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https://doi.org/10.5109/1564089

出版情報：九州大学大学院農学研究院紀要. 61 (1), pp.103-108, 2016-02-29. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **61** (1), 103–108 (2016)

Reassessment of Pigmentation Patterns used for Identifying Leptocephali (*Gnathophis heterognathos* and *G. ginanago*) Collected from the Kuroshio–Oyashio transition region

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(*Received November 4, 2015 and accepted November 19, 2015*)

Identification of leptocephali of *Gnathophis heterognathos* and *G. ginanago*, the most abundant leptocephali in Japanese water, is usually identified based on morphological characteristics such as total number of myomeres (TM), number of myomeres of anterior to the last vertical blood vessel (VBV last) and presence or absence of melanophores on the lateral surface of the posterior spinal cord. However, because some of individuals collected in the Kuroshio–Oyashio transition region differed from the norm in the above characters, the validity of the last–mentioned was examined using the mitochondrial DNA 16S rRNA gene to provide genetic evidence. A total of 394 leptocephali were classified into following four morphotypes, Types A1 (TM: 117–128, VBV last: 36–44, pigmentation: absent, Body height (BH)/Total length (TL) ratio: 0.083–0.123), A2(TM: 119–126, VBV last: 40–44, pigmentation: present, BH/TL ratio: 0.082–0.127), B1(TM: 125–134, VBV last: 41–48, pigmentation: present, BH/TL ratio: 0.093–0.137) and B2(TM: 125–135, VBV last: 43–48, pigmentation: absent, BH/TL ratio: 0.080–0.139). Group A showed lower TM, VBV last, and BH/TL ratio than those of Group B. Molecular evidence confirmed that, Groups A (Types A1 and A2) and B (Types B1 and B2) were in fact *G. heterognathos* and *G. ginanago*, respectively. In addition, relationship of total length and pre–anal myomere (PAM)/TM ratio as an indicator of metamorphosis showed that the presence of posterior spinal cord melanophores was depended on their growth. These results indicate that *Gnathophis* leptocephali obtained from the Kuroshio–Oyashio transition region were comprising only the two species, and that variations in the pigmentation pattern characters rendered them unsuitable for identification purposes. Combination of above morphometric or meristic characters is effective for identified these two species.

Key words: 16S rRNA, *Gnathophis* leptocephali, posterior spinal cord melanophores

INTRODUCTION

Elopiformes, Albuliformes, Notacanthiformes and Anguilliformes have been included in Elopomorpha on the basis of their leaf–like larval form, termed "leptocephalus" (Greenwood *et al.*, 1966). Of those orders, Anguilliformes is the largest group including about 791 species in 141 genera, representing 15 families (Nelson, 2006). As yet, a great many questions (e.g. natural habitat of leptocephali) to be solved (Smith, 1989), the lifecycle of most species being poorly known as a consequence. Although information on leptocephali collected in offshore regions is highly important in the elucidation of the lifecycles of anguilliform fishes, classification of such leptocephali is often being difficult, many species not yet being attributable to specific families (Smith, 1989).

On the other hand, of the 17 families, 69 genera and 163 species of adult Anguilliform fishes known from Japan (Hatooka, 2013), only 33 species of leptocephali have been reported from the Japan region (Mochioka *et al.*, 2014; Tawa and Mochioka, 2009; Tawa *et al.*, 2012). To clarify the lifecycle of such as anguilliform and other elopomorph fishes, it is necessary to identify leptocephali at the species level.

The most abundant leptocephali around Japan were *Gnathophis* spp., including *G. heterognathos* and *G. ginanago* (Mochioka *et al.*, 2014; Uematsu *et al.*, 1990; Uematsu *et al.*, 1992; Miya and Hirosawa, 1994; Mochioka *et al.*, 2001; Miller *et al.*, 2002; Takahashi *et al.*, 2008). Although *G. heterognathos* and *G. ginanago* have been separated on the basis of total number of myomeres (TM: 116–128 vs 123–139, respectively), number of myomeres anterior to the last vertical blood vessel (VBV last: 36–45 vs 41–50), body height, and absence (presence) of melanophores on the lateral surface of the posterior spinal cord (Tabeta and Mochioka, 1988), description about melanophores for to separate these two species have been removed in recent study (Mochioka *et al.*, 2014). It caused by some specimens collected from the Kuroshio–Oyashio transition region (transition region) that were equivocal in their affinities. But the details of such morphological features have not been mentioned.

The only consistent morphological characteristics that are transferred through metamorphosis of Elopomorpha species is the number of myomeres (in larvae), which match the number of vertebrae (in adults) (Smith, 1979). However, many species have overlapping myomere/vertebral numbers. Accordingly, matching of larvae and adults is best done by collecting series samples of each growth stage (Mochioka *et al.*, 2014; Tawa

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and Mochioka, 2009), although sample collection especially in offshore waters is sometimes difficult and at best offers only fragmentary information, making identifications difficult. In the present study, myomere numbers and pigmentation pattern were used to classify leptocephali, supported by species–specific DNA sequence. Molecular identification has frequently been applied in recent years to eggs and leptocephali of a number of anguilliform species (e.g. Aoyama *et al*., 2001; Watanabe *et al*., 2004; Ma *et al*., 2005, 2007; Kimura *et al*., 2006; Kurogi, 2008), successfully demonstrating the validity of using the mitochondrial DNA 16S rRNA gene for such species. This study focused on *Gnathophis* leptocephali, reexamined the current morphological classification of the genus using for comparison mitochondrial DNA 16S rRNA gene sequence analysis.

MATERIALS AND METHODS

Specimens

A total of 394 *Gnathophis* leptocephali were collected at night from the transition region (140° – 160°E, 35° – 40°N) (Fig. 1) with a mid water trawl net (Model JP–1, Nichimo Co., Ltd.) towed in the surface layer (< 30 m depth) at a speed of ca. 3.5 knots for 30 minutes by the T/V Hokuho Maru (664 gross tonnage, 2200 hp), chartered by the National Research Institute of Fisheries Science during 8–30 May 2002. The trawl net had a total length of 89 m, opening area of about 530 m^2, mesh sizes of 57–1000 mm, and 8 mm meshed cod end. Leptocephali specimens were initially stored in sea water at −30°C, and after thawing and measurement, preserved in 99.5% ethanol. Morphometric and meristic characters (total length: TL, pre–dorsal length: PDL, pre–anal langth: PAL, head length: HL, eye diameter: ED, maximum body height: BH, total myomere: TM, pre–dorsal myomere: PDM, pre–anal myomere: PAM, number of myomere on first vertical blood vessel: VBV 1st, number of myomere on last vertical blood vessel: VBV last) were measured or counted. Measurement and myomere counting methods followed Mochioka *et al*. (2014). PAM/TM ratio was calculated for to estimate their metamorphic stage (e.g. Tanaka *et al*., 1987; Lee and Byun, 1996). These specimens were classified into four morphotypes, Types A1, A2, B1 and B2 based on TM, VBV last, BH/TL ratio and absence (presence) of posterior spinal cord melanophores (Fig. 2). When TM or VBV last were overlapped between each type, BH/TL ratio was used for determine (details were shown in result). Types A1 (n=5), A2 (n=4), B1 (n=5), and B2 (n=6) were subjected to molecular analysis. Adult specimens of *G. heterognathos* (GH ad: n=6) and *G. ginanago* (GG ad: n=5) were collected by trawler from Tosa bay (Kochi Pref.), off Choshi (Chiba Pref.), and off Soma (Fukushima Pref.). These samples were stored at –20°C. After thawing and measurement, muscle tissue samples were excised and preserved in 99.5% ethanol for DNA analysis. Measurement and counting methods followed Hatooka (2013). All specimens used in this study were deposited in the collection of the Kyushu University Museum (KYUM).

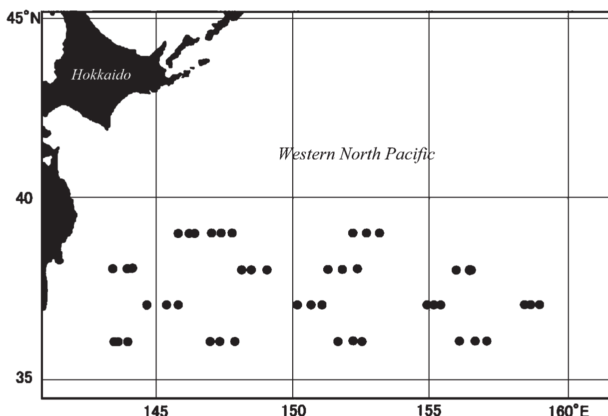

Fig. 1. Sampling locations of leptocephali in the Kuroshio–Oyashio transition region during a cruise of the T/V Hokuho Maru (8–30 May 2002).

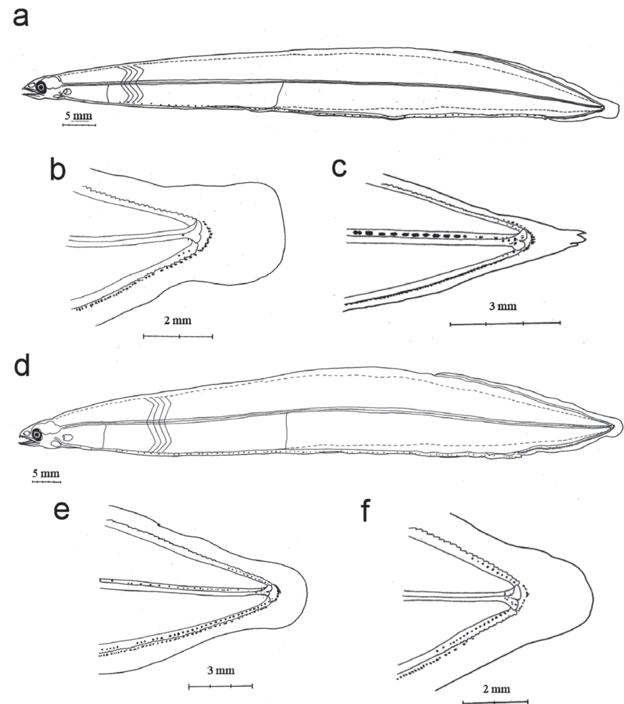

Fig. 2. Leptocephalus larvae of *Gnathophis* spp. (Types A1, A2, B1 and B2) collected from the Kuroshio–Oyashio transition region. a: Lateral view of Type A1. b: Tip of caudal region of Type A1. c: tip of caudal region of Type A2. d: Lateral view of Type B1. e: Tip of caudal region of Type B1. f: Tip of caudal region of Type B2.

DNA extraction, amplification, and sequencing

Muscle tissue samples were subjected to DNA extraction. Tissues were digested with Proteinase K at 50°C and DNA was purified by standard phenol: chloroform extraction and ethanol precipitation, or by using DNA extraction kits (DNeasy Tissue Kit; QIAGEN GmbH, Hilden, Germany). PCR (polymerase chain reaction) was used to amplify the partial mitochondrial DNA 16S rRNA gene, in a total of 50 μl volumes containing 5 μl 10 × PCR buffer, 0.2 mM each deoxynucleosyde tri-

phosphate (dNTP), 0.2 μM each primer, 2.5 units Taq DNA polymerase (TaKaRa Ex Taq; TaKaRa, Shiga Japan) and 10 ng template DNA. Primers RRNA–A (5'–GGTCCWRCCTGCCCAGTGA) and RRNA–B (5'–CCGGTCTGRACYAGATCACGT) for the mitochondrial DNA 16S rRNA gene were used. The thermal profile was 94°C/40 s (denaturing), 50°C/40 s (annealing), and 72°C/40 s (extension), for 30 cycles on a TaKaRa PCR Thermal Cycler PERSONAL (TaKaRa, Shiga Japan) or PTC–100 Programmable Thermal Controller (MJ RESEARCH, Watertown, Massachusetts, USA). Amplified DNA was purified using the QIAquick PCR Purification Kit (QIAGEN, GmbH, Hilden, Germany) and sequenced directly using the BigDye Terminator Cycle Sequencing Ready Reaction Kit Ver. 2 or 3 (Applied Biosystems, Foster City, CA, USA). They were sequenced on an automated DNA sequencer (ABI Prism 3100 Genetic Analyzer). The nucleotide sequences were deposited with GenBank, the DNA database (accession numbers being AB752344–AB752374).

Sequence analyses

DNA sequences were aligned using the computer programs Edit View ver. 1.0.1 (Applied Biosystems), Genetic Mac 8.0(Software Development Co.), and ClustalX (Thompson *et al*., 1997). A neighbor–joining (NJ) dendrogram (Saitou and Nei, 1987) based on Kimura's two parameter model (Kimura, 1980) was constructed so as to determine the genetic relationships using the MEGA 3.1 program package (Kumar *et al*., 2004). Bootstrap probabilities of nodes (P) (Efron, 1979) were calculated from 1000 replications using the MEGA 3.1 program package (Kumar *et al*., 2004). The haplotype network, estimated with the 95% statistical limits of parsimony using the algorithm in Statistical Parsimony method (Templeton *et al*., 1992) was constructed by the TCS 1.21 program package (Clement *et al*., 2000).

Materials examined

GH ad1: KYUM–PI 1837; AB752344, GH ad2: KYUM–PI 1818; AB752345, GH ad3: KYUM–PI 1827; AB752346, GH ad4: KYUM–PI 1840; AB752347, GH ad5: KYUM–PI 1842; AB752348, GH ad6: KYUM–PI 1836; AB752349, GG ad1: KYUM–PI 1900; AB752350, GG ad2: KYUM–PI 1903; AB752351, GG ad3: KYUM–PI 1906; AB752352, GG ad4: KYUM–PI 1913; AB752353, GG ad5: KYUM–PI 1908; AB752354, Type A1–1: KYUM–PI 1780–1; AB752355, Type A1–2: KYUM–PI 1780–6; AB752356, Type A1–3: KYUM–PI 1670–16; AB752357, Type A1–4: KYUM–PI 1718–18; AB752358, Type A1–5: KYUM–PI 1799–17; AB752359, Type A2–1: KYUM–PI 1695–4; AB752360, Type A2–2: KYUM–PI 1695–5; AB752361, Type A2–3: KYUM–PI 1745–8; AB752362, Type A2–4: KYUM–PI 1753–6; AB752363, Type B1–1: KYUM–PI 1696–2; AB752364, Type B1–2: KYUM–PI 1696–8; AB752365, Type B1–3: KYUM–PI 1696–10; AB752366, Type B1–4: KYUM–PI 1746–1; AB752367, Type B1–5: KYUM–PI 1746–11; AB752368, Type B2–1: KYUM–PI 1773; AB752369, Type B2–2: KYUM–PI 1781; AB752370, Type B2–3: KYUM–PI 1713–1; AB752371, Type B2–4: KYUM–PI 1713–2; AB752372, Type B2–5: KYUM–PI 1713–4; AB752373, Type B2–6: KYUM–PI 1746–17; AB752374 (Sample ID of this study: Museum reg. no.; Accession no.).

RESULTS

Morphotypes of leptocephali

Gnathophis leptocephali collected from the Kuroshio–Oyashio transition region were first classified into two groups (Groups A and B) on the basis of TM, VBV last and BH/TL ratio (Table), with further subdivision on the basis of absence (Types A1 and B2) or presence (Types A2 and B1) of posterior spinal cord melanophores (Fig. 2, Table). Types A1 and A2 had similar TM (117–128 and 119–126, respectively), VBV last (36–44 and 40–44, respectively) and BH/TL ratio (0.083–0.123

Table 1. Morphometric and meristic characters of each types of *Gnathophis* leptocephali

Leptocephalus type	n	TL	BH/TL ratio	Pigmentation
Type A1	58	69.6–98.9 (88.3±6.1)	0.083–0.123 (0.106±0.007)	Absent
Type A2	50	84.0–100.5 (94.1±3.6)	0.082–0.127 (0.109±0.008)	Present
Type B1	131	98.5–126.9 (110.7±5.0)	0.093–0.137 (0.120±0.008)	Present
Type B2	155	74.0–120.3 (106.2±8.8)	0.080–0.139 (0.118±0.009)	Absent

Leptocephalus type	n	VBV 1st	VBV last	PAM	PDM	TM	PAM/TM ratio
Type A1	58	9–12 (10.8±0.8)	36–44 (41.5±1.3)	91–106 (98.7±2.9)	70–97 (75.7±4.8)	117–128 (121.8±2.5)	0.74–0.85 (0.81±0.02)
Type A2	50	10–12 (10.7±0.7)	40–44 (41.6±1.0)	62–102 (94.9±6.8)	45–88 (72.7±5.4)	119–126 (122.0±2.2)	0.51–0.85 (0.78±0.06)
Type B1	131	10–13 (11.8±0.6)	41–48 (45.8±1.2)	64–111 (98.3±7.9)	43–90 (77.8±7.7)	126–134 (130.0±1.8)	0.50–0.83 (0.76±0.06)
Type B2	155	10–13 (11.7±0.7)	43–48 (46.0±1.1)	66–118 (103.8±4.4)	39–103 (83.2±5.3)	125–135 (129.9±2.0)	0.51–0.90 (0.80±0.03)

Parentheses show average ± standard deviation. TL: total length (mm), BH: body height (mm), Pigmentation: melanophores on the lateral surface of the posterior spinal cord, VBV 1 st: 1st vertical blood vessel myomere, VBV last.: last vertical blood vessel myomere, PAM: preanal myomere, PDM: predorsal myomere, TM: total myomere

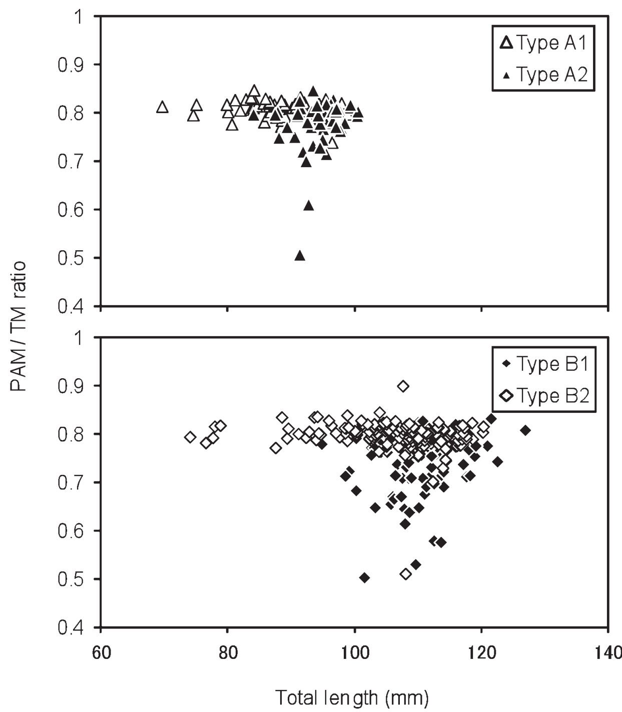

Fig. 3. Relationship between total length and pre–anal myomere (PAM) / total myomere (TM) ratio of each type of *Gnathophis* spp. leptocephali.

and 0.082–0.127, respectively) (Table). Similarly, Types B1 (TM: 126–134, VBV last: 41–48, BH/TL ratio: 0.093–0.137) and B2 (TM: 125–135, VBV last: 43–48, BH/TL ratio: 0.080–0.139) showed close morphological identities, there being no significant differences in TM ($p>0.05$, t–test), VBV last ($p>0.05$, t–test) and BH/TL ratio ($p>0.05$, t–test) between Types A1 and A2, or Types B1 and B2.

Some morphometric or meristic characters showed significant difference among each groups or types. TL of Type A2 was significantly larger than that of Type A1 ($p<0.01$, t–test). TL of Type B1 was larger than that of Type B2 ($p<0.01$, t–test). BH/TL ratio of Group B was significantly larger than that of Group A ($p<0.01$, t–test) (Table). Relationship between TL and PAM/TM ratio showed significant difference among both Types A1 and A2 or B1 and B2 (Fig. 3, Table).

Molecular genetic identification

A total of 31 specimens, including adult *G. heterognathos* (n=6) and *G. ginanago* (n=5), and Type A1 (n=5), A2 (n=4), B1 (n=5), and B2 (n=6) leptocephali were subjected to DNA analysis, a total of 495 base pairs of the mitochondrial DNA 16S rRNA gene for each specimen being determined. Among the adult *G. heterognathos* and *G. ginanago* specimens, seven instances of interspecific variation were detected, at pair of 86, 88,

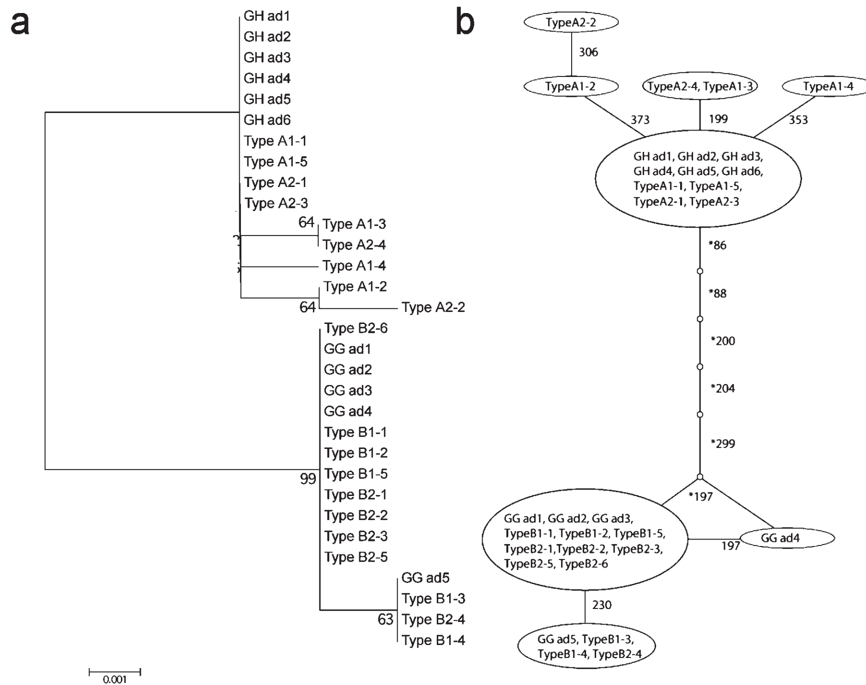

Fig. 4. a: Neighbor–Joining dendrogram for adults and leptocephali of *Gnathophis* spp. derived from mitochondrial DNA 16S rRNA gene sequences. b: Mitochondrial DNA 16S rRNA haplotype network estimated with 95% statistical limits of parsimony using the algorithm in Templeton *et al*. (1992). Number of variable sites from the 5' end indicated. * shows number of nucleotide of interspecific variation. GH ad: adult of *Gnathophis heterognathos*, GG ad: adult of *Gnathophis ginanago*.

197, 200, 204, 299, and 307. Single deletions (insertions) occurred between *G. heterognathos* and Group A (Types A1 and A2), and between *G. ginanago* and Group B (Types B1 and B2).

The neighbor–joining (NJ) dendrogram (Saitou and Nei, 1987) based on Kimura's two parameter model (Kimura, 1980), showed two clades with the 99% bootstrap probabilities (Fig. 4a). Moreover, both clades were supported by the haplotype network according to the Statistical Parsimony method (Templeton *et al*., 1992) (Fig. 4b). Adult *G. heterognathos*, and Types A1 and A2 were included in one clade, while adult *G. ginanago*, and Types B1 and B2 were clustered in the other clade. The number of nucleotide replacement (sequence divergence) in the first clade was 0–3 (0–0.61%), in the second clade, 0–1 (0–0.20%), and between each clade, 6–9 (1.21–1.81%), which was higher than that of in each clade. Therefore, the *Gnathophis* spp. leptocephali could be reasonably identified as either *G. heterognathos* or *G. ginanago*.

DISCUSSION

Gnathophis leptocephali collected from the transition region were sorted into four types (Types A1, A2, B1, and B2) based on the currently accepted morphological analysis (Fig. 2, Table). Type A1 was characterized by 117–128 TM, 36–44 VBV last, and absent of posterior spinal cord melanophores. These characters matched those of *G. heterognathos*. Type A2 had almost the same range of TM (119–126) and VBV last (40–44) as A1, but posterior spinal cord melanophores were present. The characters of Type B1 (TM: 126–134, VBV last: 41–48, presence of posterior spinal cord melanophores) matched those of *G. ginanago*, and those of Type B2 having similar TM (125–135) and VBV last (43–48) values, but lacking posterior spinal cord melanophores.

Alignment analysis of the mitochondrial DNA 16S rRNA gene of adult specimens indicated seven interspecific variations among *G. heterognathos* and *G. ginanago*. Intraspecific variations were not detected in adult of *G. heterognathos*, although one was apparent in *G. ginanago*. Clearly, adult of *G. heterognathos* and *G. ginanago* occupied different clades (Fig. 4). These results confirmed the validity of seven interspecific variations for discriminating between *G. heterognathos* and *G. ginanago*, the 16S rRNA region therefore being useful for identifying the two species. *Gnathophis* leptocephali were also divided into two groups, Groups A (including Types A1 and A2) and B (including Types B1 and B2) (Fig. 4). These two groups have been characterized by the same interspecific variations seen in adult *G. heterognathos* and *G. ginanago*. As shown in Fig. 4, Types A1 and A2, and Types B1 and B2 were included in the same clades as adult of *G. heterognathos* and *G. ginanago*, respectively. These results are indicating that the two groups of leptocephali could be identified as *G. heterognathos* and *G. ginanago*, respectively.

Pigmentation characteristics of leptocephali, traces of which persist for a time in juveniles, can provide a valid basis for identifying some species (Smith, 1979), particularly so when myomere numbers overlap (Mochioka *et al*., 2014; Smith, 1989). However the present study raised questions regarding the validity of pigmentation for identification of leptocephali in this case. Furthermore, although a single row of lateral pigment has been recognized as a discriminating character of *Conger myriaster* leptocephali (Tabeta and Mochioka, 1988), resent studies have reported pattern variations in the midlateral pigmentations of *C. myriaster* (Kurogi, 2008). Ma *et al*. (2007) suggested that these varied pigmentation patterns of *C. myriaster* leptocephali were depend on their growth. Presence of posterior spinal cord melanophores was shown in specimens with larger TL or decreasing PAM/TM ratio in each species (Fig. 3). Specimens allocated to the two types in each species in the present study with significant difference in TL and PAM/TM ratio ($p<0.01$, t–test) (Table) suggested the morphological change with metamorphosis. Therefore pigment variations of these types of *G. heterognathos* and *G. ginanago* leptocephali were considered to depend on their growth and metamorphosis. As summarized in Table, 50 of 108 specimens of *G. heterognathos* leptocephali had inconsistent pigmentation patterns. Furthermore, 155 of 286 *G. ginanago* leptocephali also could not be identified by the described pigmentation pattern. These results indicate that "absence (presence) of posterior spinal cord melanophores (Tabeta and Mochioka, 1988)" should not be considered a diagnostic character of *Gnathophis* spp. leptochali.

The present study provides further evidence of the usefulness of DNA studies for clarification of larval species' identities, although the importance of morphological characters must also receive continuing emphasis.

ACKNOWLEDGEMENTS

We are grateful to K. Mori (Fisheries Agency), M. Takahashi (Seikai National Fisheries Research Institute, Fisheries Research Agency) and Y. Shiga (Kyushu University), who provided facilities for sample collecting and genetic analysis, and Captain T. Yoshino, and the officers and crews of the training vessel Hokuho Maru for their eager assistance. We also thank G. Hardy (Ngunguru, New Zealand) for critical comments on the manuscript. This work was partially funded by projects for research of marine fisheries stock assessment and evaluation for Japanese waters from the Fisheries Agency, Japan.

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