IDENTIFICATION OF MID- TO FINAL STAGE PHYLLOSOMA LARVAE OF THE GENUS *PANULIRUS* WHITE, 1847 COLLECTED IN THE RYUKYU ARCHIPELAGO

BY

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ABSTRACT

Nucleotide sequence analysis of the mitochondrial COI gene was performed to identify mid- to final stage phyllosoma larvae of spiny lobsters of the genus *Panulirus* caught in the south of the Ryukyu Archipelago (northwest Pacific). The identified larvae were subjected to morphological investigation. All 92 larvae caught in May 2003 were late to final stage phyllosomas of *P. japonicus*, while 174 larvae in November 2004 comprised four species of phyllosoma group 1 (*P. femoristriga* ($n = 1$), *P. japonicus* (4), *P. longipes bispinosus* (110), and *P. l. longipes* (7)), one of group 2 (*P. penicillatus* (43)), and two of group 4 (*P. ornatus* (8) and *P. versicolor* (1)). Photo images of representative phyllosoma larvae, substantiated by molecular identification, are presented. Morphological investigation indicated that the ratio between the widths of cephalon and thorax (CT ratio) of the *P. japonicus* phyllosomas was the smallest, and discrimination of *P. japonicus* larvae from the other species of the "*P. japonicus* group" may be possible using the CT ratio combined with the smaller body size. Intraspecific variation was observed in the arrangement of subexopodal spines of *P. ornatus* and *P. versicolor* phyllosomas, indicating this character is not diagnostic to separate these two species.

RÉSUMÉ

L'analyse de la séquence nucléotidique du gène mitochondrial COI a été réalisée afin d'identifier les larves phyllosomes de langoustes du genre *Panulirus*, aux stades larvaires allant du milieu à la fin du développement, collectées au sud de l'archipel des Ryukyu (Pacifique nord-occidental). Les larves identifiées, ont été soumises à un examen morphologique. Les 92 larves collectées en mai 2003 étaient des phyllosomes du dernier stade de *P. japonicus*, tandis que les 174 larves de novembre 2004 comprenaient quatre espèces de phyllosomes de groupe 1 (*P. femoristriga* ($n = 1$), *P. japonicus* (4), *P. longipes bispinosus* (110) et *P. l. longipes* (7)), une du groupe 2 (*P. penicillatus* (43)) et deux du groupe 4 (*P. ornatus* (8) et *P. versicolor* (1)). Des images photo des larves phyllosomes représentatives, corroborées par l'identification moléculaire, sont présentées. L'analyse

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morphologique a montré que le rapport entre les largeurs du cephalon et du thorax (rapport CT) des phyllosomes de *P. japonicus* était le plus bas, et la séparation des larves de *P. japonicus* des autres espèces du « groupe *P. japonicus* » peut être faite en utilisant le rapport CT combiné avec la taille plus petite du corps. Une variation intraspécifique a été observée dans l'arrangement des épines subexopodiales des phyllosomes de *P. ornatus* et de *P. versicolor*, ce qui indique que ce caractère n'est pas diagnostique pour séparer ces deux espèces.

INTRODUCTION

Lobster species of the genus *Panulirus* White, 1847, have been classified into four adult species-groups (I to IV) (George & Main, 1967), and this grouping may be roughly consistent with the assignment of their phyllosoma larvae, such as phyllosoma species-groups 1 to 4 as proposed by McWilliam (1995), or Forms A to E described by Murano (1971). However, morphological species identification of the phyllosoma larvae within the species-groups has been considerably equivocal. This is partially due to the long planktonic larval period, extending from several months to more than a year (Chittleborough & Thomas, 1969), so that long-lived teleplanic larvae may drift to distant waters, where their adult forms do not exist. Larval rearing for an extended period has been extremely difficult, and complete larval development in the laboratory has been achieved in only two species, *Panulirus japonicus* (Von Siebold, 1824) and *P. longipes* (A. Milne-Edwards, 1868) (cf. Inoue, 1981; Kittaka & Kimura, 1989; Yamakawa et al., 1989; Matsuda & Yamakawa, 2000; Sekine et al., 2000). Johnson (1971a) pointed out that the phyllosoma larvae of the "*P. japonicus* group" (phyllosoma species-group 1 or Form A) may be very similar or even indistinguishable from one another in morphology. Sekiguchi (1986) suggested that the ratio between the widths of cephalon and thorax (CT ratio, see fig. 1) of the late stage *P. japonicus* phyllosomas may be smaller than in the other group 1 species in the northwestern Pacific. On the other hand, laboratory observations from complete larval development in *P. japonicus* and *P. longipes* indicated that the CT ratio may not be particularly effective to discriminate these two species. Among six species of the adult species-group IV, corresponding to phyllosoma species-group 4 or Forms D or E, five are common in tropical to subtropical regions of the Indo-Pacific or the coastal areas of the South China Sea. The phyllosoma larvae of these five species, *P. homarus* (Linnaeus, 1758), *P. ornatus* (Fabricius, 1798), *P. polyphagus* (Herbst, 1793), *P. stimpsoni* Holthuis, 1963, and *P. versicolor* (Latreille, 1804) are termed "*P. homarus* larval complex" (McWilliam & Phillips, 1992). Johnson (1971a) considered that subexopodal spines occur on pereiopods 1-3 of mid to final stage larvae of *P. ornatus* and on 1-4 of *P. versicolor* (see fig. 1), and this view has been adopted by subsequent investigators (Berry, 1974; McWilliam & Phillips, 1992;

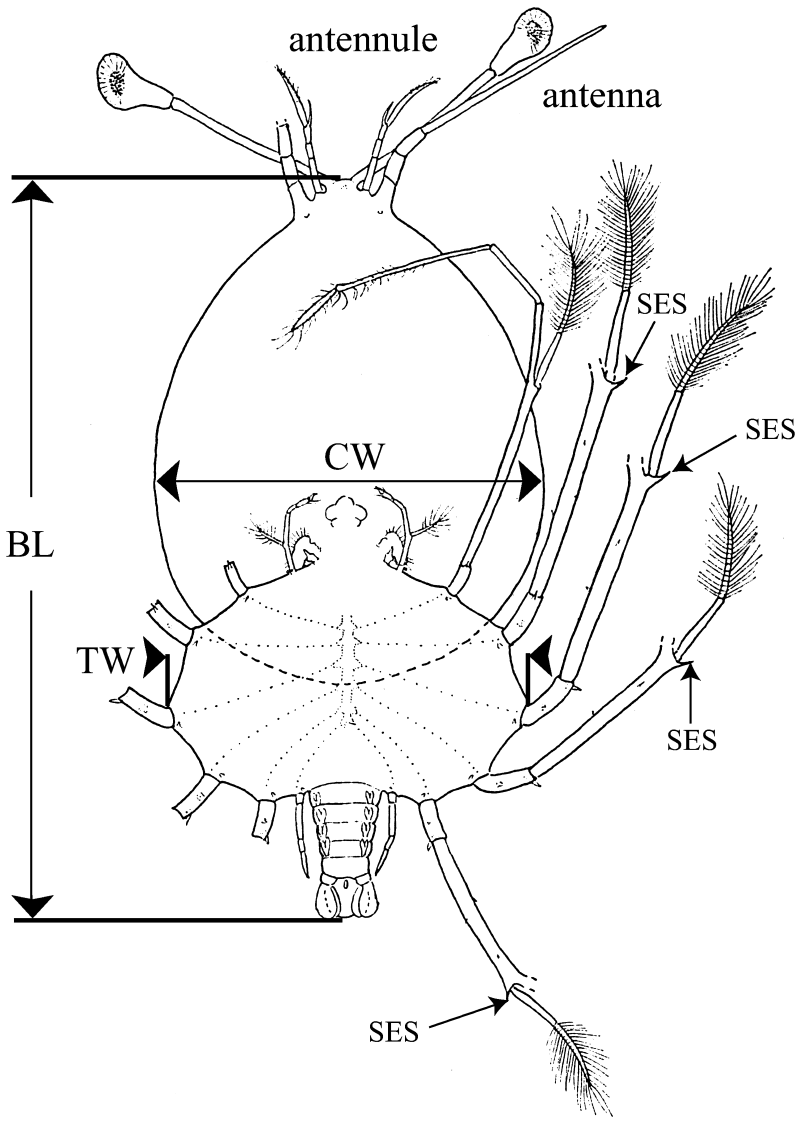

Fig. 1. Sub-final stage phyllosoma larva of *Panulirus versicolor* (Latreille, 1804) illustrated by Johnson (1971a), showing morphological features measured and recorded in this study. BL, body length; CW, cephalic width; TW, thorax width; SES, subexopodal spine.

Booth & Phillips, 1994; McWilliam, 1995). However, all these morphological descriptions rely exclusively on the distribution and relative abundance of adults, to the neglect of substantial species identification.

Molecular species identification of phyllosoma larvae was first attempted by Silberman & Walsh (1992). Using restriction fragment length polymorphism (RFLP) analysis based on PCR amplification of 28S rDNA, they demonstrated

unambiguous discrimination of phyllosoma larvae of two *Panulirus* species in the northwestern Atlantic. The technique for DNA analyses has now become common and molecular data of almost all the species of *Panulirus* are available (Ptacek et al., 2001; Yamauchi et al., 2002; Patek & Oakley, 2003; Ravago & Juinio-Meňez, 2003), but no biochemical genetic analysis has been incorporated in subsequent studies for phyllosoma identification and distribution (McWilliam & Phillips, 1992; Baisre & Alfonso, 1994; McWilliam, 1995; Yoshimura et al., 1999; Inoue & Sekiguchi, 2001; Sekiguchi & Inoue, 2002; Yoshimura et al., 2002; Inoue et al., 2004). Recently, Chow et al. (2006) developed RFLP analysis using the mitochondrial DNA COI gene to identify 10 spiny lobster species of the Indo-Pacific, and applied the technique supplemented with nucleotide sequence analysis to the mid- to final stage phyllosoma larvae collected in the northwestern Pacific. In their sample, all larvae belonging to phyllosoma species-group 2 or Form C were *P. penicillatus* (Olivier, 1791). On the other hand, larvae of phyllosoma species-group 1 or Form A were unexpectedly variable in species composition, in which mid- to subfinal phyllosoma larvae of *P. brunneiflagellum* Sekiguchi & George, 2005 (formerly known as *P. 'aka'*: see Sekiguchi & George, 2005), *P. femoristriga* (Von Martens, 1872), *P. japonicus*, *P. longipes bispinosus* Borradaile, 1899, and *P. l. longipes* (A. Milne-Edwards, 1868) were observed. Although they observed a significantly larger body size in *P. l. bispinosus* than in *P. japonicus* at a similar developmental stage, further morphological investigation was not attempted.

We have carried out plankton surveys in the south of the Ryukyu Archipelago in May 2003 and November 2004, in which *Panulirus* larvae of phyllosoma species-groups 1, 2, and 4 (corresponding to Forms A, C, and D, respectively) were collected. In this study, we report the results of both the molecular species identification and the morphological investigation on the *Panulirus* phyllosomas collected on those two cruises. We also discuss larval transportation schemes in *P. japonicus* and *P. l. bispinosus*.

MATERIALS AND METHODS

Two cruises for plankton research were performed by the R/V "Shun-yo Maru", of the Fisheries Research Agency, in May 2003 and November 2004, in which a large mid-water trawl (see Tanabe & Niu, 1998) and a frame trawl net (MOHT) (see Oozeki et al., 2004) were used, respectively. Both cruises were performed at a similar location, ranging from 21°56′N to 24°50′N and from 123°25′E to 126°12′E. Phyllosoma larvae were preserved in ethanol on board and transferred to the laboratory. Phyllosomas of *Panulirus* were sorted from other species based on the shape of the cephalic and thorax regions, and the presence of a setose exopod on

the third maxilliped. Body length (BL) and the widths of the cephalic and thoracic regions (CW and TW) were measured, and the arrangement of subexopodal spine (SES) was recorded (fig. 1). Usually appendages were used for DNA extraction, and whenever possible one pereiopod of a pair was left intact. Otherwise, a piece of thorax was dissected and used. The tissues were minced and homogenized in a 1.5 ml microcentrifuge tube using a teflon pestle. Crude DNA was extracted using a DNA extraction kit (GenomicPrep Cells and Tissue DNA Isolation Kit, Amersham Bioscience). The procedures for the polymerase chain reaction (PCR), and amplification of the mitochondrial cytochrome oxidase I gene (COI) region followed by endonuclease digestion and agarose gel electrophoresis are described in Chow et al. (2006). Direct nucleotide sequence analysis was performed for the phyllosoma larvae whose restriction profiles did not match the adult standards reported by Chow et al. (2006). The sequences determined by either using forward or reverse primers were incorporated into phylogenetic analysis, to investigate the affiliation of the phyllosoma larvae as performed by Chow et al. (2006). The definition of the developmental stages of phyllosoma larvae is arbitrary. However, based on the description for *P. longipes* larvae by Matsuda & Yamakawa (2000), we tentatively determined the developmental stages according to the relative length of antenna and antennule, the relative length of thorax and abdominal regions, and the development of pereiopods and gill buds.

RESULTS

Composition of species and developmental stages

Of the phyllosoma larvae collected, 95 larvae in May 2003 and 176 in November 2004 were morphologically identified as belonging to the genus *Panulirus*. Successful PCR amplification was performed in 92 phyllosoma larvae of May, and in 174 of the November samples. Restriction profiles of 235 out of 266 larvae matched those of the adult standards reported by Chow et al. (2006). Phylogenetic analysis based on nucleotide sequences was used to identify the remaining 31 larvae. The nucleotide sequences of these 31 larvae are available in the DDBJ (DNA Data Bank of Japan) under accession numbers AB237598 to AB237639. One Form D larva was identified to be of *P. versicolor* by RFLP analysis, then nucleotide sequence analysis was performed and the sequences (AB244283 and AB244284) obtained were nearly identical to the published data for *P. versicolor* (cf. Ptacek et al., 2001; Ravago & Juinio-Meñez, 2003).

Photo images of phyllosoma larvae of the seven species at representative stages are presented in figs. 2 to 6, showing that morphological assignment of mid- to final stage larvae into phyllosoma species-groups 1, 2, and 4 (corresponding to

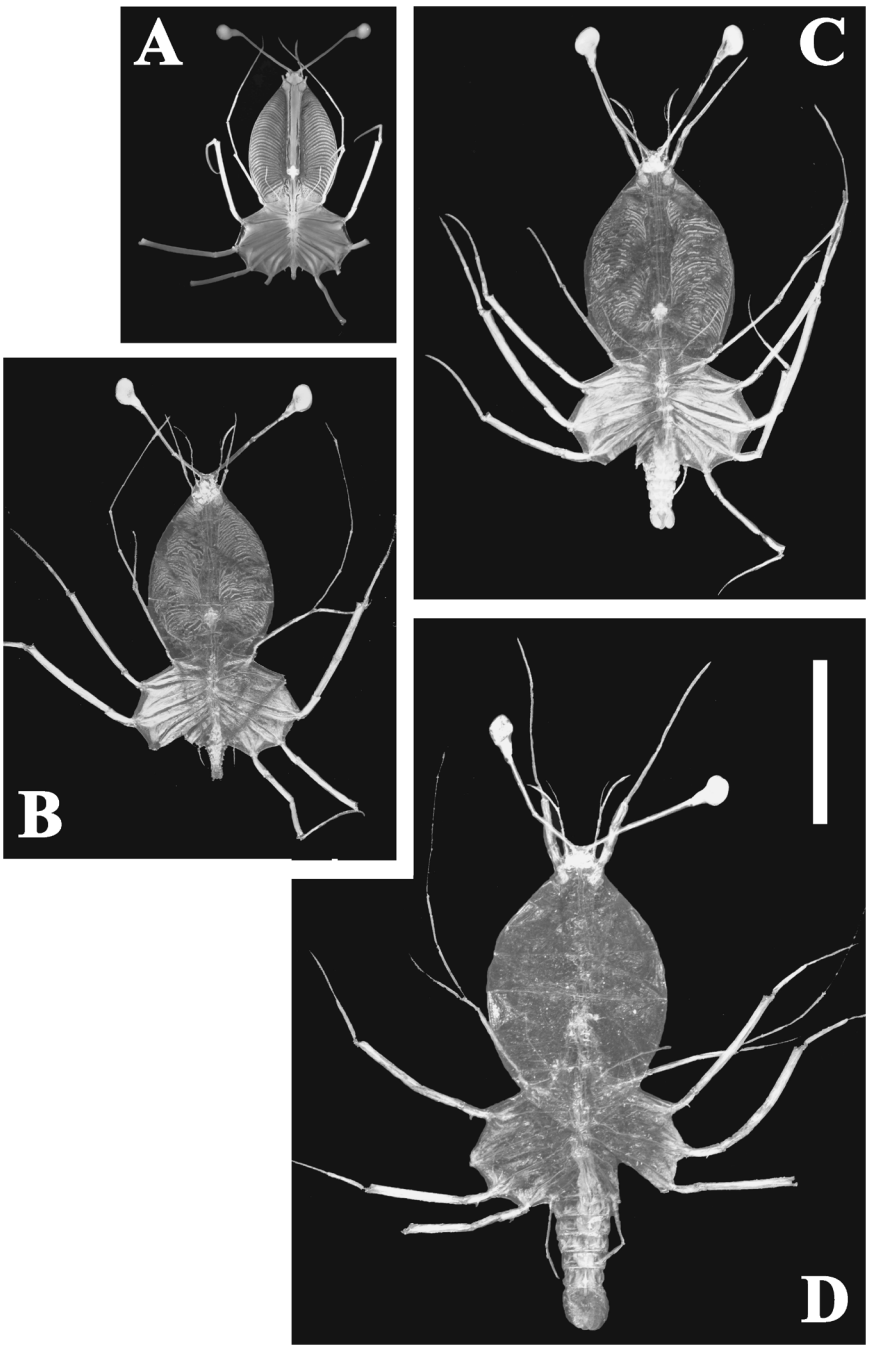

Fig. 2. Phyllosoma larvae of *Panulirus japonicus* (Von Siebold, 1824). Stages are determined as: A, VII; B, VIII; C, IX; and D, X. Scale = 10 mm.

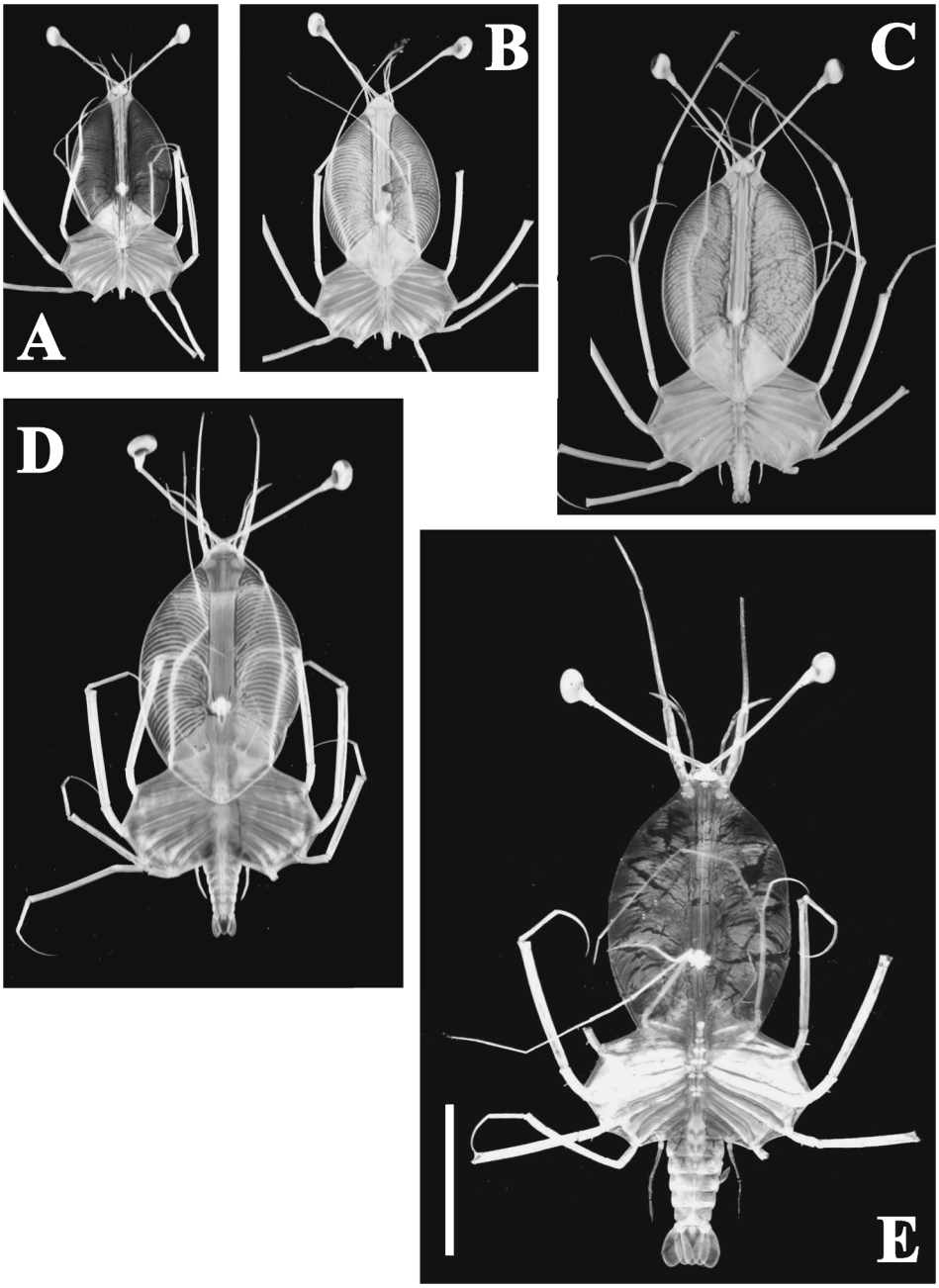

Fig. 3. Phyllosoma larvae of *Panulirus longipes bispinosus* Borradaile, 1899. Stages are determined as: A, VI ; B,VII ; C, VIII; D, IX; and E, X. Scale = 10 mm.

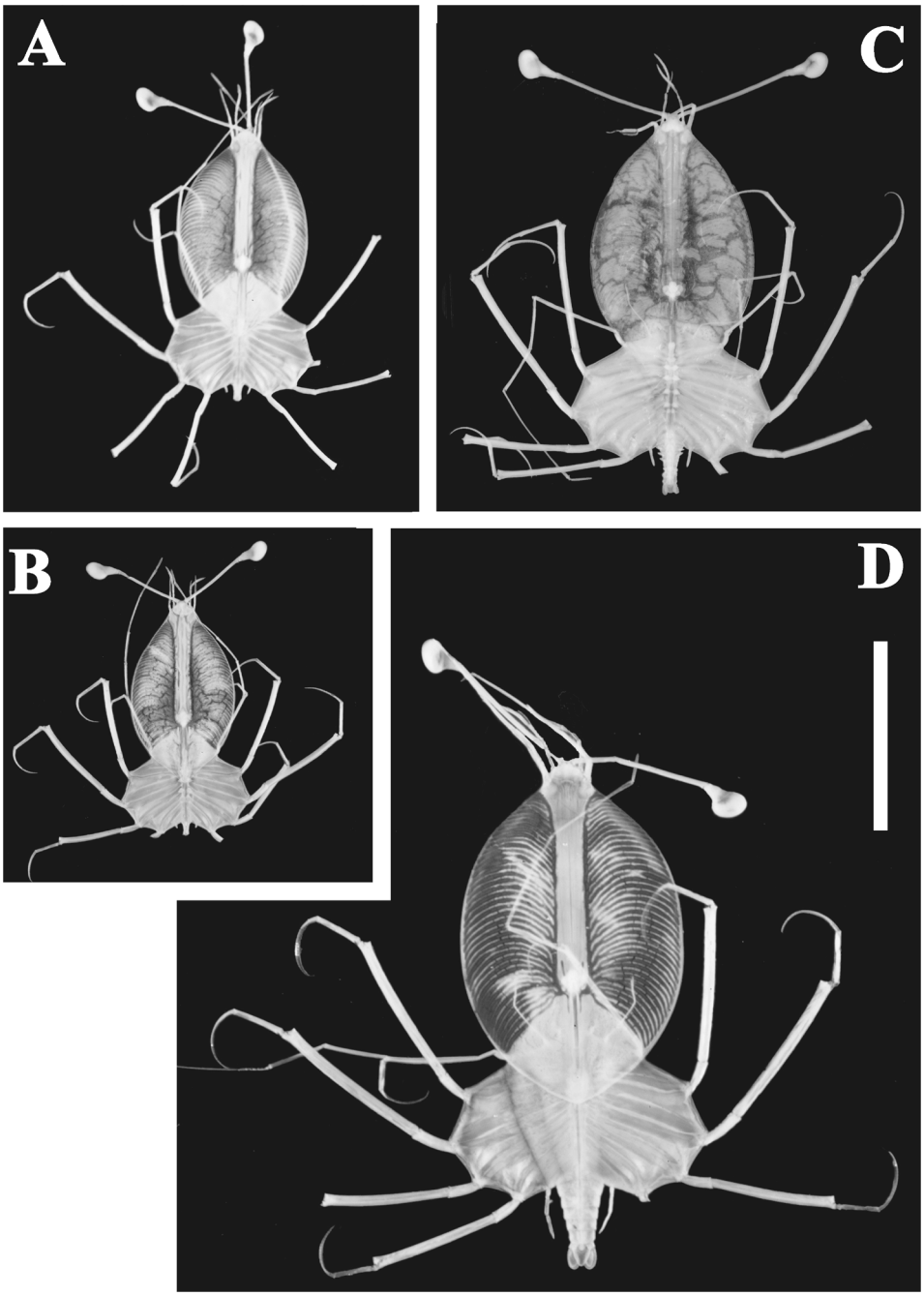

Fig. 4. Phyllosoma larvae of A, *Panulirus femoristriga* (Von Martens, 1872); and, B-D, *P. longipes longipes* (A. Milne-Edwards, 1868). Stages are determined as: A, B, VI I; C, VIII; and D, IX. Scale = 10 mm.

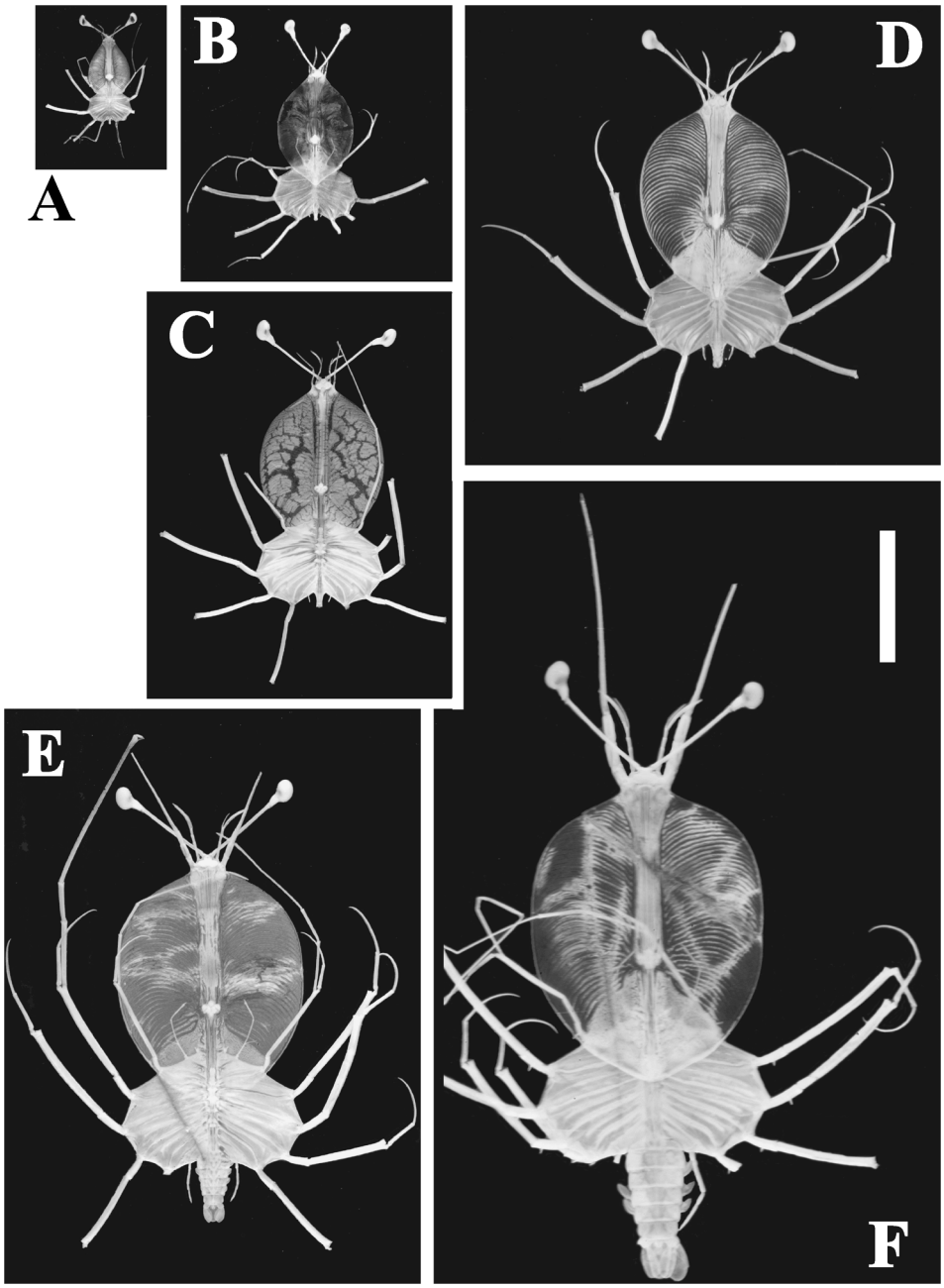

Fig. 5. Phyllosoma larvae of *Panulirus penicillatus* (Olivier, 1791). Stages are determined as: A, V; B, VI; C, VII; D, VIII; E, IX; and F, X. Scale = 10 mm.

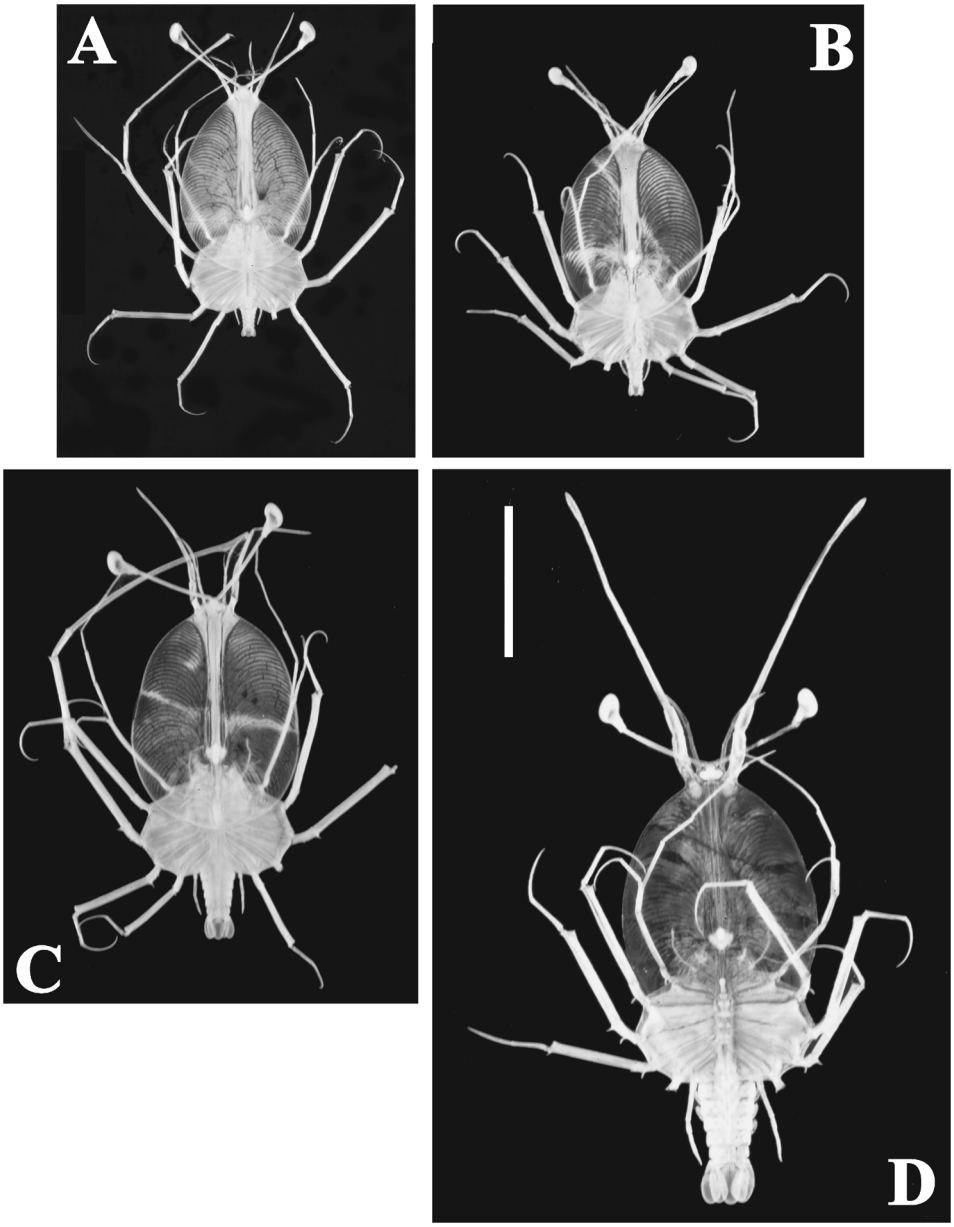

Fig. 6. Phyllosoma larvae of A, *Panulirus versicolor* (Latreille, 1804); and, B-D, *P. ornatus* (Fabricius, 1798). Stages are determined as: A, B, VIII; C, IX; and D, X. Scale = 10 mm.

Murano's (1971) Forms A, C, and D, respectively) is not difficult. The species composition and developmental stages found in the samples of the two research cruises are shown in table I. All 92 larvae in May were *P. japonicus*, and comprised

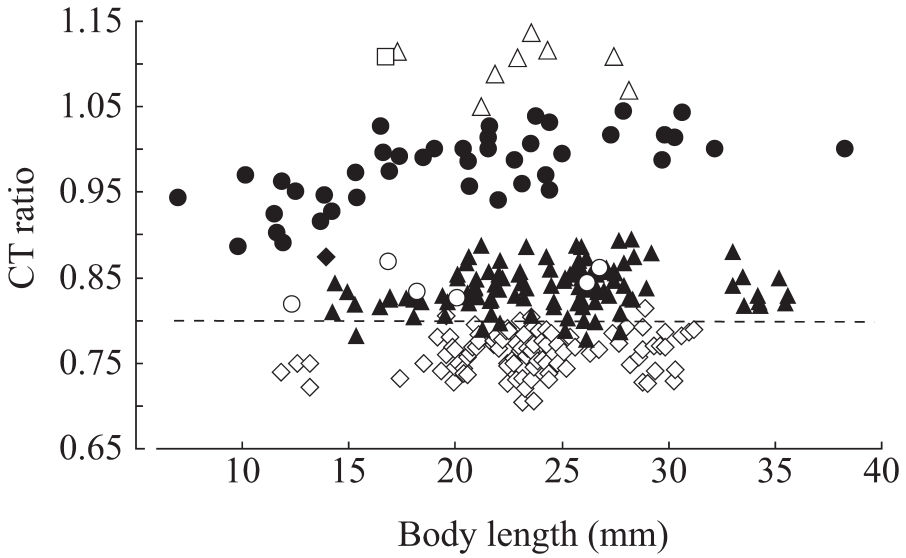

Fig. 7. The ratio between widths of cephalic and thorax regions (CT ratio) plotted against body length of phyllosoma larvae of seven *Panulirus* species. Open diamonds, *P. japonicus*; closed triangles, *P. longipes bispinosus*; open circles, *P. l. longipes*; closed diamond, *P. femoristriga*; closed circles, *P. penicillatus*; open triangles, *P. ornatus*; open squares, *P. versicolor*. Dotted line (CT ratio = 0.8) separates *P. japonicus* from the other species of the "*P. japonicus* group" at 5% error rate.

late to final stages (VIII to X). In contrast, the November sample was more variable in species composition, and phyllosoma species-groups 1, 2, and 4 were observed. Of the species-group 1 larvae, *P. longipes bisponisus* (n = 110) was the most abundant, and mid- to final stage (VI to X) larvae were observed. The other twelve larvae of species-group 1 were *P. femoristriga* (1), *P. japonicus* (4), and *P. l. longipes* (7), comprised mid-stage (VII) larvae of *P. femoristriga* and *P. japonicus*, and mid- to sub-final stage larvae (VII to IX) of *P. l. longipes*. All of the species-group 2 phyllosoma larvae were identified to be *P. penicillatus* (n = 43), comprising mid- to final stages (V to X). Nine larvae of species-group 4 were identified to be late to final (VIII to X) stage larvae of *P. ornatus* (8) and a late stage (VIII) larva of *P. versicolor* (1).

Morphological investigation

The means of CT ratio and body length with standard deviations at each stage are presented for seven species in tables II and III. The CT ratios plotted against body length are shown in fig. 7. Although a slight but significant positive correlation between body size and CT ratio was observed only in *P. penicillatus* ($r^2 = 0.445$, $P < 0.001$), the CT ratios among species or species-groups were compared without considering body size. ANOVA revealed the CT ratio among

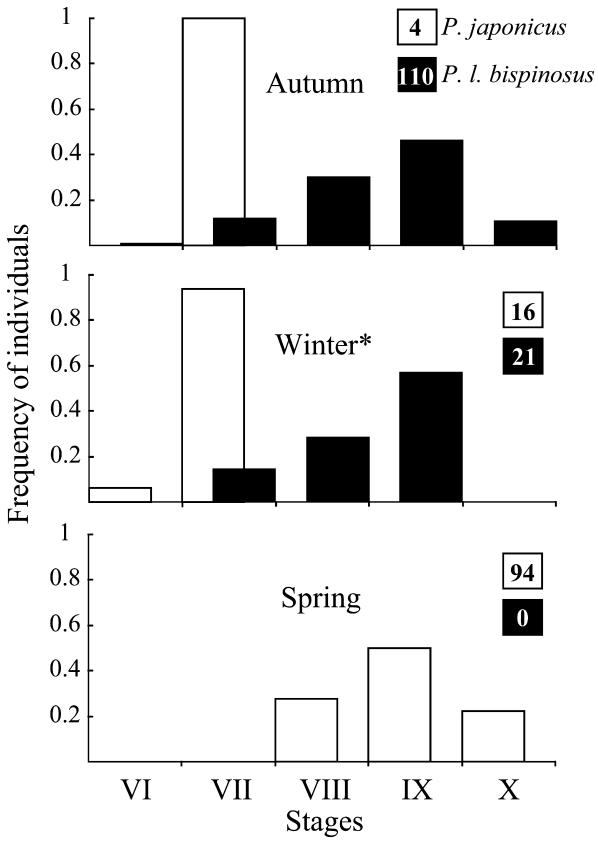

Fig. 8. Frequency of mid- to final stage larvae (VI to X) of *Panulirus japonicus* (Von Siebold, 1824) (open column) and *P. longipes bispinosus* Borradaile, 1899 (closed column) observed in the autumn, winter, and spring samples. Number of larvae is shown in legend column. *Data of the winter sample were derived from Chow et al. (2006).

phyllosoma species-groups to be significantly heterogeneous ($P < 0.001$). The subsequent Tukey test indicated that the mean CT ratio of phyllosoma species-group 4 (*P. ornatus* and *P. versicolor*) was significantly larger than for the other species, and that of species-group 2 (*P. penicillatus*) was significantly larger than that of species-group 1 ($P < 0.05$). The CT ratio between species within species-group 1 was also heterogeneous ($P < 0.001$), in which *P. japonicus* phyllosomas had a significantly smaller CT ratio than the other species (without *P. femoristriga*) ($P < 0.05$). No significant difference in CT ratio was observed between *P. l. bispinosus* and *P. l. longipes*. Even when *P. japonicus* data were removed, the CT ratio of species-group 1 was significantly smaller than that of the other groups ($P < 0.05$). No larvae of *P. l. bispinosus* and *P. l. longipes* had CT ratios smaller than 0.77 or larger than 0.9.

TABLE I
Number and stage of phyllosoma larvae of seven species of *Panulirus* collected in two research cruises performed by R/V "Shunyo-Maru"

Species	May 2003	Nov. 2004	Stage V	VI	VII	VIII	IX	X
P. femoristriga (Von Martens, 1872)	0	1	0	0	1	0	0	0
P. japonicus (Von Siebold, 1824)	92	4	0	0	4*	27	45	20
P. longipes bispinosus Borradaile, 1899	0	110	0	1	13	34	52	11
P. l. longipes (A. Milne-Edwards, 1868)	0	7	0	1	1	3	2	0
P. ornatus (Fabricius, 1798)	0	8	0	0	0	1	5	2
P. penicillatus (Olivier, 1791)	0	43	1	10	8	16	7	1
P. versicolor (Latreille, 1804)	0	1	0	0	0	1	0	0

* All stage VII larvae of *P. japonicus* were from the November sample.

Body lengths of phyllosoma larvae at the same developmental stages were observed to be heterogeneous among species (table III). In phyllosoma species-group 1, larvae of *P. japonicus* were significantly smaller than those of *P. l. bispinosus* at all stages and also than that of *P. l. longipes* at stage IX ($P < 0.05$). No sub-final (IX) or final (X) stage phyllosoma larvae of *P. japonicus* were observed to reach 27 and 32 mm, respectively. No significant difference in body length was observed between *P. l. bispinosus* and *P. l. longipes* larvae, and sub-final (IX) and final (X) stage phyllosoma larvae of these species never exceeded 30 or 36 mm, respectively. Larvae of *P. penicillatus* may be the largest amongst the species examined, exceeding 30 and reaching 38 mm at stages IX and X, respectively, while those of *P. ornatus* and *P. versicolor* may be the smallest.

Discriminant analysis indicated that the CT ratio of 0.8 may be a boundary line to clearly separate *P. japonicus* from the other species of the "*P. japonicus* group" at a 5% error rate (fig. 7, dotted line). All phyllosoma larvae showing CT ratios smaller than 0.77 may be *P. japonicus*, although this criterion may lose 30% of the *P. japonicus* larvae that have relatively larger CT ratios. Thus, mid- to late stage phyllosoma larvae of *P. japonicus* may be discriminated from the other species of the "*P. japonicus* group" by the smaller CT ratio, combined with the relatively smaller body size at the same developmental stage.

Intraspecific and intra-individual variation of the SES (subexopodal spines) were observed in the phyllosomas of *P. ornatus* and *P. versicolor*. Six out of eight *P. ornatus* larvae had sufficient pereiopod setae arrangements for this investigation. Of six *P. ornatus* larvae, four had SES on the 1st to 3rd pereiopods but not on 4th, one final stage larva was observed to possess SES on a 4th pereiopod, and one sub-final larva had no SES on any of its pereiopods. One late stage (VIII) *P. versicolor* larva had an intact array of pereiopods on one side, and an SES was

TABLE II

Means of ratios between cephalon and thorax width (CT ratio ± SD) at each stage of phyllosoma larvae of seven species of *Panulirus*; range is shown in parentheses

Species	Stage						Total
	V	VI	VII	VIII	IX	X	
P. femoristriga (Von Martens, 1872)	–	–	0.874	–	–	–	0.874
P. japonicus (Von Siebold, 1824)	–	–	0.740 ± 0.013^{c} (0.722 ~ 0.75)	0.761 ± 0.019^{c} (0.727 ~ 0.806)	0.761 ± 0.024^{d} (0.704 ~ 0.803)	0.766 ± 0.024^{c} (0.726 ~ 0.815)	0.761 ± 0.023^{d} (0.722 ~ 0.815)
P. longipes bispinosus Borradaile, 1899	–	0.810	0.821 ± 0.015^{b} (0.782 ~ 0.843)	0.838 ± 0.024^{b} (0.789 ~ 0.887)	0.852 ± 0.066^{c} (0.778 ~ 0.895)	0.836 ± 0.019^{b} (0.816 ~ 0.880)	0.838 ± 0.025^{c} (0.778 ~ 0.895)
P. l. longipes (A. Milne-Edwards, 1868)	–	0.819	0.868	0.833 ± 0.007^{b} (0.826 ~ 0.839)	0.852 ± 0.013^{c} (0.843 ~ 0.861)	–	0.842 ± 0.020^{c} (0.819 ~ 0.861)
P. ornatus (Fabricius, 1798)	–	–	–	1.115	1.100 ± 0.033^{a} (1.050 ~ 1.137)	1.089 ± 0.029^{a} (1.069 ~ 1.109)	1.099 ± 0.028^{a} (1.050 ~ 1.137)
P. penicillatus (Olivier, 1791)	0.943	0.924 ± 0.030 (0.885 ~ 0.970)	0.987 ± 0.024^{a} (0.943 ~ 1.000)	0.992 ± 0.030^{a} (0.940 ~ 1.038)	1.017 ± 0.021^{b} (0.987 ~ 1.044)	1.000	0.979 ± 0.041^{b} (0.885 ~ 1.044)
P. versicolor (Latreille, 1804)	–	–	–	1.107	–	–	1.107

Mean CT ratios carrying different superscripts at the same stages are significantly different from one another ($P < 0.05$).

TABLE III

Mean body length (mm ± SD) and range (parentheses) at each stage of phyllosoma larvae of seven species of *Panulirus*

Species	Stage					
	V	VI	VII	VIII	IX	X
P. femoristriga (Von Martens, 1872)	–	–	13.9	–	–	–
P. japonicus (Von Siebold, 1824)	–	–	12.7 ± 0.6^{b} (11.8 ~ 13.2)	20.3 ± 1.0^{c} (17.4 ~ 22.1)	23.7 ± 0.9^{c} (22.2 ~ 26.3)	29.1 ± 1.1^{b} (26.7 ~ 31.2)
P. longipes bispinosus Borradaile, 1899	–	14.2	16.8 ± 1.4^{a} (14.2 ~ 18.4)	21.5 ± 1.2^{b} (19.4 ~ 23.5)	26.3 ± 1.3^{b} (22.2 ~ 29.2)	33.7 ± 1.8^{a} (29.0 ~ 35.6)
P. l. longipes (A. Milne-Edwards, 1868)	–	12.3	16.9	20.1 ± 0.7^{bc} (19.5 ~ 20.9)	26.5 ± 0.4^{b} (26.2 ~ 26.8)	–
P. ornatus (Fabricius, 1798)	–	–	–	17.3	22.8 ± 1.2^{d} (21.2 ~ 24.3)	27.8 ± 0.1^{c} (27.4 ~ 28.1)
P. penicillatus (Olivier, 1791)	7.0	12.1 ± 1.5 (9.9 ~ 14.2)	16.9 ± 1.3^{a} (15.3 ~ 19.0)	22.6 ± 1.5^{a} (20.4 ~ 25.0)	29.7 ± 1.7^{a} (27.3 ~ 32.2)	38.3
P. versicolor (Latreille, 1804)	–	–	–	16.7	–	–

Means of body length carrying different superscripts at the same stages are significantly different ($P < 0.05$).

observed on the 1st and 2nd but not on 3rd and 4th pereiopods. This larva had lost the 1st and 4th pereiopods of the other side, and the SES was observed on the 2nd and 3rd pereiopods. Thus, the results indicate that the SES arrangement may not be a diagnostic characteristic for discriminating between phyllosoma larvae of *P. ornatus* and *P. versicolor*. All these phyllosoma larvae of *P. ornatus* and *P. versicolor* were deposited in the Natural History Museum and Institute, Chiba, Japan under specimen numbers CBM-ZC 8534 to 8542.

DISCUSSION

Identification of phyllosoma larvae

The results of the present investigation support the phyllosoma species-groups proposed by McWilliam (1995), and indicate these to be a valid system, but all the same showed that morphological species identification of larvae within the phyllosoma species-groups may be very difficult.

Larvae of *P. penicillatus* categorized to phyllosoma species-group 2 (corresponding to Murano's (1971) Form C) is morphologically unique (see fig. 5). Since this species is the only one of the adult species-group II in the Indo-Pacific (McWilliam, 1995) and the larvae appear to be morphologically distinct from those of *Panulirus interruptus* (Randall, 1840) (cf. Johnson, 1956, 1971b) belonging to the same phyllosoma species-group 2 (McWilliam, 1995), the external morphology alone may discriminate mid- to final stage phyllosoma larvae of *P. penicillatus* from those of the other species in the Indo-Pacific. Furthermore, Murano's (1971) Form B (stage X in his study but determined as IX in the present study) may be of *P. penicillatus*, because of the large body size (32.1 mm) and CT ratio (0.95).

Sekiguchi (1986) presented a key character to separate late stage phyllosoma larvae of *P. japonicus* and *P. longipes*, having cephalic shields "distinctly" or "slightly" narrower than the thorax region, respectively. Although this vague description appears to be invalid for discriminating *P. japonicus* from *P. longipes* sspp. and seems to be abandoned in Sekiguchi & Inoue (2002), the present investigation indicates the implication proposed by Sekiguchi (1986) to be correct. This morphological characteristic of *P. japonicus*, substantiated by the present molecular analysis, may be used to re-examine Form A phyllosoma specimens collected and preserved in previous studies. Nevertheless, identification of phyllosoma larvae of *P. longipes* sspp. definitely requires molecular techniques. Form D larvae described by Murano (1971) are morphologically very similar to our final stage larva of *P. ornatus* (fig. 6 D) but require molecular techniques to be identified, as we have observed intraspecific and intra-individual variation in SES arrangement. Although the present study indicates that the arrangement of the SES (subexopodal

spine) is not a reliable key character for separating *P. ornatus* and *P. versicolor*, a more comprehensive analysis using larger numbers of specimens is necessary to investigate the aspect of intraspecific variation in the SES arrangement.

Implications for larval transportation in *P. japonicus* and *P. longipes bispinosus*

Although many efforts have been made to delineate the larval transportation process of *P. japonicus* around Japan (Yoshimura et al., 1999; Inoue & Sekiguchi, 2001; Sekiguchi & Inoue, 2002; Yoshimura et al., 2002; and references therein), uncertainty in the identity of Form A phyllosoma larvae has been a primary obstacle. Data obtained from November (autumn) and May (spring) samples of *P. japonicus* and *P. l. bispinosus* in the present study, and those from the January (winter) sample reported by Chow et al. (2006) are still fragmentary, but may implement larval series to outline the transportation process (fig. 8). The autumn sample was dominated by mid- to final stage larvae (VI to X) of *P. l. bispinosus* ($n = 110$), with only four *P. japonicus* larvae of stage VII. One puerulus was observed on this cruise, which was later molecularly identified to be of *P. l. bispinosus* (unpubl. data). Chow et al. (2006) observed a larger number of *P. japonicus* larvae ($n = 16$) comparable with *P. l. bispinosus* (21) in the winter sample collected in the north-east of the Ryukyu Archipelago (27°30′-30°27′N 133°20′-135°E). More advanced stage larvae were observed in *P. l. bispinosus* (VII to IX) than in *P. japonicus* (VI and VII) in both the autumn and winter samples, but the stage compositions in each species were similar between autumn and winter samples. Again, one puerulus of *P. l. bispinosus* was observed in the winter sample (unpubl. data). It was not expected to observe no larvae of *P. longipes* sspp. in the spring sample, while aggregation of more advanced stage larvae of *P. japonicus* may be reasonable. Settlement of *P. longipes* has been observed to occur after October in Japanese waters (Tanaka et al., 1984; Tanaka, 1987; Yoshimura et al., 1999; Inoue et al., 2002). Our data coincide well with these field observations and indicate that the metamorphosis and settlement of *P. longipes* sspp. must be completed by spring. Settlement of *P. japonicus* pueruli in Japanese waters occurs from April to October (Fushimi, 1978; Yoshimura et al., 1999), and the large aggregation of late to final stage phyllosoma larvae of this species observed in the south of the Ryukyu Archipelago may be the reservoir. Thus, the results obtained in this study support hypotheses for the larval transportation processes of *P. japonicus* proposed in previous studies (Sekiguchi, 1997; Yoshimura et al., 1999; Inoue & Sekiguchi, 2001; Sekiguchi & Inoue, 2002). However, it is entirely unknown how phyllosoma larvae stay in a certain body of water for a significant period and leave that water at the appropriate timing. Since the problem of species identification of *Panulirus* phyllosoma larvae is now almost solved, it is necessary to design a

sampling scheme for a better understanding of the larval transportation process in *P. japonicus*.

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