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Benthic platyctenid ctenophore, *Vallicula multiformis* Rankin, 1956, found in an aquarium on Palawan Island, the Philippines

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Abstract: The benthic ctenophore *Vallicula multiformis* Rankin, 1956 (Ctenophora, Platyctenida) was originally described from Jamaica. We found the species in an aquarium during our survey of jellyfish from Palawan Island, the Philippines. Molecular analysis of the species confirmed the morphology-based identification of the samples. This discovery is the first report of the occurrence of species from the western Pacific area.

Key words: Benthic ctenophore, Coeloplanidae, *Vallicula*, Palawan, the Philippines

Introduction

There are two types of life forms in the phylum Ctenophora, planktonic and benthic, with most of the ctenophore species being planktonic, and only some benthic. Except for deep-sea species (Lindsay & Miyake 2007), benthic ctenophores belong to the order Platyctenida, which has five families: Coeloplanidae, Ctenoplanidae, Lyroctenidae, Tjalfiellidae, and Savangiidae (Mills 2017). Coeloplanidae is the largest family with two genera by *Coeloplana* (33 species) and the monotypic *Vallicula* (*V. multiformis* Rankin, 1956) (Mills 2017). A morphological identification of coeloplanid species is challenging because of their flexible morphs (Alamaru et al. 2017). However, *V. multiformis* can be easily distinguished from other coeloplanid species by a unique character—the anchor-shaped tentacle sheath (Rankin 1956).

Vallicula multiformis has been found on rocks and the surfaces of benthic animals and algae, and it has been recorded from the Gulf of Mexico, the Caribbean Sea, Atlantic Ocean, northeastern Pacific Ocean, northern Indian Ocean, and the Red Sea (Alamaru et al. 2016, Glynn et al. 2017, Prasade et al. 2015, Rankin 1956, World Register of Marine Species (WoRMS), 2017).

We found *V. multiformis* in an aquarium during a survey trip for jellyfish research to Palawan Island, the Philippines. Until now, there has been no record of *V. multiformis* from East or Southeast Asia. This may be the first finding of this species in the western Pacific area. The morphology of the species is briefly described on the basis of the specimens collected from Palawan Island, along with its molecular identification.

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Materials and Methods

Sampling was conducted from 24 to 27 August, 2017, at El Nido and Western Philippines University-Binduyan Marine Research Station (WPU-BMRS) on Palawan Island, the Philippines. A number of specimens of *Vallicula multiformis* were found in aquarium tanks of WPU-BMRS. Sampling was conducted with permission from the Palawan Council for Sustainable Development (PCSD), Republic of the Philippines.

Ten specimens were kept alive and brought back to Japan in a 500 mL plastic jar. Some specimens were fixed *in situ* in 99.5% ethanol for DNA analysis. Live specimens were placed in a small cubic tank (17 cm L×17 cm W ×17 cm H) with flowing seawater filtered through an undergravel filter and fine-grained coral sand in the laboratory at Kitasato University. Plastic Petri dishes with attached benthic ctenophores were set on sand. Water temperature was kept at approximately 30℃ using a water bath and salinity was 34. All animals were provided with fresh food daily using live *Artemia* sp. nauplii (A&A Marine, Utah, USA). A few individuals were also kept in plastic Petri dishes with still water. Three fully grown individuals were anesthetized with $MgCl_2$ and then fixed in about 3% formalin for morphological study.

Morphological identification was based on Rankin (1956), Gershwin et al. (2010), Alamaru et al. (2016), and Mills & Haddock (2007). Live specimens were observed noting the distribution and number of aboral papillae. The tentacular axial size and height were measured during the folding of the whole body in the floating phase, as described by Rankin (1956), under a stereoscopic microscope (Olympus SMZ-10) (Fig. 1).

Three specimens were utilized for a genetic analysis using cytochrome oxidase subunit I (COI), because COI is more effective in discriminating between species of benthic ctenophores compared with ITS1, 18S rRNA, and 28S rRNA (Alamaru et al. 2017). DNA extraction from a small tissue section removed from animals preserved in 99.5% ethanol was conducted using a DNeasy Blood & Tissue Kit (QIAGEN, Germany) in accordance with the manufacturer's instructions. Before PCR, the extracted genomic DNA was purified using a QIAquick PCR purification Kit (QIAGEN, Germany). The mitochondrial COI gene was amplified in reactions containing 1.0 μL of Mighty Amp DNA Polymerase (Takara Bio, Japan), 100 μL of 2× Mighty Amp Buffer (Takara Bio, Japan), 66 μL of Milli-Q water, 2.0 μL of DMSO, 3.0 μL of 5M betaine, 1.0 μL of DNA template, and 1.2 μL of each primer, LCOI490 (5′GGT CAA CAA ATC ATA AAG ATA TTG G3′) and HCO2198 (5′TAA ACT TCA GGG TGA CCA AAA AAT CA3′) (Alamaru et al. 2017). PCR was conducted in a thermal cycler (T100 Thermal Cycler, Bio-Rad) as follows: 95℃ for 3 min for initialization, 35 cycles of thermal denaturation for 1 min at 95℃, annealing for 1 min at 40℃, and elongation for 1.50 min at 72℃, followed by a final elongation at 72℃ for 20 min (Alamaru et al. 2017). The resulting PCR products were cleaned using ExoSAP-IT (Thermo Fisher, USA), sequenced using a Big Dye Terminator v3.1 Cycle Sequence Kit (Applied Biosystems, USA), and concentrated by ethanol precipitation. The gene sequence was determined using an ABI PRISM 3130x1 genetic analyzer (Applied Biosystems, USA).

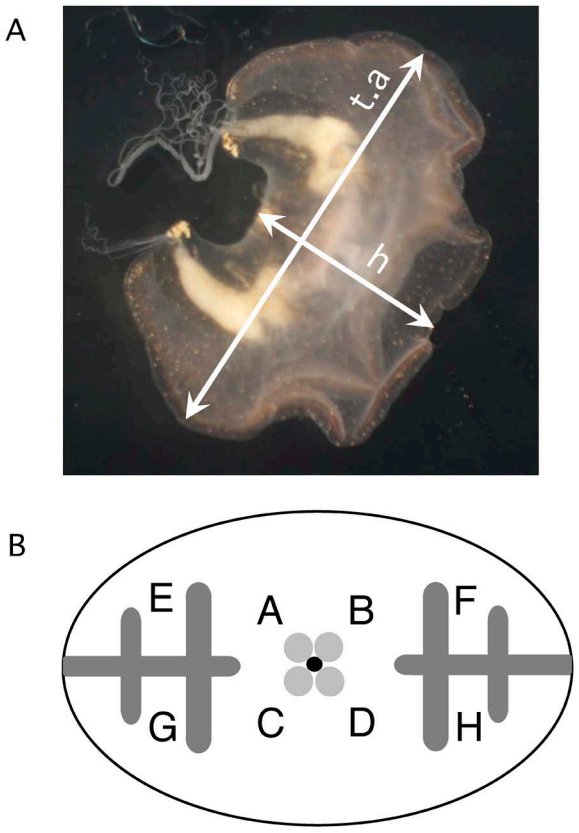

Fig. 1. Measurement of *Vallicula multiformis*.
A: Measurement of *V. multiformis* when contracted in the floating phase. t.a-tentacular axial size, h-height.
B: Schematic *V. multiformis* in aboral view. The alphabet characters indicate the area of aboral papillae.

The analyzed sequences were aligned in ClustalW incorporated in MEGA 7.0.26 (Kumar et al. 2016). Phylogenetic analysis and pairwise distance (K2P) measurements were performed with the maximum likelihood method in MEGA 7.0.26.

Results and Discussion

Species identification

Based on morphological and genetic data, Alamaru et al. (2016) arrived at the same conclusion as Rankin (1956) that the genera *Coeloplana* and *Vallicula* can be distinguished by the shape of the tentacle sheath (the flask-shaped tentacle sheath in *Coeloplana* and the anchor-shaped tentacle sheath in *Vallicula*). Whereas the species of *Coeloplana* can be discriminated based on their color, pigment pat-

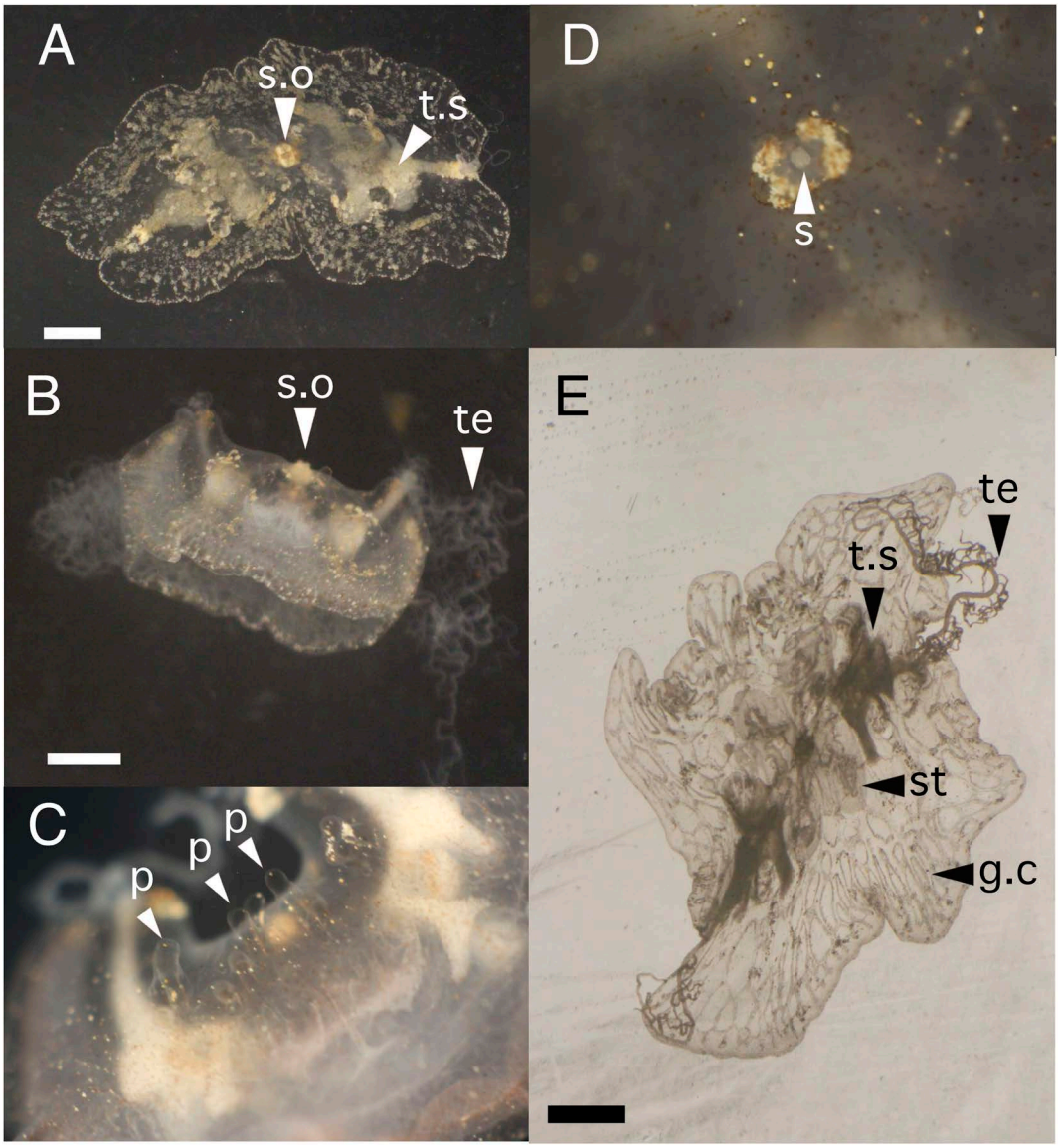

Fig. 2. *Vallicula multiformis*. Live specimen.
A: Aboral view of creeping phase. B: Lateral view of floating phase. C: Aboral papillae. D: Aboral sense organ. E: Gastrovascular canal system and tentacles. Scale bar = 1 mm. g.c-gastrovascular canal, p-papilla, s-statolith, s.o-sense organ, st-stomach, te-tentacle, t.s-tentacle sheath.

tern, oral groove, oral lappets, and host identity. The present benthic ctenophore was easily identified morphologically as *Vallicula multiformis* because of the anchor-shaped tentacle sheath (indicated by t.s in Fig. 2A, E) and blind ending of the gastrovascular canals (indicated by g.c in Fig. 2E). These are highly differentiated features between *Coeloplana* and *Vallicula* (Alamaru et al. 2016, Prasade et al. 2015).

Partial gene sequences of COI (380 bps) were analyzed for two specimens, whereas the sequence from the third individual could not be obtained. A BLAST search of homologous sequences showed a 99% similarity between our specimens and *V. multiformis* (recorded from the Red Sea). The maximum likelihood analysis of the sequences resolved our samples within the monophyletic *V. multiformis* clade, which was sister to the genus *Coeloplana*, and thus further corroborated their affiliation with *V. multiformis* (Fig. 3). The pairwise distance (K2P) between coeloplanid species ranged from 0.027 to 0.428, and that between the genera *Coeloplana* and *Vallicula* was between 0.329 and 0.428 (Table 1). The pairwise distance (K2P) between *V. multiformis* and our sample was 0.008 to 0.021, suggesting that our samples belonged to *V. multiformis*. The sequences were submitted to DDBJ under the accession numbers LC434631 and LC434632.

Behavior and morphology

Vallicula multiformis kept in plastic Petri dishes with

Table 1. Interspecific and intraspecific COI sequence diversity (K2P distance) in Coeloplanid species.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	*Coeloplana lineolata* (KT886000)																	
2	*Coeloplana fishelsoni* (KT885974)	0.053																
3	*Coeloplana bannwarthi* (KT886007)	0.047	0.050															
4	*Coeloplana anthostella* (HQ435811)	0.070	0.085	0.064														
5	*Coeloplana huchonae* (KT886017)	0.061	0.070	0.049	0.027													
6	*Coeloplana yulianicorum* (KT886020)	0.137	0.164	0.137	0.137	0.144												
7	*Coeloplana loyai* (KT886005)	0.123	0.143	0.111	0.124	0.120	0.137											
8	*Coeloplana* sp. 2 (KT885996)	0.143	0.136	0.120	0.130	0.114	0.143	0.069										
9	*Coeloplana astericola* (KT885991)	0.146	0.136	0.130	0.146	0.136	0.163	0.081	0.055									
10	*Coeloplana bocki* (HQ435815)	0.152	0.159	0.136	0.146	0.143	0.163	0.115	0.130	0.134								
11	*Vallicula multiformis* (KT886022)	0.385	0.394	0.376	0.386	0.372	0.423	0.329	0.338	0.354	0.355							
12	*Vallicula multiformis* (KT886026)	0.399	0.399	0.381	0.400	0.376	0.428	0.338	0.342	0.359	0.364	0.011						
13	*Vallicula multiformis* (KT886027)	0.399	0.399	0.381	0.400	0.376	0.428	0.338	0.342	0.359	0.364	0.011	0.000					
14	*Vallicula multiformis* (KT886023)	0.399	0.390	0.372	0.391	0.368	0.418	0.334	0.333	0.359	0.360	0.008	0.008	0.008				
15	*Vallicula multiformis* (KT886025)	0.399	0.390	0.372	0.391	0.368	0.418	0.334	0.333	0.359	0.360	0.008	0.008	0.008	0.000			
16	*Vallicula multiformis* (KT886024)	0.399	0.390	0.372	0.391	0.368	0.418	0.334	0.333	0.359	0.360	0.008	0.008	0.008	0.000	0.000		
17	*Vallicula multiformis* (This study, Philippine (1))	0.386	0.386	0.376	0.405	0.381	0.427	0.338	0.346	0.363	0.373	0.021	0.016	0.016	0.019	0.019	0.019	
18	*Vallicula multiformis* (This study, Philippine (2))	0.386	0.394	0.376	0.405	0.381	0.423	0.338	0.346	0.363	0.373	0.013	0.008	0.008	0.011	0.011	0.011	0.008

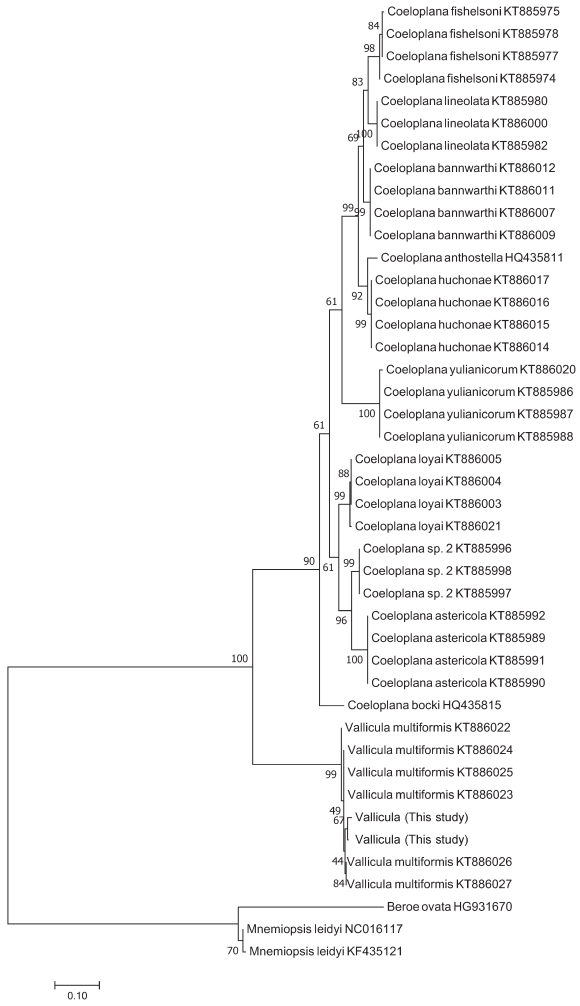

Fig. 3. Molecular phylogenetic analysis in coeloplanid species by maximum likelihood method using COI (380 bps).

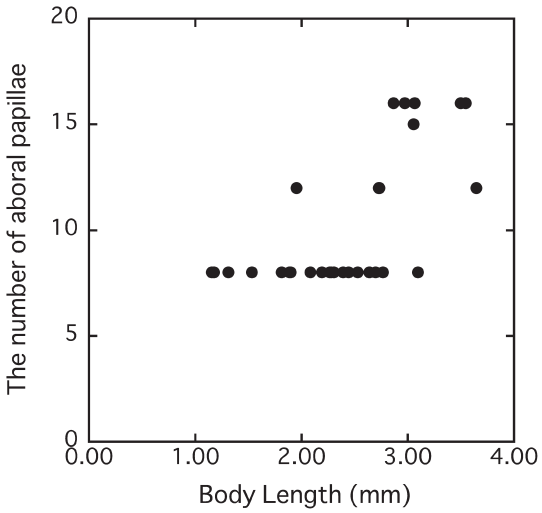

Fig. 4. Relationship between body size and the number of aboral papillae.

still water died within a week, indicating that *V. multiformis* required water current to thrive. *Vallicula multiformis* extended its tentacles in the early morning and at night; similar behavior has been observed in other coeloplanid species (Alamaru et al. 2016). The population of 16 individuals kept in a small cubic tank with flowing seawater increased by asexual reproduction due to fission to 19, 27, 33, and 51 individuals at 5, 7, 22, 47 days, respectively. The tentacular axial size and height (Fig. 1A) when contracted in the floating phase were 1.18~3.55 mm and 0.73~1.95 mm, respectively. The number of aboral papillae ranged from 8 to 16 (Fig. 4). The tentacular axial size in individuals that had more than 8 aboral papillae was more than 2.9 mm. The number of aboral papillae located at A : B : C : D : E : F : G : H (Fig. 1B) was 1 : 1 : 1 : 1 : 1 : 1 : 1 : 1 in small individuals (1.16~3.10 mm, N=21), 2 : 2 : 2 : 2 : 1 : 1 : 1 : 1 in medium individuals (1.95~3.65 mm, N=4) and 2 : 2 : 2 : 2 : 2 : 2 : 2 : 2 in large individuals (2.87~3.55 mm, N=5). The number of aboral papillae varied in similar sized individuals (about 3.00 mm). This phenomenon derives from the growth and degrowth of the individuals. The individuals that have many aboral papillae may be degrown individuals. Aboral papillae developed from near the statocyst to the edge of the body. Rankin (1956) noted the possibility that the aboral papillae may play a role in respiration with the expanded surface area helping oxygen absorption. Many particles were found in the aboral papillae after feeding and digestion of *Artemia* nauplii. When a papilla contracted, those particles were pushed into the peripheral region of the gastrovascular canal. The remains of digested food were evacuated through the anal pores near the sensory organ, as reported in Presnell et al. (2016), when all aboral papillae around the sensory organ contracted. It is easy to transport digested food to the peripheral region in small-sized individuals with a small number of aboral papillae. However, it is difficult in large-sized individuals without aboral papillae around the peripheral region. The increase of the number of papillae is needed for large-sized individuals to transfer nutrients throughout the body. The aboral papillae seem to play a part in transporting digested nutrients to all areas of the body with internal ciliary currents and in the evacuation of food remains.

Habitat and Distribution

Vallicula multiformis were attached to the walls of concrete tanks used for abalone seed production near the surface and on corrugated plastic sheets in the tanks at WPU-BMRS (Fig. 5). All our live individuals were reddish in body color (Fig. 5A, B). However, the reddish body color faded and became a translucent sorrel during their culture in the aquarium (Fig. 2A–E). Though the body color of jellyfish is also likely to change depending on their food and its nutrients, the lack of body coloration phenomenon in this species was similarly observed by Rankin (1956), suggesting that *V. multiformis* can change its body color.

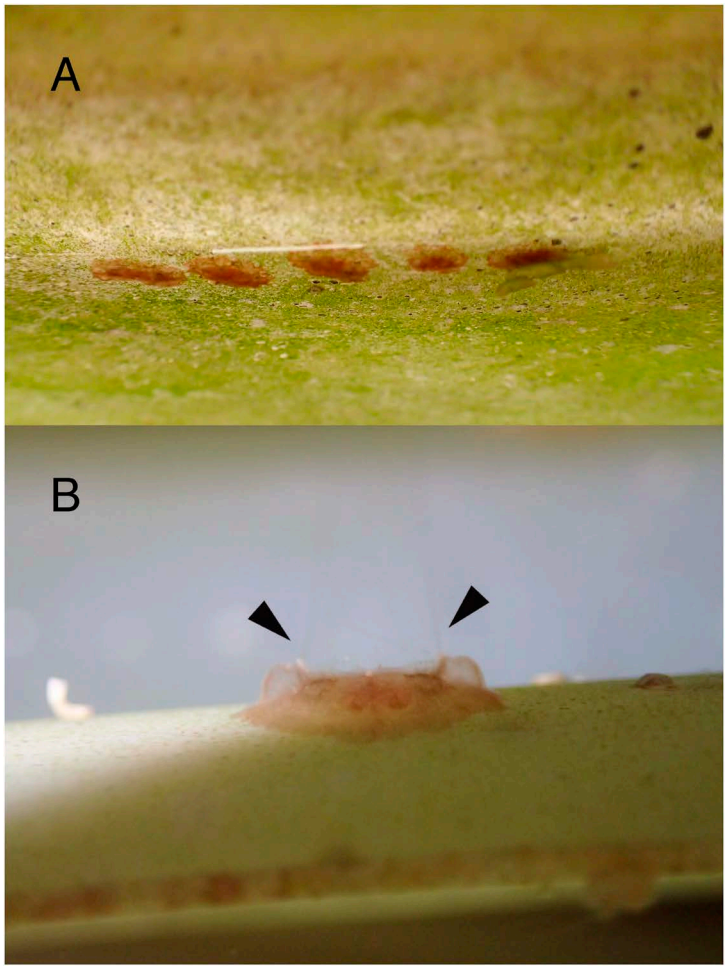

Fig. 5. *Vallicula multiformis* attached to the wall of a concrete tank (A) and on corrugated plastic sheeting (B). Specimen in B with elongated tentacles.

However, Parasade et al. (2015) argued that the color and pattern of Coeloplanidae species do not change. *Vallicula multiformis* has white, brown, pink, green, and purple pigments (Alamaru et al. 2016, Glynn et al. 2017) and its color varies from colorless to reddish, green, yellow, dark brown, and purplish brown (Alamaru et al. 2016, Glynn et al. 2017). The body color of *V. multiformis* attached to the green algae *Halimeda opuntia* (Linnaeus) J. V. Lamouroux in the Indian Ocean was translucent green with white spots (Parasade et al. 2015), but that of *V. multiformis* attached to the red alga *Acanthophora spicifera* (M. Vahl) Børgesen was dark-brown or purplish brown (Glynn et al. 2017). It appears that *V. multiformis* can mimic the color of its substrate using color pigments and, although it cannot change its body color drastically (i.e., from green to red), it can alternate between dark and light coloring. Our individuals of *V. multiformis* were reddish in color and did not match the color of the substrate. Their color probably imitated the color of many calcareous algae from Rhodophyta, which are numerous on rocks, corals, and sea grasses in tropical waters.

Vallicula multiformis has so far been recorded from a number of locations around the world: Jamaica (Emson & Whitfield 1991, Rankin 1956), Bermuda (Freeman 1967), Cuba (Moro et al. 2010), Brazil (Marcus 1956), Hawaii (Carlton & Eldredge 2009, Mills & Haddock 2007, Wirtz 1998), California (Carlton & Eldredge 2009, Mills & Haddock 2007, Wirtz 1998), Florida (Glynn et al. 2017), the Canary Islands (Moro et al. 2010), Portugal (Wirtz 1998), the Gulf of Kutch on the coast of western India (Prasade et al. 2015), the Gulf of Aqaba in the Red Sea (Alamaru et al. 2016), and Palawan Island (present study). *Vallicula multiformis* have been found attached to sea grasses, algae, hydrozoan polyps, soft corals, bryozoans, sea urchins, sea cucumbers, sea stars, ascidians, and rocks (Alamaru et al. 2016, Marcus 1956, Prasade et al. 2015, Rankin 1956, Wirtz 1998). *Vallicula multiformis* was the only benthic ctenophore collected at WPU-BMRS. There is no record of introduction into the aquaria of any artificial organisms or rocks coming from commercial dealers since the beginning of operation of WPU-BMRS, except for the red alga *Gracilaria firma* Chang & Xia, which is collected regularly from the coastal areas of Quezon, Palawan, as a natural food for cultured abalone. The red algae for feed-

ing the brood stock was also taken from Taytay Bay, Palawan. *Vallicula multiformis* may have been present on these red algae and have been introduced into WPU-BMRS. On the other hand, WPU-BMRS is located on the coast of the Sulu Sea, and natural sea water is pumped up into the hatchery facility. Thus, the larvae of this benthic ctenophore may have settled in the tanks through introduction through running natural sea water. However, we were unable to find *V. multiformis* in the field around Palawan. *Vallicula multiformis* in Palawan may be an introduced species, as also seen in Hawaii and California (Carlton & Eldredge 2009, Mills & Haddock 2007). To assess its status, we must find it in the field around the Philippines in the future.

Miglietta & Lessios (2009) showed that the hydrozoan *Turritopsis dohrnii* (Weismann, 1883) is an invasive species worldwide, based on the mitochondrial 16S gene sequence comparisons. This species has morphological differences between tropical and temperate populations. However, they have the same haplotype and no genetic difference (K2P distance was 0.0031). In this study, the average of the intraspecific genetic difference in the same locality was 0.006 (range: 0.00–0.11) in the Red Sea population and 0.008 in the Philippines population (Table 1). However, the genetic difference between the Red Sea and the Philippine populations was 0.014 on average (range: 0.011-0.021). Alamaru et al. (2017) suggested that for species delimitation, the K2P distance of the COI gene in benthic ctenophores must differ by more than 3%. The genetic difference between Red Sea and Philippine population was high compared with this 3% figure for species delimitation. This suggests that the Philippines population is not introduced. We must analyze other genes and haplotypes to study population genetics to clarify this point in the future.

Regardless of whether *Vallicula multiformis* in Palawan is a native or invasive species, the new find of *V. multiformis* in Southeast Asia indicates that the species has a world-wide distribution in tropical waters. This is aided by the species' ability to inhabit a variety of substrates, such as algae, animals, and rocks (Alamaru et al. 2016), as well as anthropogenic materials such as plastics (this study). The behavior of *V. multiformis* includes four phases: gliding, creeping, floating, and sessile phases (Rankin, 1956). Among these, the floating phase is characterized by elongate tentacles in the presence of water currents. In the laboratory, some smaller individuals detached from the substrate and floated in the water by expanding the tentacles and then re-attached on other substrates such as air tubes, air pipes, and the walls of the tank. In nature, not only its planktonic larvae, but also the floating phase of adult *V. multiformis* may contribute to the species' dispersal. In addition, it is likely that its non-specific preference for substrates could facilitate anthropogenic introduction. The world-wide distribution of this species may have been made possible by natural dispersion and/or anthropogenic introduction. Future ecological and population genetics studies are warranted to assess this status.

Acknowledgements

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