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Distribution pattern of GFP (green fluorescent protein) in a bivalve-inhabiting hydrozoan, *Eutima japonica* (Leptomedusae: Eirenidae)

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Bright green auto-fluorescence was observed in the umbrellar margin, umbrellar marginal warts, tentacular bulbs, tentacles, and manubrium of laboratory-reared immature (1–14 days old) medusae of Eutima japonica *from Japan and China.* In vivo *microscopic fluorescence spectra showed that the green fluorescence was similar to that of green fluorescent protein (GFP) found in* Aequorea victoria*, although the maximum emission wavelength (503 nm) was slightly bluer. No fluorescence was detected in the cirri, statocysts, radial canals, velum, or subumbrella of the medusae. The fluorescence distribution pattern in* E. japonica *more closely resembles that of* Eugymnanthea inquilina *from the Mediterranean Sea than that of Japanese* Eugymnanthea japonica*, which is the derived species of* E. japonica*. This suggests that the common fluorescence pattern is convergently evolved in the former two species, perhaps owing to the as yet unclarified physiological and/or ecological function of GFP and/or GFP-like proteins.*

Keywords: fluorescence pattern and spectra, green fluorescent protein (GFP), hydromedusa, *Eutima*, *Eugymnanthea*, systematics

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INTRODUCTION

The two species of the genus *Eugymnanthea* are commensal hydroids associated mainly with *Mytilus galloprovincialis* and other bivalves inhabiting shallow waters. The medusae of *E. inquilina* occurring in the Mediterranean Sea and *E. japonica* from Japanese waters are morphologically similar and sometimes hard to distinguish (Kubota 2000, 2004; Govindarayan *et al.*, 2005). Kubota *et al.* (2008), however, found that these medusae are remarkably different in their microscopic fluorescence patterns and thus are completely separable; namely, bright green auto-fluorescence was detected in the umbrellar margin of a spent medusa of *E. inquilina*, in contrast to subumbrellar fluorescence in *E. japonica*. The fluorescence distribution patterns were thus shown to be a reliable taxonomic character in some hydroids.

In the present study, we examined the fluorescence pattern of *Eutima japonica*, one of the ancestral and most closely related species to *Eugymnanthea japonica* (Kubota, 2000). We assumed that the fluorescence pattern of *Eutima japonica* would be more similar to that of *Eugymnanthea japonica* inhabiting the same geographical region than to that of *E. inquilina* remotely isolated from both of them. Moreover, we checked whether the auto-fluorescence is due to green fluorescent protein (GFP) or another fluorescent substance, as essential data for the future study of this novel character.

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MATERIALS AND METHODS

Two populations of *Eutima japonica* associated with *Mytilus galloprovincialis* were observed, one from Minami-Sanriku town, Miyagi Prefecture, Japan, collected in August 2004, and the other from Tsingtao, China, collected in September 2007. Polyps were cultured at the Seto Marine Biological Laboratory in Shirahama town, Wakayama Prefecture, Japan, being kept in filtered seawater and fed with newly hatched *Artemia* larvae for two weeks in the former case, and for seven months in the latter. The released medusae were identified on the basis of their morphology as the northern form of *Eutima japonica* (Kubota, 1992, 2008). Seven one-day-old medusae (six Japanese specimens and one Chinese specimen) and eight 14-day-old immature medusae (seven Japanese specimens and one Chinese specimen) were observed under a fluorescent microscope (BX51, Olympus, Japan) with excitation of ultraviolet (330–385 nm) and blue-violet (420–440 nm) wavelength, at the Kobe University Research Center for Inland Seas. *In vivo* fluorescence spectra were measured with a fluorescence microscope equipped with an ultra-high sensitivity multi-channel photodiode array detector (PMA-11, Hamamatsu Photonics, Japan) (Murakami *et al.*, 2004). The fluorescence pattern of a medusa of *Eugymnanthea japonica* originating from *Mytilus galloprovincialis* collected at Shirahama, Japan, in June 2007 was also confirmed for comparison (Kubota *et al.*, 2008).

RESULTS

Bright green auto-fluorescence was detected in different parts of body such as the umbrellar margin, umbrellar marginal

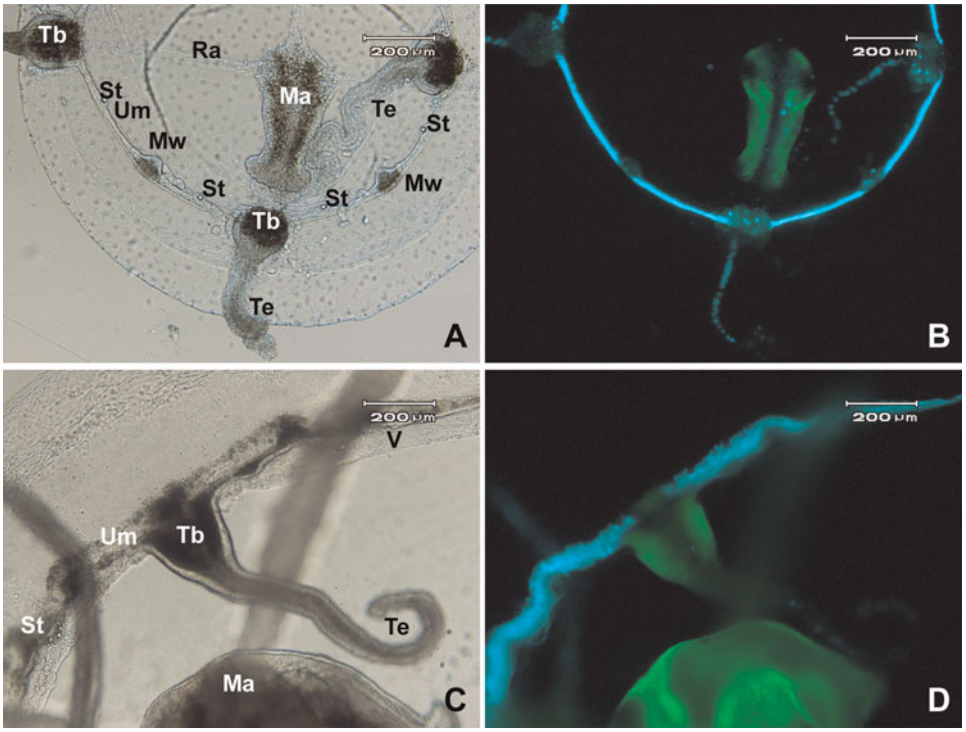

Fig. 1. Photomicrographs of immature medusae of *Eutima japonica* from Miyagi Prefecture, Japan (A, B: 1-day-old; C, D: 14-day-old), showing various body parts: tentacles (Te), tentacular bulbs (Tb), umbrellar marginal wart (Mw), manubrium (Ma), umbrellar margin (Um), velum (V), and statocysts (St). Auto-fluorescence images under blue light excitation (B, D) and transmitted light images (A, C) of the same two individuals.

warts, tentacular bulbs, tentacles, and manubrium of the laboratory-reared immature (1–14 days old) medusae of *Eutima japonica* from Japan (13 specimens) and China (two specimens) (Figure 1A–D). Fluorescence emission spectra with an emission maximum at 503 nm indicated the contribution of GFP (Figure 2). No fluorescence was observed in the remaining body parts such as cirri, statocysts, radial canals, or subumbrella of these medusae (Figure 1A–D). In contrast, in the subumbrella of a mature medusa of *Eugymnanthea japonica* from Japan (Figure 3A–B), green fluorescence was observed as has been described previously (Kubota *et al.*, 2008), but in a different pattern than that shown in a photomicrograph in the previous paper (Kubota *et al.*, 2008; Figure 1D).

DISCUSSION

Microscopic fluorescence spectra indicated that the green auto-fluorescence of *Eutima* and *Eygymnanthea* may be derived from GFP. Green fluorescent protein was first discovered in the hydromedusan *Aequorea* (Shimomura *et al.*, 1962) and has also been found in other cnidarians, i.e. sea anemones and corals (Ward, 1998; Shimomura, 2006), as well as amphioxus (Deheyn *et al.*, 2007). The physiological and ecological functions of GFP and GFP-like proteins (Belogurova *et al.*, 2008) have not been resolved to date, but these substances are presumed to function in the generation of warning or masking coloration and can be used as fluorescent markers in cell and molecular biology (Yanushevich *et al.*, 2005). In bioluminescent medusae emission spectra will differ according to ecological factors, as was shown by Haddock & Case (1999), but bivalve-inhabiting hydrozoans such as *Eutima japonica*, *Eugymnanthea japonica* and *Eugymnanthea inquilina* are not bioluminescent species (Kubota, unpublished data).

Green fluorescent protein distribution patterns in tissues and organs of some hydromedusans are variable among closely related species, as reported by Kubota *et al.* (2008) and this study. The fluorescence distribution pattern of *Eutima japonica* more closely resembles that of *Eugymnanthea inquilina* from the Mediterranean Sea than that of the Japanese *Eugymnanthea japonica*, even though

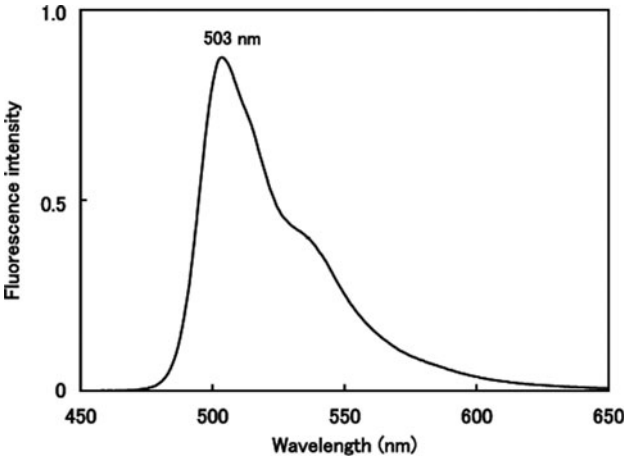

Fig. 2. *In vivo* fluorescence emission spectra of green fluorescent protein in a 14-day-old immature medusa of *Eutima japonica* from Tsingtao, China; excitation wavelength: 435 nm.

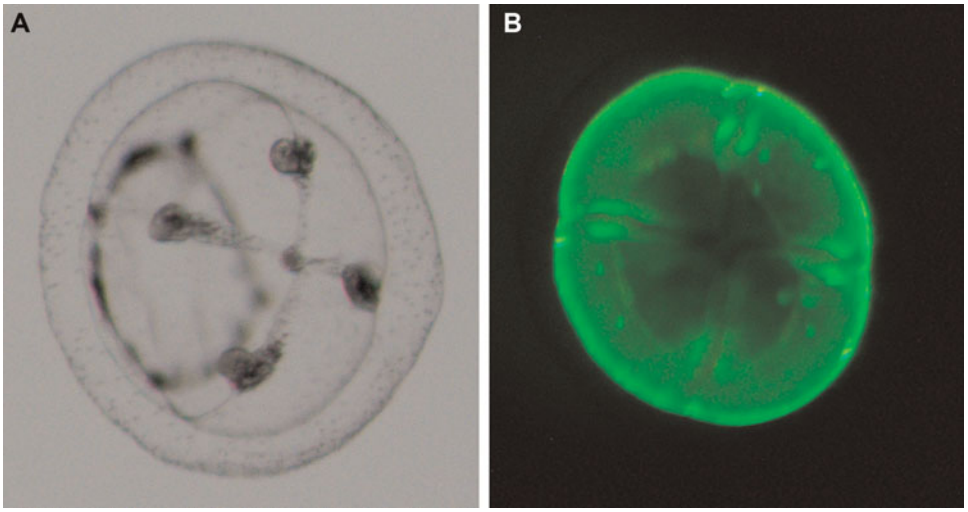

Fig. 3. Microphotographs of a spent medusa of *Eugymnanthea japonica* from Shirahama, Wakayama Prefecture, Japan. Bright-field image (A) and auto-fluorescence image under blue light excitation (B) of the same individual of which umbrellar diameter is 0.93 mm.

the latter represents an advanced form derived from the present ancestral species. This is against our expectation and implies that the similar fluorescence pattern may be a convergent character related to its physiological and/or ecological role, although the real function of the fluorescence has almost never been resolved. Although the distribution patterns of GFP may have no phylogenetic significance, they are useful and important as taxonomic markers.

The maximum emission wavelength of GFP is different in each of the three geographically widely separated species of amphioxus studied by Deheyn *et al.* (2007). Similarly, the maximum emission wavelength of GFP in the present species (503 nm) is slightly different from that of *Aequorea victoria* and *Renilla reniformis* (508–509 nm), as well as those of the hydromedusae of *Halistaura*, *Mitrocoma*, and *Phialidium* (497–498 nm) (Shimomura *et al.*, 1962; Ward, 1998; Shimomura, 2006). Such differences among species imply that the maximum emission wavelength of the GFP is species-specific, at least within a limited taxonomic group such as Leptomedusae. Further study is required, including a comparison of the maximum emission wavelength of GFP and the fluorescence pattern among ordinary medusae with tentacles and a mouth like the present species.

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