . . G	[626]
B5	. . . . A . . . . . G	[626]
B6	. . . . A . . . . . G	[626]
B7	. . . . A . . . . . G	[626]
B8	. . . . A . . . . . G	[626]
B9	. . . . A . . . . . G	[626]
B10	. . . . A . . . . . G	[626]
B11	. . . . A . . . . . G	[626]