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Cypris Larvae (Cirripedia: Balanomorpha) Display Auto-fluorescence in Nearly Species-specific Patterns

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The identification of balanomorph larvae plays an important role in ecological study and for protection against biofouling. However, it is difficult to identify species of cyprids (settling larvae) of balanomorph barnacles, as they show remarkably similar morphology. Some authors have suggested distinguishing cyprids of different species by carapace length, pigmentation, and fine carapace detail. However, such criteria are only applicable to a narrow range of balanomorph species. Recently, we were have serendipitously found species-specific distribution of fluorescent substances in cultured cyprids obtained from adult balanomorph barnacles, collected near the coast of Japan. Fluorescent patterns (FPs) of cyprids from 11 species were classified into five major groups. Cyprids specimens collected from the field were estimated, based on the FPs and other morphological characteristics (pigmentation and carapace length), after which their species were identified using the following two criteria: the morphology of adults derived from the field cyprids that adhered to a culture dish, and cyprid 12S rRNA gene sequence. The results of species estimation by FPs largely corresponded to the correct species identification. Other FP groups were found in the field cyprids. This study of FPs should be helpful for identification of cyprid species.

Key words: auto-fluorescent pattern, balanomorph barnacle, cyprid, species identification, culture of cyprids, 12S rRNA gene

INTRODUCTION

Balanomorph barnacles are one of the most abundant sessile organisms, living widely throughout the world's oceans and having an active swimming larval stage. Balanomorph larvae of various species grow and settle in different seasons. Seasonal fluctuation of populations derived from the phytoplankton dynamics or temperature of habitat (Lang and Ackenhusen-Johns, 1981). Immigrant barnacles that arrived on ships have been dispersed new habitats (Yamaguchi et al., 2009). Excessive growth of barnacles beginning from the settlement of larvae in coastal areas causes severe biofouling of artificial structures that make up shore protection works, fisheries facilities, and sluices of costal plants, including electric power stations. Gaines and Bertness (1922) estimated the dynamics of balanomorph larvae though the study of settling juveniles, but it is still necessary to study the population dynamics of balanomorph larvae directly, including the identification of each species, not only for ecological studies, but also for protection from biofouling.

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Balanomorph barnacle eggs are fertilized endosomatically and after hatching, barnacle nauplius larvae are released from the female parent (Rainbow, 1984; Anderson, 1994). The nauplius repeats ecdysis five times and develops to the stage of cyprid (settling larva). The cyprid displays exploration behavior and temporary adhesion, and finally adheres permanently onto a basis and metamorphoses to a juvenile barnacle, beginning sessile life. Most balanomorph barnacles, with some exceptions, are hermaphroditic (Charnov, 1987), but are cross-fertilized and need a sympatric habitat for breeding (Rainbow, 1984). Therefore, the free-living larvae of balanomorph must select a suitable settlement location for their survival and propagation (Crisp, 1961; Crisp and Meadows, 1962).

Balanomorph nauplii in many species have been studied by their morphological features: cephalic shield, a pair of frontal filaments in the anterior region, three pairs of appendages that are the antennules, antennae and mandibles, and the labrum in the thoracic region (Walley, 1969; Walker et al., 1987; Korn, 1995). Morphological characters in detail were used for interspecific identification of nauplii occurring in Korean coastal waters (Lee et al., 1999), although these are not adequate for routine use.

The most prominent morphological traits of cyprids are an enclosing bivalve carapace that was rounded anteriorly and tapering posteriorly to a blunt point, a pair of antennules

equipped with attachment organs in the anterior region, six pairs of biramous thoracic limbs, and a pair of caudal appendages (Fig. 1S) (Walley, 1969; Walker et al., 1987). Cyprids of all balanomorph barnacles show remarkably similar morphology. Some authors advocate distinguishing cyprids of two different species by carapace length (Power et al., 1999), but others have reported that there were differences in carapace length in different habitats or environments (Crisp, 1962; Barns, 1953). The identification of cyprid species was possible only one from six species in Korean coastal waters, based on numerous small denticulate processes on the surface, not found in other species (Lee et al., 1999). Accordingly, it is difficult to identify many species in field cyprids using morphological criteria alone.

DNA analyses have been used to distinguish individuals and species of balanomorph larvae: restriction fragment length polymorphism (Power et al., 1999); sequences of 16S ribosomal DNA (Begum et al., 2004) in the mitochondrial genome; hybridization using species specific probe (Goffredi et al., 2006); and amplification of DNA using a species-specific primer set to estimate the individual numbers of 13 species by real-time PCR (Endo et al., 2010). However, all of these methods require special equipment and are time-consuming. Immunostaining (Miller et al., 1991) and immunochromatography (Yamashita, unpublished data) can readily identify species, but libraries of specific antibodies against many barnacle species are incomplete. In this study, we report on the autofluorescent emission from most species of balanomorph larvae living along the coast of Japan. This study of FPs should be helpful for identification of cyprid species.

MATERIALS AND METHODS

Collection of adult balanomorph barnacles and culture of cyprids

Seven species of adult balanomorph barnacles were collected from the intertidal and subtidal zones along the coastal region of the Seto Inland Sea (34°77′N; 134°72′E): *Amphibalanus amphitrite*, *A. improvisus*, *Balanus trigonus*, *Fistulobalanus albicostatus*, *Megabalanus rosa*, *Chthamalus challengeri*, *Tetraclita japonica.* Adults of *A. eburneus* were collected from the Seto Inland Sea, and the Pacific Ocean, Shizuoka (34°43′N; 137°34′E). Adults of *A. reticulatus* and *M. volcano* were collected from the East China Sea, Okinawa (26°43′N; 127°84′E). Adults of *Tetraclitella chinensis* were collected from the East China Sea, Nagasaki (32°67′N; 128°87′E) and identified by Yamaguchi. Other adult barnacles were identified by the morphological features of shell, terga, and scuta (Yamaguchi, 1995; Pitombo, 2004).

Each species of adult balanomorph barnacle was maintained in separate tanks and all were fed with nauplii of *Artemia salina*. We collected the larvae that hatched spontaneously from the adults or from fertilized egg masses that we removed from the adults. Larvae were cultured in 1–2 L beakers (0.5–2 larvae/ml) filled with the seawater filtered through a 0.22 or 0.45 μm membrane (Advantec Toyo, Japan) and with antibiotics added (streptomycin 6.6 mg/l, penicillin G 3 mg/l: Wako Pure Chemical, Japan). The beakers were maintained at 23–25°C under 12L:12D light con-

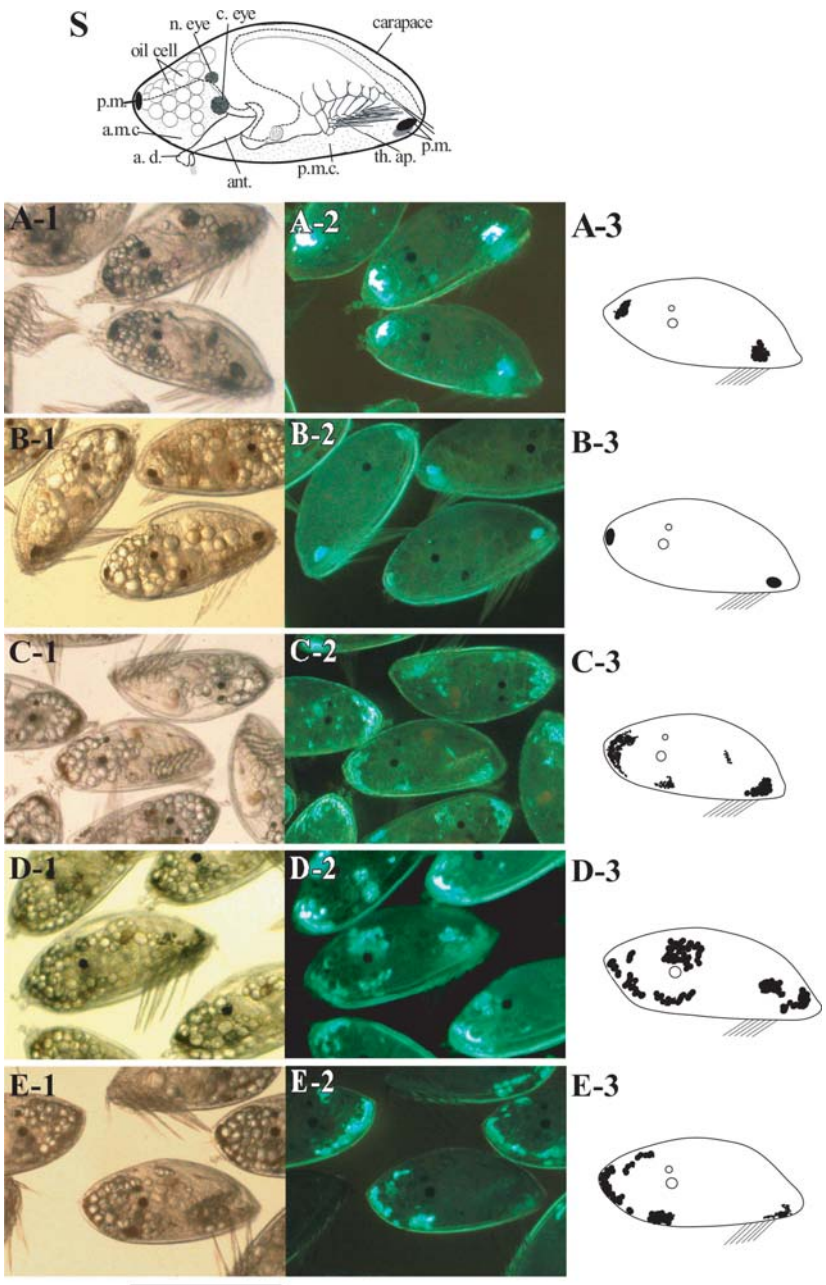

Fig. 1. Fluorescent patterns of balanomorph cyprids in group A. (S) A schematic of cyprid general features (After Walley, 1969; Anderson 1994), a.d., adhesive disk; ant, antennule, a.m.c, anterior mantle cavity; c.eye, compound eye; n.eye, nauplius eye; p.m, pigment mass; p.m.c., posterior mantle cavity; th, ap.; thoracic appendages. **(A)** *A. amphitrite*, **(B)** *A. improvisus*, **(C)** *A. eburneus*, **(D)** *A. reticulates*, **(E)** *F. albicostatus*. **(A-1)** – **(E-1)** are photographs under incident light, **(A-2)** – **(E-2)** are photographs under a fluorescent SZX-FBV filter unit. **(A-3)** – **(E-3)** are schematics of FPs. The fluorescent areas are black dots and black parts. Scale bar, 500 μm.

ditions with aeration. The nauplii of *C. challengeri* were fed with *Isochrysis galbana.* The nauplii of *M. rosa* and *M. volcano* were fed with *Chaetoceros gracilis* and *Skeletonema costatum*, and the others were fed with *C. gracilis*.

Collection of the field cyprids

The cyprids were collected from the field with a NXX13 plankton net (scale spacing = 0.1 mm) at coastal areas: Himeji (34°77′N; 134°72′E), Hyogo facing the Seto Inland Sea in July, August and October 2002, June and July 2009 and May 2010: Tosa-Bay (33°47′N; 133°50′E) and Uranouchi-Bay (33°43′N; 133°44′E) of Kouchi, in the Pacific Ocean in August 2010: Naka-umi (35°50′N; 133°13′E) and Owashi-Bay (35°55′N; 133°04′E) of Shimane, in the Japan Sea in August 2010. Plankton, including the cyprids, were kept alive in a cool box while being taken to the laboratory.

Observation of auto-fluorescence in larvae

The cyprids were anesthetized by exposing them to a mixture of equal volumes of seawater and 400 mM $MgCl_2$. They were viewed under an SZX-FBV filter set (Excitation 400–440/Emission 470–nm). In the case of the field plankton, cyprids were easily distinguished from other plankton at a lower magnification, as the whole carapace of all cyprids emitted a weak fluorescence. Subsequently, the cyprids were more closely observed and photographed at higher magnification.

Fluorescent patterns and species identification of the field cyprids

For cyprids collected in 2002, their fluorescent patterns were observed individually, while each larva was put into a petri dish made of polymethylpentene (PMP) with filtered seawater, and its adhesion and metamorphosis were urged by gentle agitation. The juveniles that metamorphosed on the PMP dishes were fed with *C. gracilis*, *I. galbana*, *S. costatum* and nauplii of *A. salina*. They were cultured to the adult stage, at which point the resulting balanomorphs species could be identified.

In the case of cyprids collected in 2009 and 2010, the cyprid fluorescent patterns were observed and individually transferred into 95% ethanol. PCR was applied directly to a whole cyprid by reference to the studies of bivalve (Hosoi et al., 2004). That is, an alcohol-fixed cyprid was dried in a PCR tube, added with 10.4 μL diluted water and vortexed. 12S rRNA genes were amplified using primers 12S-Foward (GAACCA GGATTAGATACCC) and 12S-Reverse (TTTC-CCGCGAGCGACGGGCG) according to Begum et al. (2004), and 9.6 μL of the PCR reaction solutions from the Advantage2 PCR Kit (TAKARA BIO INC. Shiga, Japan). PCR conditions were modified from those of Endo et al. (2010) to: 1 min at 95°C; 35 cycles of 95°C for 30 s and 56°C for 30 s; and 68°C for 1 min with the Gene Amp PCR System 2720 (Applied Biosystems, CA, USA). Direct sequencing was performed on an ABI PRISM 3130 using the BigDye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA).

Partial nucleotide sequences of 12S rRNA genes were aligned using CLUSTAL W (Thompson et al., 1994). Phylogenetic analyses were performed with the neighbor-joining method (Saitou and Nei, 1987) and the 1000 bootstrap replicates (Felsenstein, 1985), using MEGA version 4 software (Tamura et al., 2007). Evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and were in the units of the number of base substitutions per site. Codon positions included were 1^{st} + 2^{nd} + 3^{rd} + noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete-deletion option). Confidence in the phylogenetic grouping was evaluated by the bootstrap value.

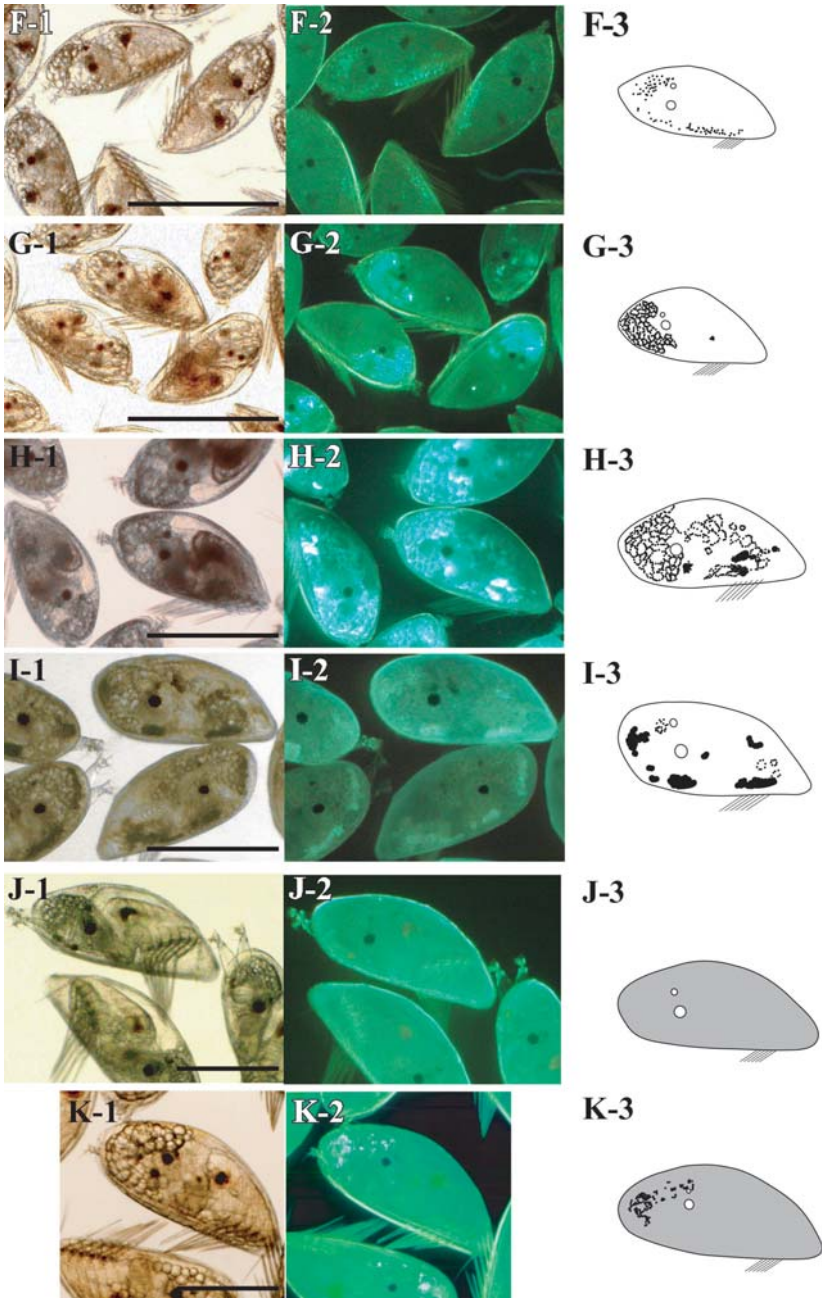

Fig. 2. Fluorescent patterns of balanomorph cyprids in groups B, C, D, and E. **(F)** *T. chinensis*, **(G)** *C. challengeri*, **(H)** *T. japonica*, **(I)** *M. volcano*, **(J)** *M. rosa*, **(K)** *B. trigonus*. **(F-1)** – **(K-1)** are photographs under incident light, **(F-2)** – **(K-2)** are photographs under a fluorescent SZX-FBV filter unit. **(F-3)** – **(K-3)** are schematics of FPs. The fluorescent areas are black dots and parts. Scale bars, 500 μm.

RESULTS

Fluorescent patterns of cyprids

All eleven species of cultured cyprids exhibited fluorescent substances (FS). Fluorescence was strongest when viewed under an SZX-FBV filter unit. Most of the fluorescent masses in the cyprids were symmetrically located on the median plane of the bivalve carapace. The distribution patterns of FS, the brown pigments and oil cells of each species were shown in Fig. 1 and Fig. 2 and described as follows. Fig. 1S shows a schematic figure of cyprid. We classified five major groups (A, B, C, D, and E) of fluorescent patterns (FPs), based on the observation results of the cultured cyprids.

Group A included five Amphibalaninae species (Fig. 1), characterized by fluorescent masses in the cephalon and thorax. In case of *A. amphitrite* (Fig. 1A) and *A. improvisus* (Fig. 1B), two pairs of fluorescent masses were associated with masses of dark brown pigments. The fluorescent masses of *A. amphitrite* (Fig. 1A) were compact, but irregular. A pair of posterior masses was in the middle thoracic part of lower rims of the carapace. The fluorescent masses of *A. improvisus* (Fig. 1B) were uniform ellipsoids. A pair of posterior masses was in near the posterior end of the carapace.

The fluorescent masses of *A. eburneus* (Fig. 1C) and *A. reticulates* (Fig. 1D) were loose aggregates. The posterior fluorescent masses of the former were associated with light brown pigments and the latter with dark or light pigments. It was difficult to distinguish *A. eburneus* (Fig. 1C) and *A. reticulatus* (Fig. 1D).

Fistulobalanus albicostatus (Fig. 1E) was characterized by intermittently fluorescent masses from the cephalic end toward the posterior end along lower rims of carapace. The fluorescence was associated with light brown pigments.

Group B was *T. chinensis* (Fig. 2F), which has fine fluorescent granules, dispersed between the oil cells in the cephalon and arrayed along the thoracic rim.

Group C included *C. challengeri* (Fig. 2G) and *T. japonica* (Fig. 2H), and was characterized by fine fluorescent granules occupying the interspaces between oil cells in cephalon. *T. japonica* had also large fluorescent masses in thorax, associated with dark brown pigment masses.

Group D was *M. volcano* (Fig. 2I) with large body (carapace length of cultured cyprid > 680 μm). Its fluorescent masses were associated with dark brown pigments.

Group E included *M. rosa* (Fig. 2J) and *B. trigonus* (Fig. 2K). The fluorescent granules were very few and the whole carapace emitted weak fluorescence. The fluorescent granules were never found in *M. rosa* (Fig. 2J). In the case of *B. trigonus* (Fig. 2K), fluorescent granules were observed very rarely in the anterior region of some cyprids. Accordingly, *M. rosa* was difficult to distinguish from *B. trigonus*.

Estimation by FPs and species identification by morphology

Twenty-eight cyprids were collected in 2002, and classified into three groups (A, C and E) based on FPs; 12 individuals of *A. amphitrite* (A-group), three of *C. challengeri* (C-group), and 13 of E-group. These were allowed to develop into adult balanomorphs, and then the species was identified by morphology. The first estimation corresponded to the final morphological identification (Table 1).

Estimation by FPs and species identification by genome

Ninety-two cyprids were collected in 2009 and 2010 and classified into the known groups of FPs (78 individuals), and others were tentatively classified into five unknown groups (UKa-e, 14 individuals) based on FP. The known groups were estimated as follows: 19 individuals as *A. amphitrite*, 2 as *A. improvisus*, 11 as *A. eburneus* or *A. reticulatus*, 1 as *F. albicostatus*, 13 as *T. chinensis*, 17 as *C. challengeri*, 15 as E-group (Table 1).

A phylogenetic tree was made from 136 sequences of partial 12S rRNA, derived from adult data, the cultured larvae data (Endo et al., 2010, and this study: AB673054–AB673067) and the field cyprids from this study (AB673068–AB673102). The phylogenetic tree (Fig. 3) showed 13 distinct clades corresponding to the known species, and other three unknown types. From high bootstrap values (91 to 100%) of these clades, it was decided that the field cyprids were identical with the species-identified barnacles belonging to the same clades.

Seventy-eight field cyprids belonging to the known groups of FPs were included in the expected nine clades of the phylogenetic tree (Fig. 3).

UK types may be distinguished tentatively from the identified species, although there were not a sufficient number of them to confirm their FPs. The cyprids that were assigned to the UK-e (two individuals) and UK-b (four individuals) by FPs were *Fistulobalanus kondakovi* and *Capitulum mitella,* respectively. The species of eight cyprids (UK-a, UK-c and UK-d) could not be identified.

FPs of *F. kondakovi* (UK-e): Fluorescent masses were located along the carapace of the cephalic end and posterior end, and scattered throughout the body (Supplementary Fig. S1). FPs of *C. mitella* (UK-b): The whole carapace emitted

Table 1. Species estimation using FP followed by species identification using morphology or 12S rRNA sequences, based on field cyprids.

FPs group	speices	FPs	adult morphology	FPs	DNA
A	*Amphibalanus amphitrite*	12	12	19	19
	Amphibalanus improvisus	0	0	2	2
	Amphibalanus eburneus	0	0	11	11
	Amphibalanus reticulatus		0		0
	Fistulobalanus albicostatus	0	0	1	1
B	*Tetraclitella chinensis*	0	0	13	13
C	*Chthamalus challengeri*	3	3	17	17
	Tetraclita japonica	–	–	–	–
D	*Megabalanus volcano*	–	–	–	–
E	*Balanus trigonus*	13	13	15	14
	Megabalanus rosa		0		1
others	UK-a			2	
	UK-b: *Capitulum mitella*			4	
	UK-c			1	
	UK-d			5	
	UK-e: *Fistulobalanus kondakovi*			2	
	total	28		92	

T. japonica and *M. volcano* were not found in field cyprids.

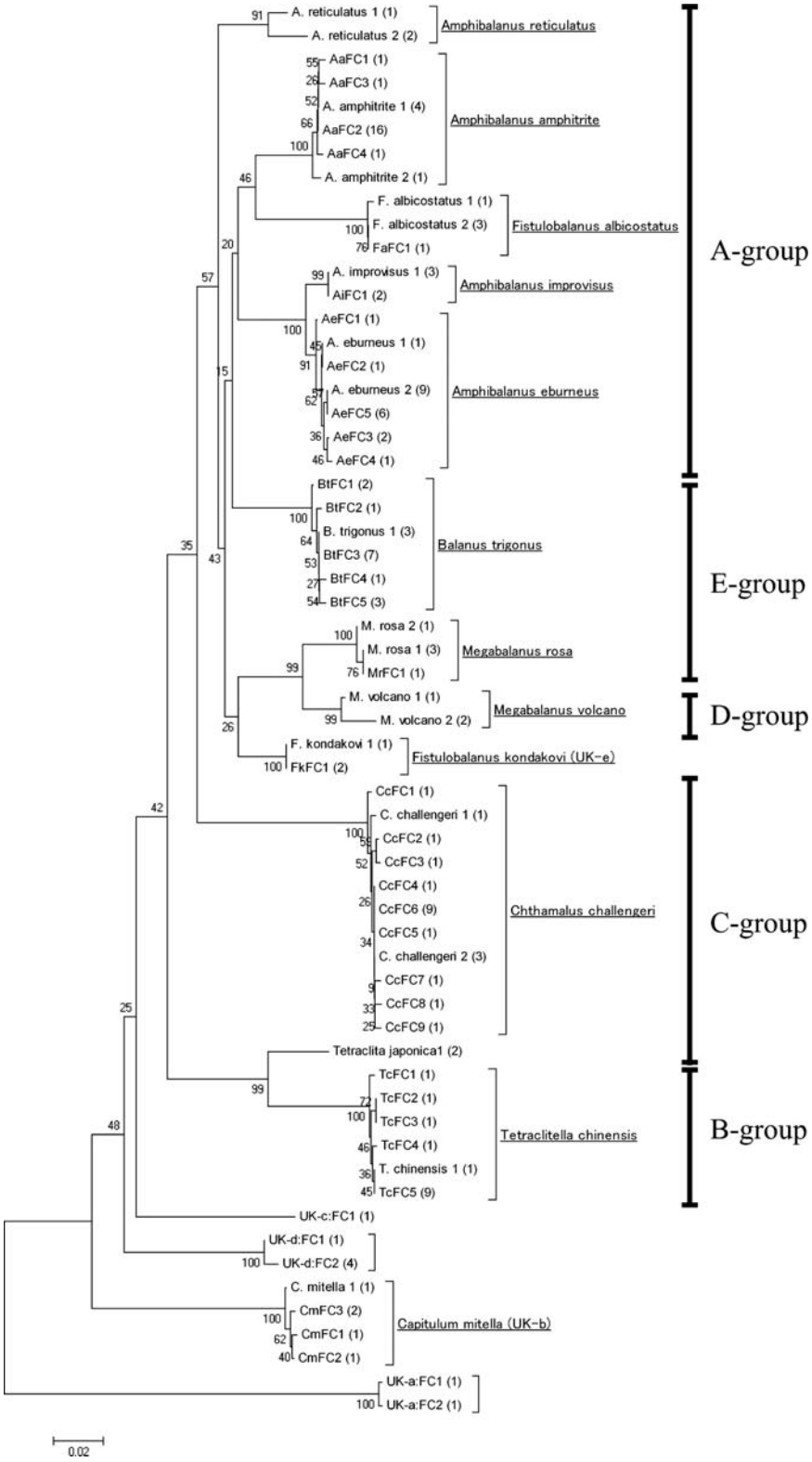

Fig. 3. Phylogenetic tree of barnacles based on 12S rRNA gene. The phylogenetic analysis of identified barnacles and field-collected cyprids was made based on the sequence data of a partial region of the 12S rRNA gene. The optimal tree with the sum of the branch length (0.98789754) is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. There were a total of 289 positions in the final dataset. The haplotypes are expressed such as A. reticulates1 in the case of an identified barnacle, and AaFC1 in case of a field cyprid. Individual number is included in parentheses.

a weak fluorescence, and there were numerous dark brown pigments, except around the nauplius eye. The morphology of their carapace was distinctly different from the cyprids of the other twelve species; the posterior end was narrow and pointed (Supplementary Fig. S1). UK-a was equipped with thick antennules. The fluorescent masses in this type were scant in the thorax. In UK-c, smear of fluorescent masses were spread over ventral thoracic region. Numerous dark brown pigments were distributed all over the thorax. UK-d: the blunt corner was present at thoracic posterodorsal carapace.

Species estimation using FPs types was judged to be largely accurate. Furthermore, we were able to find UK types from the field cyprids, corresponding to other species beyond the first 11 standard species. However, we were unable to distinguish between *A. eburneus* (Fig. 1C) and *A. reticulatus* (Fig. 1D), and between *M. rosa* (Fig. 2J) and *B. trigonus* (Fig. 1K) by FPs.

DISCUSSION

Balanomorph FPs are stable during cyprid stage

Auto-fluorescence was observed in the cultured cyprids and in the field cyprids as well, and their FPs were characteristics to each species. Cultured cyprids adhered on the substratum about two to seven days. Their FP characteristics did not change before settlement. This was also right in case of C-group that was characterized by fine fluorescent granules among the oil cells, though the size of the fluorescent area in the cephalon of cyprid was deceased by consumption of the oil cell with ageing. Whereas, Walley (1969) and also this paper reported that FPs did change at the time of molting from nauplii to cyprid. FPs of cyprids are retained in chilled (6.5°C) condition for several days, or under anesthesia with $MgCl_2$, whereas they are lost after fixation with 4% formalin or ethanol. So we have used chilled condition for transport of field cyprids and anesthesia condition for observation. Under these conditions, cyprid FPs are stable and reliable characteristics of each species, and is applicable to species estimation.

FPs and identification of species

Estimation of cyprid species based on FPs closely corresponded to the identification based on the adult morphology and DNA sequence. Estimation of the field cyprid species suggested that they belonged to the known FPs groups (A, B, C, and E) and to the other groups (UK-types). Two of the other UK-types were identified as *F. kondakovi* (Sessilia, Balanomorpha) and *C. mitella* (Pedunculata, Scalpellomorpha) from DNA sequences. The *C. mitella* cyprids had various features that differed from Balanomorpha with respect to FPs, distribution of pigments and morphology of the carapace (Supplementary Fig. S1); which might be a signature of Scalpellomorpha. UK-a, UK–c and UK-d may have originated out of the balanomorph groups from their position on the phylogenetic tree (Fig. 3).

The present study of FPs overcomes the difficulty of cyprid species identification based on the morphology. The observation of FPs is a much more convenient first step. If identification cannot be accomplished by FPs, DNA analysis should be the next step to confirm the species. On the whole, utilization of FPs should save time for species identification of balanomorphs and neighboring species in field studies.

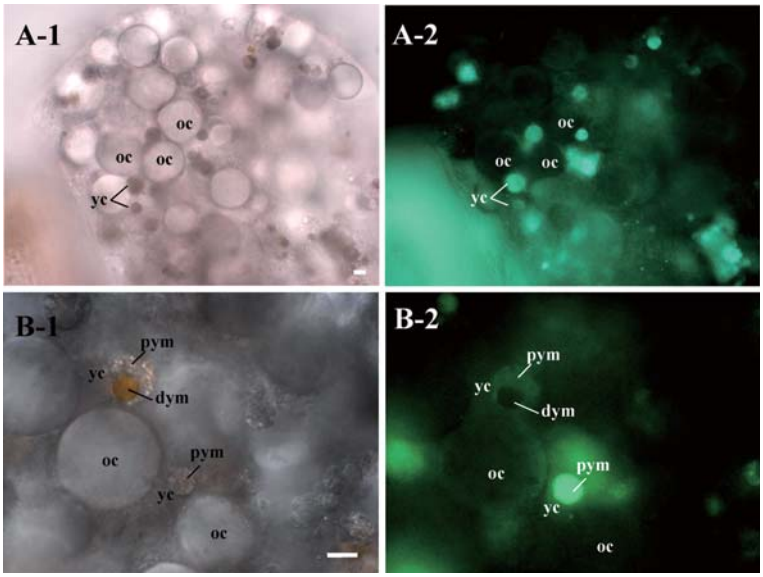

Fig. 4. Fluorescent substances (FS) in cyprid of *Megabalanus volcano*. **(A-1)** Yellow cells (yc) resided among the oil cells (oc). **(A-2)** Yellow cells (yc) showed fluorescence. **(B-1)** Yellow cells with pale yellow materials (pym) and with dark yellow material (dym). **(B-2)** Pale yellow material was composed of strongly fluorescent granules. Large dark yellow material (dym) was not fluorescent. **(A-1)** and **(B-1)** under incident-light, **(A-2)** and **(B-2)** under a fluorescent SZX-FBV filter unit. Scale bars, 10 μm.

Some insights about fluorescent granules and fluorescent cells

Fluorescent granules were found in the cells (Fig. 4), and fluorescent cells were clearly observed in the thoracic limbs of *M. volcano* cyprids where they were associated with oil cells. Fluorescent cells had a cytoplasm full of numerous vacuoles containing a pale yellow fluorescent material and 7–8 μm of dark yellow material without fluorescence (pigment). The appearance of these cells was similar to the yellow cells that Walley reported (1969). In our observation of

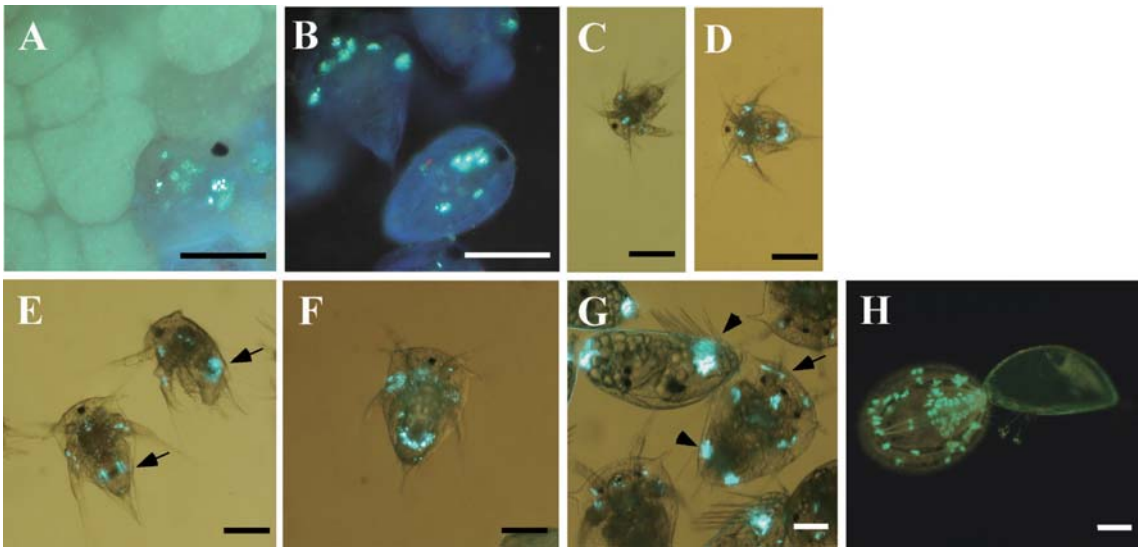

Fig. 5. Fluorescent substances (FS) in developing *A. Amphitrite.* Embryos before hatching **(A)** and post-hatching larva **(B)** already possessed FS in the anterior half of the body. A stage II nauplius larva **(C)** equipped with FS at the labrum and antennae. Stage III **(D)**, IV **(E)** and V **(F)** nauplius larvae show strong FS (arrow) observed at the mid-gut constriction, and in stage VI **(G)**, they were trans-located to the base of the cyprid thoracic appendages (arrow head). FS (arrow) also appeared in front of the compound eyes. In the period when the cyprid settled and metamorphosed, the FS moved from the cephalon to the nearby basal plate and from the thorax to the region underlying the terga and scuta of the shell plates **(H)**. The dorsal view of a juvenile just after metamorphosis **(H)**. Left transparent structure is an exavia of the cyprid. On the right side is a juvenile. The masses of fluorescent substances are dispersed into a numerous small fluorescent granules in a juvenile. Scale bars, 100 μm.

about 11 species, fluorescent masses or granules were frequently associated with brown pigments. In other cases, however, fluorescent granules and brown pigments appeared independently. It is likely that the yellow cells change their cytological properties in response to their situation. Furthermore, yellow cells (FS) in *A. amphitrite* were present in the pre-hatching larva, nauplius, cyprid and the adult. Distribution of the FS changed from stage to stage (Fig. 5), though this was relatively stable in the cyprid stage, as described above.

We have sometimes found an intimate association between yellow cells and oil cells. The oil cells may work as a food reserve in barnacles (Walley, 1969; Holland and Walker, 1975) and center for energy metabolism as fat bodies in insects (Arrese and Soulages, 2010). It remains unclear whether or not yellow cells cooperate with oil cells in fat metabolism, and whether or not yellow cells have some unknown roles in the larvae and adults.

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