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Tima nigroannulata (Cnidaria: Hydrozoa: Eirenidae), a New Species of Hydrozoan from Japan

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***Tima nigroannulata* sp. nov. is described from medusae collected in shallow waters of four prefectures on the Pacific coast of Japan (Miyagi, Fukushima, Kanagawa, and Miyazaki), as well as from cultures maintained at two aquaria (Enoshima Aquarium, Kanagawa Prefecture; Tsuruoka City Kamo Aquarium, Yamagata Prefecture). Adult medusae differ from those of other known species of the genus *Tima* Eschscholtz, 1829 in the following combination of characters: (1) umbrella usually hemispherical or higher, (2) marginal tentacles up to 50 or more in number; and (3) black pigment granules form a ring around the umbrella rim, and sometimes extend onto the tentacles and radial canals. Their hydroids, from aquarium cultures, have stolonal colonies with pedicels of varied length, vestigial hydrothecae, slender and vase- to club-shaped hydranths, and a whorl of about 20 filiform tentacles with an intertentacular web basally. Medusa buds develop singly within gonothecae that arise from the hydrothecal pedicels. The cnidomes of both hydroid and medusa stages comprise heteronemes, provisionally identified as microbasic mastigophores. Medusae of *T. nigroannulata* are confirmed as a unique, cohesive lineage by comparing mtDNA COI sequence fragments with those from two congeners, resulting in three well-supported reciprocally monophyletic clades, one representing each species. Records of the western Atlantic medusa *Tima formosa* L. Agassiz, 1862 from Japan overlap those of *T. nigroannulata*, and are believed to have been based on the new species described herein.**

Key words: Hydroidolina, hydromedusae, Leptothecata, marine biology, marine invertebrates, Medusozoa, plankton, taxonomy

INTRODUCTION

Hydromedusae comprise a diverse faunal group in the marine plankton of Japan (Kubota and Gravili, 2007). In the leptothecate family Eirenidae Haeckel, 1879 alone, seven described and one incompletely identified species have been reported from coastal waters of the country: *Eirene hexanemalis* (Goette, 1886) from Amakusa, *Eirene lacteoides* Kubota and Horita, 1992 from Toba Aquarium, *Eirene menoni* Kramp, 1953 from Amakusa, *Eutima japonica* Uchida, 1925 from off Hokkaido, *Eutonia indicans* (Romanes, 1876) from the north of Japan to Onagawa Bay, *Tima formosa* L. Agassiz, 1862 from Hokkaido to Onagawa Bay, *Eugymnanthea japonica* Kubota, 1979 from central Japan (see Kubota and Horita, 1992), and *Helgicirrha* sp. from an unstated location (Kubota and Gravili, 2007). *Tima saghalinensis* Bigelow, 1913 was originally described from waters off a sector of Saghalin Island (= Sakhalin Island) then occupied by Japan (as Karafuto Prefecture) but assimilated into Russia after 1945. As for *T. formosa*, the species was originally described from Massachusetts Bay, USA (Agassiz L, 1862), with a distribution likely restricted to northeastern North America (Petersen, 1962: p. 107). Records of this hydrozoan from Japan (and China) were considered by Petersen to be "very doubtful".

This study was undertaken to establish the identity of medusae from Japan, earlier assigned to *Tima formosa*, by examining specimens from four locations in the country. These medusae were compared with accounts of the species from Japan (Uchida, 1925, 1938), and with descriptions

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of *T. formosa* from the northeastern United States (Agassiz L, 1862; Agassiz A, 1865; Mayer, 1910; Petersen, 1962). Molecular analyses were undertaken to complement morphological studies in establishing the identity, distinctness, and genetic affinities of the species.

In being distinct from *T. formosa*, and from all other known species of the genus, the Japanese hydromedusa population studied here is recognized and described as a new species. Also described for the first time is its hydroid stage. Hydroids referable to *Tima* are otherwise very poorly known, with the only previous account being a brief description of the hydroid of *T. formosa* from Massachusetts, USA, by A. Agassiz (1865).

MATERIALS AND METHODS

Collecting and culturing

Hydromedusae examined here were collected using a dipnet, or a submerged open-top container on a stick, from shallow waters (0–1 m) at four locations on the Pacific coast of Japan (Fig. 1; Table 1). Specimens were cultured either in a pseudokreisel or in kreisel aquaria (Hamner, 1990; Raskoff et al., 2003) at both the Enoshima Aquarium and the Tsuruoka City Kamo Aquarium. Monoculture holding tanks at the Kamo Aquarium were maintained for multiple generations utilizing hydroid cultures established from known medusae, collected at Iwaki, Fukushima Prefecture, in 2005. Hydroids at the Enoshima Aquarium and the Kamo Aquarium were fed a Vietnamese strain of *Artemia* nauplii once a day. Water quality was maintained with weekly water changes for hydroids, and medusae were housed in open system separate aquaria. Hydroids and medusae were maintained at an average temperature of 20°C and at salinities of 30–35‰. Specimens for morphological and nematocyst studies were preserved in 3–4% formalin. Those for molecular analyses were preserved in > 85% ethanol.

Morphology and systematics

Preserved specimens from four locations in Japan were examined to characterize their morphology and to establish their identity. Medusae of *Tima* from the country had earlier been assigned to *T. formosa*, a species with its type locality in Massachusetts Bay on the Atlantic coast of the United States (Agassiz L, 1862). Medusae from the collections were compared morphologically with descriptions of those assigned to the species from Japan (Uchida, 1925, 1938) and with original accounts of *T. formosa* from the eastern USA (Agassiz L, 1862; Agassiz A, 1865; Mayer, 1910; Petersen, 1962). Specimens of the hydroid stage of the species, from cultures at the Kamo Aquarium, were examined and described. Characters employed in descriptions of medusae and hydroids follow those listed in Bouillon et al. (2006: p. 83–88).

Type specimens, and others, have been deposited in collections within the Section of Invertebrate Zoology, Department of Natural History, Royal Ontario Museum, Toronto, Canada. Collection numbers have been assigned the prefix ROMIZ (Royal Ontario Museum, Invertebrate Zoology).

Illustrations of nematocysts herein, all to the same magnification, are from photomicrographs originally taken at ×1000 using a Zeiss Axioscop microscope. Nematocyst classification follows Weill (1934) and Östman (1979a, b, 1982, 1999).

All cited references have been examined, unless otherwise

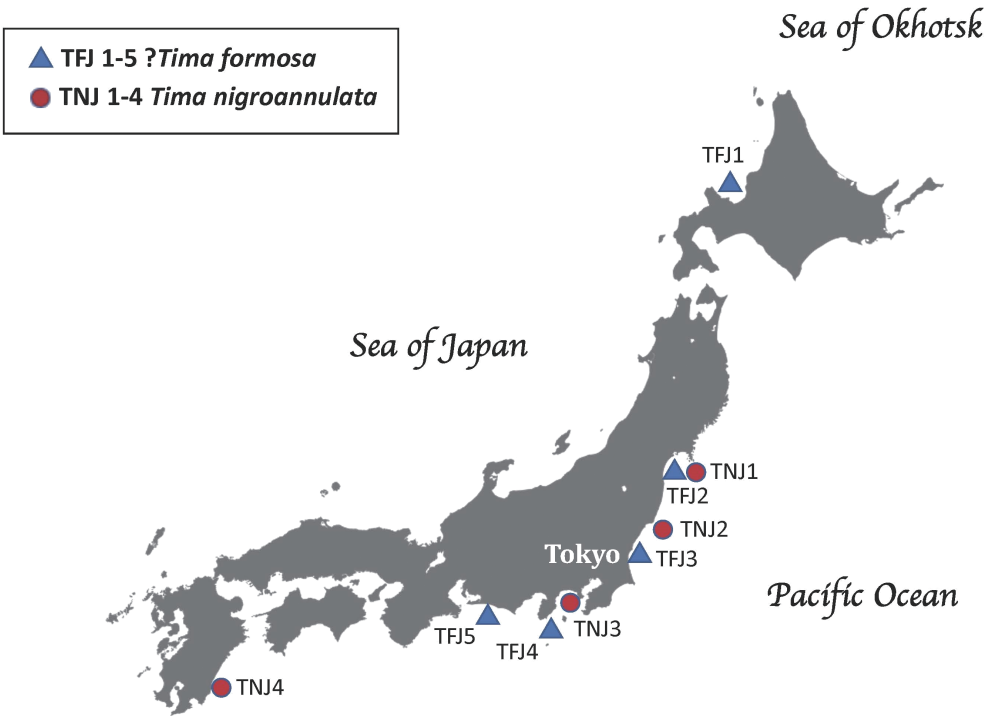


Fig. 1. Map of Japan, showing historical distributional records of medusae identified as ?*Tima formosa* (TFJ), and sampling localities of *Tima nigroannulata* (TNJ). ▲ TFJ = ?*Tima formosa* (literature records of medusae, Japanese waters): **TFJ1** - Oshoro, Hokkaido (Uchida, 1925), collected in 1922 (43.3°N, 140.7°E); **TFJ2** - Onagawa Bay, Mangoku-ura (Uchida, 1938), collected in 1936 (38°N, 141°E); **TFJ3** - Oarai Fishing Port, Ibaraki (Saito and Shiba, 2008), collected 2007 (36°18′N, 140°34′E); **TFJ4** - Between Katsura-Jima Island and Mahahashi-Jima Island (Kubota and Yamazaki, 2007), collected in 1931 (34.5°N, 139°E); **TFJ5** - Lake Hamana (brackish), Shizuoka (Okamoto et al., 2016), (34°44′N, 137°34′E). ● TNJ = *Tima nigroannulata* (medusae from this study, Japanese waters): **TNJ1** – Sendai, Miyagi Prefecture (38°16′24.9″N, 141°00′19.7″E); **TNJ2** – Iwaki, Fukushima Prefecture (36.9°N, 140.9°E); **TNJ3** – Fujisawa, Kanagawa Prefecture (35°18′24.3″N, 139°28′53.8″E); **TNJ4** - Miyazaki, Miyazaki Prefecture (31°54′29″N, 131°27′29.3″E).

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specified, and illustrations are based on specimens examined as part of his study.

DNA extraction, PCR amplification, and DNA sequencing

Whole specimens of hydromedusae from Japan (Table 2) were removed from the ethanol storage buffer and rinsed in deionized distilled autoclaved water. Genomic DNAs were extracted from each whole organism using a Macherey-Nagel NucleoSpin® Mini kit for DNA from cells and tissue (Takara Bio, Inc. USA, 1290 Terra Bella Ave., Mountain View, CA 94043, USA; Reference #740952.50) according to the manufacturer's protocol. Genomic DNA was eluted in 200 μl of deionized autoclaved water and stored at −20°C.

Fragments of 613 basepairs (bp) of the mitochondrial DNA (mtDNA) cytochrome *c* oxidase I (COI) gene were amplified by polymerase chain reaction (PCR) using the primers LCO1490/HCO2198 (Folmer et al., 1994). Target fragments were amplified using a MyCycler™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) with Conquest PCR Master Mix Optimizing Pack reagents and buffers from Lamda Biotech (catalog # D911-Mix1234). PCRs were conducted at Hawaii Pacific University's Oceanic Institute, in individual volumes of 20 μl each under the following conditions: 4 min at 94°C, 33 cycles of 94°C for 40 s, 52°C for 1 min and 72°C for 90 s, with a final 72°C extension for 5 min.

PCR fragments were purified with Macherey-Nagel NucleoSpin® Gel and PCR Clean-up columns (Takara Bio, Inc. USA, 1290 Terra Bella Ave., Mountain View, CA 94043, USA; Reference #740609.50), per the manufacturer's protocol, and visualized via agarose minigel electrophoresis. Amplified mtDNA fragments were sequenced using the same primer pair as the initial PCRs. DNA sequencing was performed at the Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB) at the University of Hawaii, Manoa.

Phylogenetic analysis

RAxML (Randomized Axelerated Maximum Likelihood) (Stamatakis, 2014) and MEGA X (Molecular Evolutionary Analysis) (Kumar et al., 2018) were used for phylogenetic reconstruction; statistical support was assessed with 1000 bootstrap replicates. We compared topologies among maximum parsimony, minimum evolution, and maximum likelihood. In instances where different methods resulted in identical tree topologies, we have high confidence that the results are representative of the evolutionary history of the sequences comprising the data set (Holland and Hadfield, 2004), whereas differences in results indicate that more than one interpretation is possible. Tree topologies were extremely consistent, well resolved, and well supported.

RESULTS

Taxonomic account

Phylum **Cnidaria** Verrill, 1865
Subphylum **Medusozoa** Petersen, 1979
Class **Hydrozoa** Owen, 1843
Subclass **Hydroidolina** Collins, 2000
Order **Leptothecata** Cornelius, 1992
Family **Eirenidae** Haeckel, 1879

Irenidae Haeckel, 1879, p. 199 [emended to Eireninae by Mayer (1910)].

Diagnosis (after Kramp, 1961; Calder, 1991; Cornelius, 1995; Bouillon et al., 2006).

Hydroids typically forming stolonal or erect colonies arising from a creeping hydrorhiza, or occurring as simple, solitary commensals; one unusual species (*Eirene hexanemalis*) planktonic, without a hydrorhiza, with hydroid metamorphosing directly into a medusa. Hydrothecae when present pedicellate with a basal diaphragm; comprising either a vestigial collar at hydranth base or with cylindrical walls and an operculum of folded flaps indistinctly separated from hydrothecal rim, later reduced to a collar surrounding hydranth base. Hydranths extensile, with a whorl of amphicoronate filiform tentacles having an intertentacular web; hydranths of commensal species sessile, naked, usually solitary, attached to host by a basal disc.

Gonophores free medusae or medusoids, arising from hydranth, hydrocaulus, hydranth pedicel, or hydrorhiza; gonothecae present or absent. Medusae with small or smallish manubrium at tip of a distinct gastric peduncle; radial canals simple, four–six in number; marginal

Table 1. Collection data for specimens of *Tima nigroannulata* sp. nov. examined here.

Location	Prefecture	Geographic Coordinates	Date	Collector	Depth	Type
Miyazaki	Miyazaki	31°54′29″N, 131°27′29.3″E	29-Apr-20	Y Kawagoe	0–1 m	wild
Fujisawa	Kanagawa	35°18′24.3″N, 139°28′53.8″E	29-Apr-20	G Yamamoto	0–1 m	wild
Iwaki	Fukushima	36.9°N, 140.9°E	2005	S Mizutani	cultured	aquarium
Sendai	Miyagi	38°16′24.9″N, 141°00′19.7″E	12-Jan-20	S Ikeda	0–1 m	wild

Table 2. Taxa included in phylogenetic analyses, with GenBank accession numbers, sampling locations, and GPS coordinates.

ID #	Species	GenBank Accession #	Location	Geographic Coordinates
TBS1	*Tima bairdii*	MF00509	Fanafjord, Norway	60.2°N, 5.2°E
TBS2	*Tima bairdii*	MG935038	Skagerrak, Sweden	58.3°N, 10.5°E
TFC1	?*Tima formosa*	JQ716166	Changjiang River Estuary, China	31.5°N, 122.15°E
TFC2	?*Tima formosa*	JQ716168	Jiaozhou Bay, China	36.1°N, 120.25°E
TFC3	?*Tima formosa*	JQ716169	Jiaozhou Bay, China	36.1°N, 120.25°E
TFC4	?*Tima formosa*	JQ716170	Jiaozhou Bay, China	36.1°N, 120.25°E
TNJ1	***Tima nigroannulata*****,** sp. nov.	MW490678	Sendai, Japan	38°N, 141°E
TNJ2	***Tima nigroannulata*****,** sp. nov.	MW490677	Fukushima, Japan	36.9°N, 140.9°E
TNJ3	***Tima nigroannulata*****,** sp. nov.	MW490679	Fukushima, Japan	36.9°N, 140.9°E
AA	*Alatina alata*	KM200330	Waikiki Beach, Hawaii	21.276°N, 157.827°W

Note: Sequences obtained in this study are in bold. Hydrozoans identified as ?*Tima formosa* are of questionable specific identity.

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tentacles hollow; bases of tentacles with or without excretory pores; marginal cirri and marginal warts present or absent; statocysts closed, eight or more in number; gonads on radial canals, not extending onto manubrium. Ocelli lacking.

Remarks. Hydrozoans of the family Eirenidae Haeckel, 1879 are much better known from the medusa stages than from their hydroids. The group is typically distinguished by having (1) medusae with a typically well-developed gastric peduncle; gonads occurring along the entire length of four–six simple radial canals; eight to many epidermal closed statocysts; hollow marginal tentacles; (2) hydroids with diaphanous gonothecae and reduced to vestigial hydrothecae (Cornelius, 1995; Bouillon et al., 2006). Following Bouillon (1985), the family name Eutimidae Haeckel, 1879 is currently included as a synonym of Eirenidae (e.g., Cornelius, 1995; Bouillon et al., 2006; Schuchert, 2021). However, Eirenidae as presently constituted has been shown to be polyphyletic, with two groups, "Eirenids I" and "Eirenids II", distinguished in molecular analyses by Maronna et al. (2016). Further refinement of eirenid systematics is warranted. See also remarks below.

Genus ***Tima*** Eschscholtz, 1829

Tima Eschscholtz, 1829, p. 103.

Type species. *Tima flavilabris* Eschscholtz, 1829, by monotypy.

Diagnosis (after Kramp, 1961; Cornelius, 1995; Bouillon et al., 2006). Hydroids inadequately described; where known, occurring as stolonal colonies with characters of other free-living species of the family.

Gonophores free medusae. Where known, gonothecae pedicellate, clavate, with thin, filmy perisarc, arising from hydrothecal pedicel. Medusae with characters of the family, with a well-developed gastric peduncle, four radial canals, and numerous (> eight) marginal vesicles and marginal warts; without lateral and marginal cirri and excretory pores; tentacle bases and tentacles with an abaxial longitudinal furrow; gonads extending along entire length of radial canals.

Remarks. Four species are currently assigned to *Tima* Eschscholtz, 1829 besides *T. nigroannulata* sp. nov. from Japan. Their type localities and reported distributions are as follows. (1) *Tima flavilabris* Eschscholtz, 1829, type species of the genus (type locality: North Atlantic Ocean northeast of the Azores); reported from the central and northeast North Atlantic (Petersen, 1957, 1962; Winkler, 1982; Bleeker and van der Spoel, 1988). (2) *Tima bairdii* (Johnston, 1833) (type locality: Berwick Bay, UK, surface); reported from the North Sea and vicinity from southern Norway to Belgium, and eastwards to the Baltic Sea off Germany (Cornelius, 1995). (3) *Tima formosa* L. Agassiz, 1862 (type locality: Massachusetts Bay, USA); reported from the Atlantic coast of North America from southern New England (Petersen, 1962) to Nova Scotia (Stafford, 1907); as noted earlier, records from Japan and China have been considered doubtful (Petersen, 1962), as is a record from Bermuda (Fewkes, 1883). (4) *Tima saghalinensis* Bigelow, 1913 (type locality: off Sakhalin Island, Russia); reported from the Sea of Okhotsk (Naumov, 1960; Petersen, 1962; Zavolokin, 2010, as *Tima sachalinensis*). Medusae identified as *T. formosa* from China may be different from both the Atlantic species of that name and from the Japanese *T. nigroannulata*.

Conflicting results exist in previous phylogenetic studies regarding whether *Tima* should be assigned to "Eirenids I" or "Eirenids II" of Maronna et al. (2016). Zheng et al. (2014) included a medusa identified as *T. formosa* from China in a clade with *Eirene kambara* Agassiz and Mayer, 1899 and *Eirene hexanemalis* (Goette, 1886), species included by Maronna et al. (2016) in "Eirenids I". Schuchert et al. (2017) included *T. bairdii* in a clade with *Eucheilota menoni* Kramp, 1953, listed in "Eirenids II" by Maronna et al. (2016). The phylogenetic tree presented herein includes the cubozoan outgroup *Alatina alata* (Reynaud, 1830), and consists of three distinct, reciprocally monophyletic clades each with 100% bootstrap support (1000 replicates) (Fig. 2). The COI sequences for *T. bairdii* and *T. nigroannulata* also form a well-supported clade, suggesting that these two lineages are sister species. A combined molecular systematic and

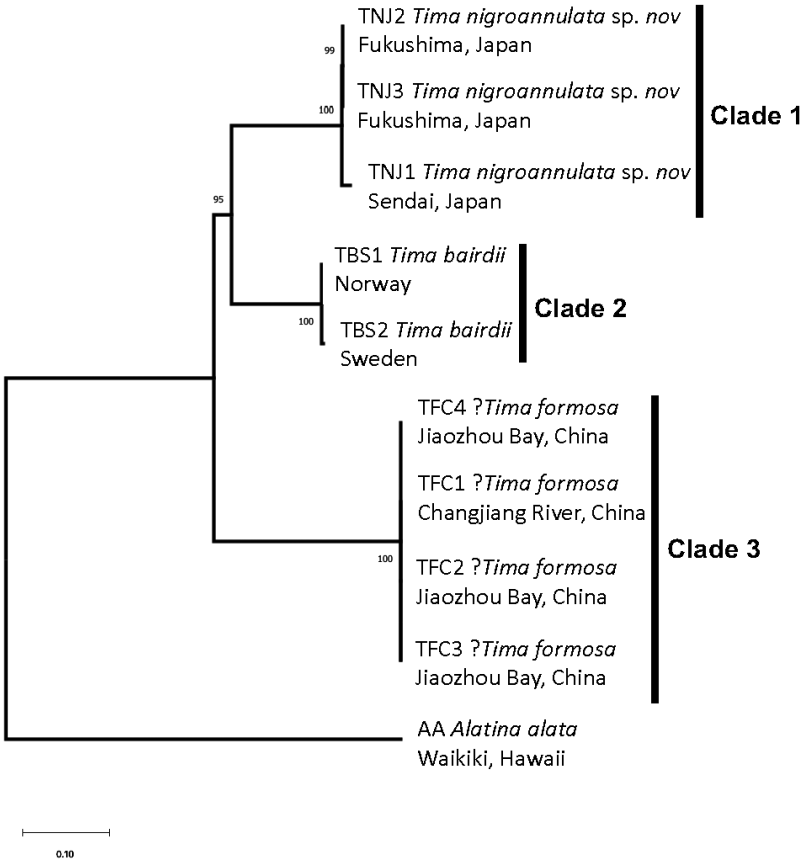


Fig. 2. Maximum likelihood tree based on 10 cytochrome *c* oxidase I (COI) mtDNA sequence fragments from nine specimens of *Tima* spp. and the outgroup box jellyfish *Alatina alata* collected at Waikiki Beach, Oahu, Hawaii. Bootstrap support was generated via 1000 replicates using the GTR + I substitution model.

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morphological comparison of the species included here, together with *T. flavilabris* Eschscholtz, 1829 (type species of *Tima*) and species assigned to both *Tima* and *Eirene* Eschscholtz, 1829, would be taxonomically informative. Published tree topologies present conflicting results in the generic assignment of species within this group.

Medusae of the genus sometimes occur in significant numbers. Blooms of *T. bairdii* have been reported to interfere at times with fishing efforts in the Skagerrak, Scandinavia, and the southern Baltic Sea (Russell, 1970; Cornelius, 1995), and they occasionally strand on British shores in substantial numbers (Russell, 1953). According to Mayer (1910), *T. formosa* is common in Buzzards Bay, Massachusetts, and Newport Harbor, Rhode Island, USA, in April and May. *Tima saghalinensis* was found to be a dominant medusa in the central Sea of Okhotsk, Russia (Zavolokin, 2010).

Because of their large size, species of *Tima* have been used in exhibits at aquaria worldwide. Juveniles examined here were from cultures maintained at both the Tsuruoka City Kamo Aquarium and the Enoshima Aquarium, Japan.

Tima nigroannulata sp. nov.
Figs. 3–9

?*Tima formosa*: Uchida (1925), p. 92; Uchida (1938), p. 53; Uchida (1940), p. 281; Kubota (1997), pp. 496, 534, figs. 4–40; Kubota and Yamazaki (2007), p. 6, pl. 2, fig. 5; Kubota and Gravili (2007), p. 194; Saito and Shiba (2008), p. 8; Murakami (2016), p. 71; Okamoto et al. (2016), p. 32; Yamamori et al. (2017), p. 175 [not *Tima formosa* L. Agassiz, 1862].

Type locality. Japan: Kyushu, Miyazaki Prefecture, Miyazaki, 31°54′29.0″N, 131°27′29.3″E, surface (0–1 m), < 1 m from shore.

Material examined. *Holotype*. ROMIZ B5390, Japan, Kyushu, Miyazaki Prefecture, Miyazaki, 31°54′29.0″N, 131°27′29.3″E, surface (0–1 m), < 1 m from shore, 29 April 2020, coll. Yoshiki Kawagoe, one medusa: umbrella 4.5 cm wide, 3 cm high, with 53 marginal tentacles, adult female.

Paratypes (13 medusae and several colonies of hydroids, in five lots). ROMIZ B5391, Japan, Miyazaki Prefecture, Miyazaki, 31°54′29.0″N, 131°27′29.3″E, surface (0–1 m), < 1 m from shore, 29 April 2020, coll. Yoshiki Kawagoe, three medusae: (1) umbrella 4.6 cm wide, 3.8 cm high, with 45 marginal tentacles, adult male; (2) umbrella 3.6 cm wide, 2.1 cm high, with 44 marginal tentacles, adult male; (3) umbrella 2.3 cm wide, 1.2 cm high, with 30 marginal tentacles, adult female. ROMIZ B5392, Japan, Kanagawa Prefecture, Fujisawa, 35°18′24.3″N, 139°28′53.8″E, surface (0–1 m), < 1 m from shore, 15.1°C, 29 April 2020, coll. Gaku Yamamoto, five medusae: (1) umbrella 4.5 cm wide, 2.5 cm high, with 38 marginal tentacles, adult male; (2) umbrella 2.9 cm wide, 1.5 cm high, with 30 marginal tentacles, adult male; (3) umbrella 2.0 cm wide, 1.2 cm high, with 22 marginal tentacles, juvenile; (4) umbrella 5.0 cm wide, 2.0 cm high, with 42 marginal tentacles, adult female; (5) umbrella 3.8 cm wide, 1.8 cm high, with 34 marginal tentacles, adult male. ROMIZ B5393, Japan, Fukushima Prefecture, Iwaki, 36.9°N, 140.9°E, in cultures at Kamo Aquarium, preserved 11 June 2020, cultures originating from medusae coll. 2005 by S. Mizutani, five medusae: (1) umbrella 2.5 cm wide, 1.5 cm high, with 36 marginal tentacles, juvenile; (2) umbrella 2.7 cm wide, 1.6 cm high, with 34 marginal tentacles, juvenile; (3) umbrella 2.6 cm wide, 1.4 cm high, with 36 marginal tentacles, juvenile; (4) umbrella 2.5 cm wide, 1.5 cm high, with 36 marginal tentacles, juvenile; (5) umbrella 2.1 cm wide, 1.5 cm high, with 38 marginal tentacles, juvenile. ROMIZ B5395, Japan, Kanagawa Prefecture, Fujisawa, 35°18′24.3″N, 139°28′53.8″E, fragments of a hydroid colony or colonies, with one gonophore, raised at Enoshima Aquarium from medusae collected 29 April 2020; coll. Gaku Yamamoto. ROMIZ B5396, Japan, Miyazaki Prefecture, Miyazaki, 31°54′29.0″N, 131°27′29.3″E, dense hydroid colony or colonies, with gonophores, raised at Enoshima Aquarium from medusae collected 29 April 2020, coll. Yoshiki Kawagoe.

Other material (four medusae and several colonies of hydroids, in five lots). ROMIZ B5394, Japan, Fukushima Prefecture, Iwaki, 36.9°N, 140.9°E, in cultures at Kamo Aquarium, cultures originating from medusae coll. 2005 by S. Mizutani, four medusae: (1) umbrella 2.3 cm wide, 1.7 cm high, with 33 marginal tentacles, juvenile; (2) umbrella 2.2 cm wide, 1.2 cm high, with 38 marginal tentacles, juvenile; (3) umbrella 2.0 cm wide, 1.4 cm high, with 41 marginal tentacles, juvenile; (4) umbrella 2.3 cm wide, 1.4 cm high, medusa fragmentary and missing tentacles, juvenile. ROMIZ B5397, Japan, Fukushima Prefecture, Iwaki, in cultures at Kamo Aquarium, cultures originating from medusae coll. 2005 by S. Mizutani, several hydroid colonies or colony fragments, to 1.2 mm high, without gonothecae.

Etymology. The specific name is an adjective derived from the Latin words *niger* (black) and *annulus* (ring), in reference to the ring of black pigment occurring around the periphery of the umbrella of this species.

Common name. Elegant jellyfish (Fig. 3), a name applied to medusae of the species in Japan; giyamankurage in Japanese.

Description of advanced juvenile medusae (based

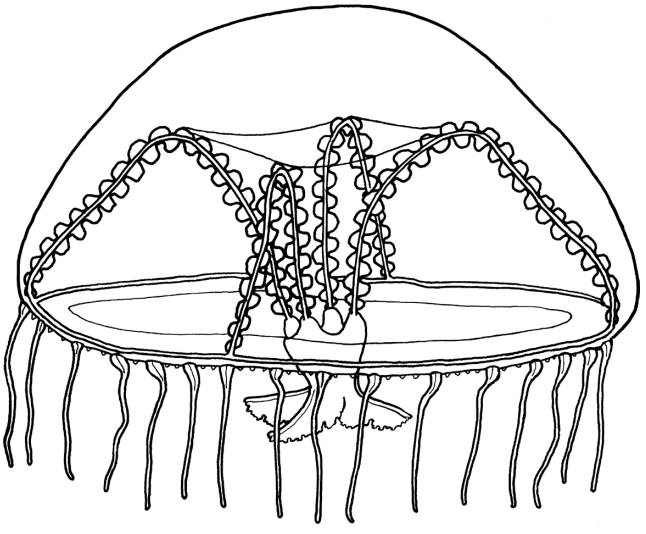


Fig. 3. *Tima nigroannulata*, adult medusa (non-type). Drawn from a photograph (by S. Ikeda) of a specimen collected 12 January 2020 at Sendai, Japan (38°16′24.9″N, 141°00′17.9″E).

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on paratype material, ROMIZ B5393, and non-type material, ROMIZ B5394. Fig. 4, non-type; Fig. 5A, B, non-type). Umbrella higher than hemisphere, with regular curvature, diameter at margin 20–27 mm, height 12–17 mm, mesoglea thick at apex, gradually thinning towards periphery. Gastric peduncle well-developed, conical, gradually tapering, 6–11 mm long, 9–15 mm in diameter at base, varied in length but usually extending beyond subumbrellar cavity. Tentacles varying from 33–41 in number, with four of them perradial and given off immediately below bases of radial canals, each with median abaxial groove; proximal ends of tentacles oblong-oval, elongated, fleshy, laterally compressed, projecting inwards, unevenly spaced, usually separated but occasionally arising side-by-side, outer edges partly adhering to exumbrella; part of tentacles beyond base varying greatly in length but usually quite long, wrinkled, thread-like, widest basally, thinnest at tip, irregularly segmented, densely armed with nematocysts throughout. Lateral cirri and marginal cirri lacking; excretory pores lacking. Marginal warts conical with rounded tips, varying in size and number, with zero–five between tentacles, appearing to increase in number with increasing size of medusa, occasional larger ones appearing to develop tentacles. Statocysts ectodermal, closed, oval to almost spherical, varied in size, 65–90 in number but numbers appearing to increase with increasing size of medusae, usually alternating with tentacles and marginal warts, but their spacing uneven; number of statoliths indeterminable in preserved specimens. Manubrium located at tip of gastric peduncle, short in preserved specimens, 1.5–3 mm long, cruciform in cross section, with bulbous pouches extending onto bases of radial canals, hanging partly or completely below velar opening; lips four, perradial, long, folded, curtain-like, with frilly edges. Radial canals four, slender, straight, widening slightly at insertion with manubrium, elsewhere nearly equal in diameter. Circular (ring) canal distinct. Velum thin, with wide velar opening. Gonads lacking. Color of tentacles, marginal warts, radial canals, manubrium, and lips whitish in preserved specimens; umbrella margin in all medusae with prominent ring of black pigment granules, extending over bases of tentacles and sometimes continuing outwards onto tentacles, pigmentation of ring becoming denser towards periphery; umbrella, gastric peduncle, and velum glassy and transparent. Living medusae from cultures with tan tentacular bases, with color likely influenced by digested prey.

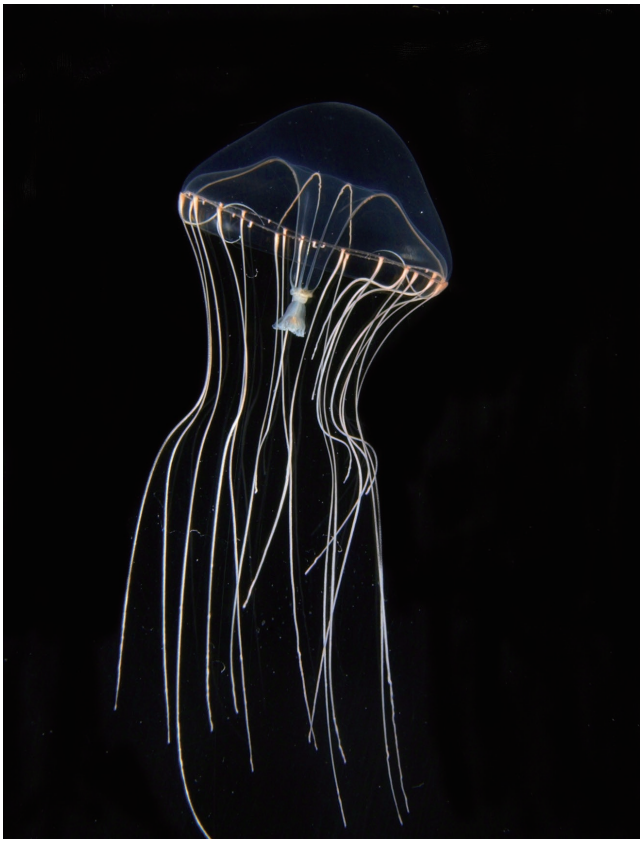


Fig. 4. *Tima nigroannulata*, advanced juvenile medusa (non-type), raised at the Enoshima Aquarium (photograph by G. Yamamoto).

Cnidome on tentacles (undischarged), Fig. 6A–C; non-type, ROMIZ B5394. Large microbasic mastigophores 16.4–20.2 μm long × 2.6–2.9 μm wide (n = 6); medium microbasic mastigophores 12.0–15.0 μm long × 2.1–2.6 μm wide (n = 10); small microbasic mastigophores 9.0–10.8 μm long × 1.7–2.1 μm wide (n = 10).

Cnidome on oral arms (undischarged), Fig. 6D; non-

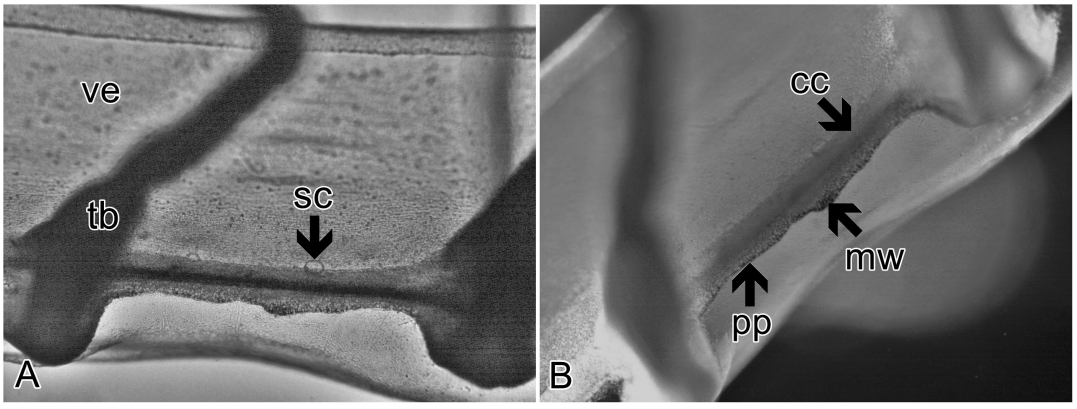


Fig. 5. *Tima nigroannulata*. **(A, B)** oral view of part of rim of umbrella (non-type, ROMIZ B5394), showing bases of tentacles (tb), circular canal (cc), incipient marginal wart (mw), statocysts (sc), pigment particles (pp), and part of velum (ve), Iwaki, Fukushima Prefecture, Japan.

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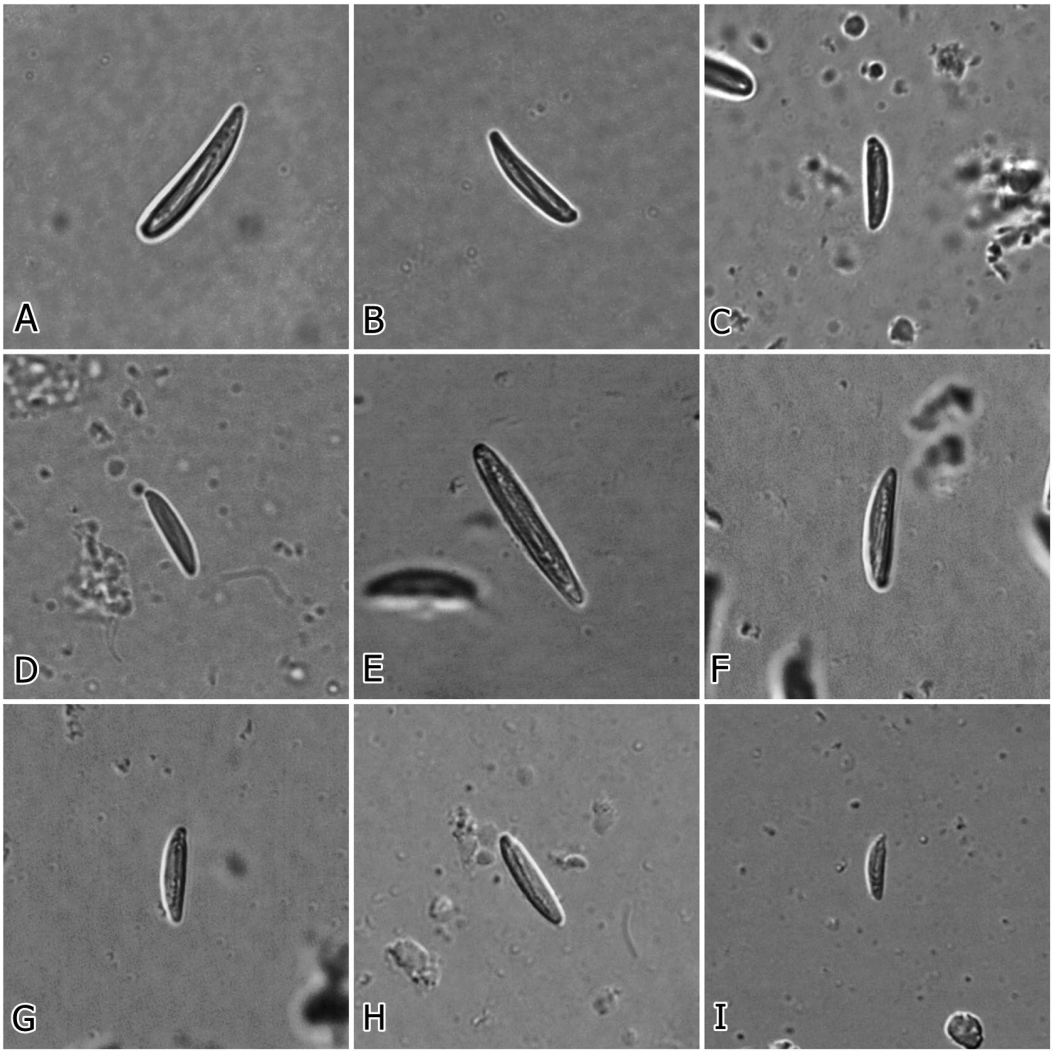


Fig. 6. *Tima nigroannulata*, nematocysts of juvenile and adult medusae, and hydroids, all to the same magnification. **(A)** large microbasic mastigophore, juvenile medusa (non-type, ROMIZ B5394), tentacle, Fukushima Prefecture, Japan. **(B)** medium microbasic mastigophore, juvenile medusa (non-type, ROMIZ B5394), tentacle, Fukushima Prefecture, Japan. **(C)** small microbasic mastigophore, juvenile medusa (non-type, ROMIZ B5394), tentacle, Fukushima Prefecture, Japan. **(D)** small microbasic mastigophore, juvenile medusa (non-type, ROMIZ B5394), oral arm, Fukushima Prefecture, Japan. **(E)** large microbasic mastigophore, adult medusa (paratype, ROMIZ B5391), tentacle, Miyazaki Prefecture, Japan. **(F)** medium microbasic mastigophore, adult medusa (paratype, ROMIZ B5391), tentacle, Miyazaki Prefecture, Japan. **(G)** small microbasic mastigophore, adult medusa (paratype, ROMIZ B5391), tentacle, Miyazaki Prefecture, Japan. **(H)** small microbasic mastigophore, adult medusa (paratype, ROMIZ B5391), oral arm, Miyazaki Prefecture, Japan. **(I)** small microbasic mastigophore, hydranth of hydroid (paratype, ROMIZ B5396), Miyazaki Prefecture, Japan.

type, ROMIZ B5394. Small microbasic mastigophores 10.6–11.8 μm long × 1.9–2.5 μm wide (n = 10).

Description of adult medusae (based on holotype, ROMIZ B5390, and paratype material, ROMIZ B5391, ROMIZ B5392. Fig. 3, non-type). Umbrella usually higher than hemisphere, with regular curvature; diameter at margin 23–46 mm, height 12–38 mm, mesoglea thick at apex, gradually thinning towards periphery. Gastric peduncle well-developed, conical, gradually tapering, 7–17 mm long, 11–21 mm in diameter at base, of varied length but usually extending to or beyond umbrella margin. Tentacles varying in number from 30 to 53, with four of them perradial and given off immediately below bases of radial canals, each with median abaxial groove; proximal ends of tentacles oblong-oval, elongated, fleshy, laterally compressed, projecting inwards, unevenly spaced, usually separated but occasionally arising side-by-side, outer edges partly adhering to exumbrella; part of tentacles beyond base varying greatly in length but usually quite long, wrinkled, thread-like, appearing irregularly segmented, widest basally, thinnest near tip, densely armed with nematocysts throughout. Lateral cirri and marginal cirri lacking; excretory pores lacking. Marginal warts conical with rounded tips, varying considerably in size, 42–115 in number, with zero–five between tentacles, increasing in number with increasing size of medusa, occasional larger ones appearing to develop tentacles. Statocysts ectodermal, closed, varied in size, oval to almost spherical, 71–141 in number, tending to increase in number with increasing medusa size, varying in number between tentacles and marginal warts, spacing uneven, occasionally

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with two side-by-side; number of statoliths indeterminable in preserved material. Manubrium located at tip of gastric peduncle, short and wide, cruciform in cross section, with bulbous pouches extending onto bases of radial canals, hanging partly or completely below velar opening; mouth cruciform, with four perradial channels leading into lips; lips four, perradial, 8–13 mm long, folded, curtain-like, with frilly edges. Radial canals four, slender, straight to partly zigzag due to gonadal folds, widening slightly at insertion with manubrium, elsewhere nearly equal in diameter. Circular canal narrow, distinct. Velum thin, with wide velar opening. Gonads ribbon-like, nearly straight to tightly folded, occurring continuously along radial canals from tip of peduncle to circular canal; medusae dioecious. Color of tentacles, marginal warts, radial canals, manubrium, and lips whitish in preserved specimens; umbrella margin with prominent ring of black pigment granules, extending along and sometimes over bases of tentacles and occasionally continuing outwards onto tentacles, pigmentation of ring becoming denser towards periphery, black pigment also usually present along edge of gonads next to radial canals; umbrella, gastric peduncle, and velum glassy and transparent. When exposed to black light, bases of tentacles glow light blue.

Cnidome on tentacles (undischarged), Fig. 6E–G; paratype, ROMIZ B5391. Large microbasic mastigophores 17.0–23.3 μm long × 2.7–3.4 μm wide (n = 10); medium microbasic mastigophores 13.0–16.7 μm long × 2.3–2.8 μm wide (n = 10); small microbasic mastigophores 8.7–12.5 μm long × 1.7–2.5 μm wide (n = 10).

Cnidome on oral arms (undischarged), Fig. 6H; paratype, ROMIZ B5391. Small microbasic mastigophores 10.0–12.2 μm long × 1.9–2.7 μm wide (n = 10).

Description of hydroids and newly liberated medusae (based on paratype material, ROMIZ B5396. Fig. 7A, D, non-types; Fig. 7B, C, paratypes; Fig. 8A, B, paratypes; Fig. 9A–C, non-types). Colonies stolonal, arising from creeping hydrorhiza. Hydrorhizal stolons long, slender, smooth or with occasional wrinkles, giving rise to hydrothecal pedicels of varied length, most of them quite short. Hydrothecal pedicels usually with a few irregular annulations or wrinkles at base, otherwise partly to mostly smooth, branched only when supporting pedicellate gonotheca below base of

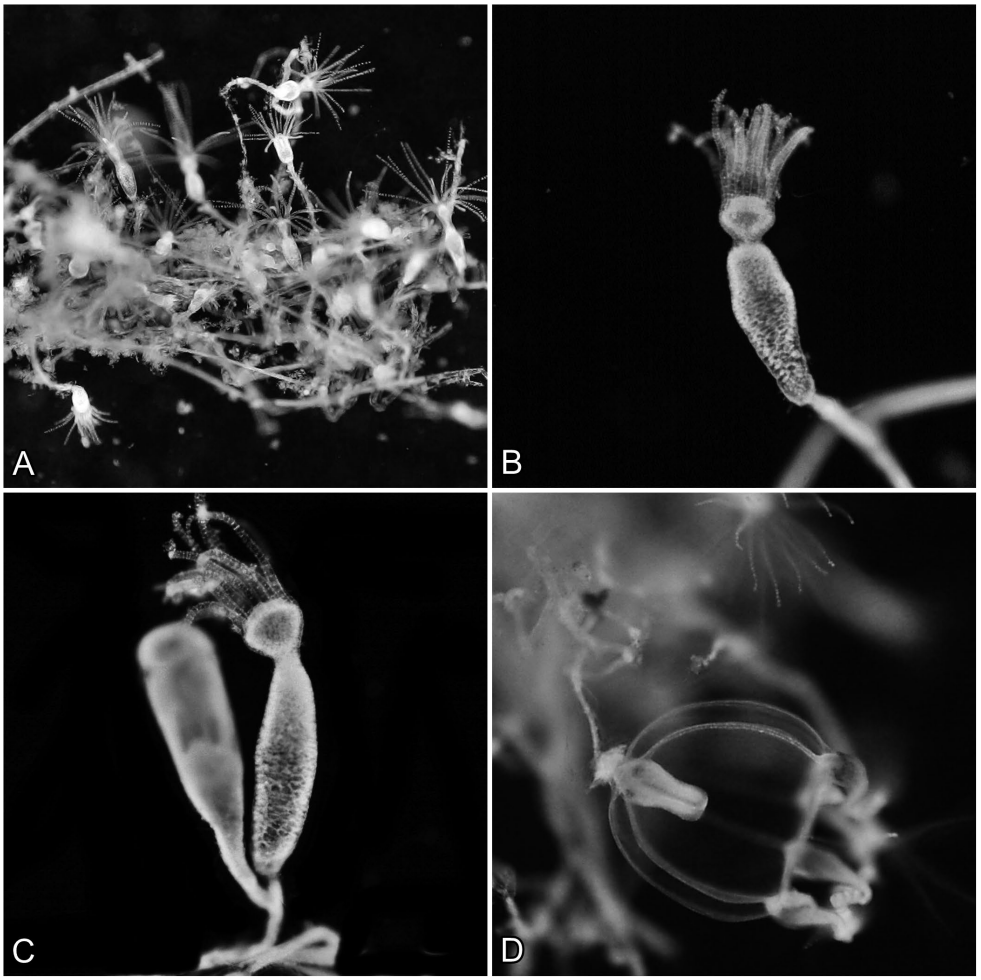


Fig. 7. **(A)** hydroid colony, Fukushima Prefecture, Japan, raised at the Kamo Aquarium (photograph by S. Ikeda). **(B)** hydroid, Miyazaki Prefecture, Japan (paratype, ROMIZ B5396), raised at the Enoshima Aquarium (photograph by D. Calder). **(C)** Hydroid hydranth and gonotheca with a medusa bud, Miyazaki Prefecture (paratype, ROMIZ B5396), raised at the Enoshima Aquarium (photograph by D. Calder). **(D)** Medusa bud, 1.22 mm high, 1.32 mm wide, with four tentacles, about to be released from hydroid, specimen from Enoshima Aquarium (photograph by G. Yamamoto).

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hydranth; perisarc of hydroid colonies very thin. Hydrotheca reduced to short, irregular, filmy collar enveloping only extreme base of hydranth, proximal end with distinct diaphragm below ring of desmocytes; operculum lacking. Hydranths quite large, naked except at proximal end, narrowest at base, gastric region typically vase- to club-shaped, constricted short distance below tentacular whorl, constriction usually pronounced but sometimes indistinct, hydranth expanding again at distal end, here becoming subspherical to knob-shaped, supporting whorl of tentacles; hypostome of varied shape, sometimes flaring but usually curved and flatter than hemisphere, with central, round mouth. Tentacles filiform, in one whorl, about 20 in number, their bases with intertentacular web. Nematophores absent.

Gonophores free medusae. Gonothecae long, slender, filmy, with rounded distal end, gradually tapering towards proximal end, arising from hydranth pedicels by gonothecal pedicels, each gonotheca with single medusa bud. Advanced medusa buds filling much of gonotheca, each bud with four large tentacles, four radial canals, circular canal, and tubular manubrium. Medusa bud nearing release higher than a hemisphere, 1.2 mm high, 1.3 mm wide, tentacles four, perradial, tentacular bases tan-coloured with black pigment spots (apparent in color photographs); manubrium short, without peduncle (Fig. 7D). Newly liberated medusae higher than a hemisphere; umbrella 1.2 mm high, 1.4 mm wide; perradial tentacles four, well-developed; interradial tentacles four, some little more than marginal warts; radial canals four; circular canal present, peduncle undeveloped; manubrium short, not reaching velar opening; lips four, perradial, distinct, with frilly edges; velum well-developed, with radiating folds, velar opening small; statocysts with statoliths present (Fig. 9A–C).

Cnidome on hydranths of hydroid, including tentacles (undischarged), Fig. 6I; paratype, ROMIZ B5396. Small microbasic mastigophores 7.0–7.9 μm long × 1.5–1.8 μm wide (n = 10).

Remarks. Medusae of *T. nigroannulata* sp. nov. differ from those of other described species of the genus (Mayer, 1910; Kramp, 1959, 1961; Petersen, 1962), as summarized in the following key:

1. Umbrella distinctly flatter than a hemisphere 2
   Umbrella typically hemispherical or higher 3
2. Umbrella much flattened, resembling a watch glass; tentacles as many as 250–300, gastric peduncle short ..... *T. saghalinensis*
   Umbrella less flattened but not hemispherical; tentacles 60–80, gastric peduncle relatively long ..... *T. flavilabris*
3. Tentacles normally ca. 16; umbrella diameter 50–65 mm; warts ca. 12 between tentacles; marginal vesicles ca. six between tentacles ..... *T. bairdii*
   Tentacle number many more than 16 4
4. Tentacles ca. 32, sometimes up to 40; marginal warts usually 96; statocysts ca. 128; umbrella diameter 63–100 mm; without ring of black pigment around umbrella margin ..... *T. formosa*
   Tentacles 30–53; marginal warts 40–115; statocysts 71–141; umbrella diameter at least 20–46 mm; margin with ring of black pigment particles..... *T. nigroannulata*

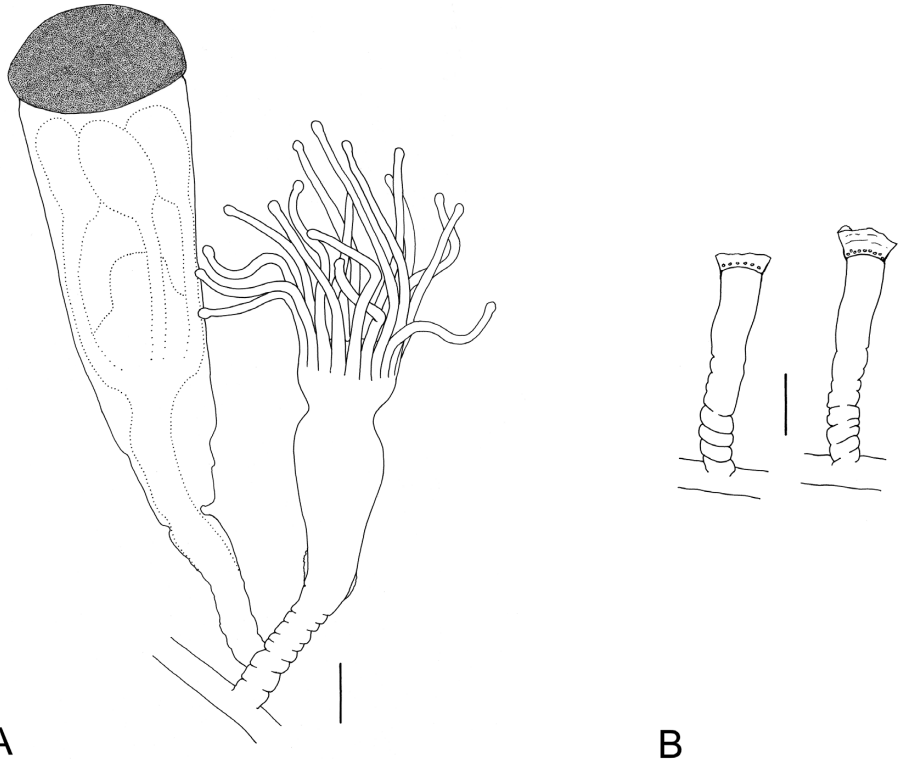


Fig. 8. *Tima nigroannulata*, hydroid stages (paratypes, ROMIZ B5396), Miyazaki, raised at the Enoshima Aquarium from a medusa collected at Miyazaki, April 2020. **(A)** hydranth, and gonotheca with a medusa bud. **(B)** chitinous exoskeletons, showing stolons, hydrothecal pedicels, and vestigial hydrothecae.

Medusae identified as *T. formosa* have been reported from several locations in Japan (Fig. 1), including Oshoro, Hokkaido Prefecture, collected 1922 (Uchida, 1925); Onagawa Bay, Miyagi Prefecture, collected 1936 (Uchida, 1938, 1940); between Katsura-jima Island and Mahanashi-jima Island, Miyagi Prefecture, collected 1931 (Kubota and Yamazaki, 2007); Oarai Fishing Port, Ibaraki Prefecture, collected 2007 (Saito and Shiba, 2008); Lake Hamana (a semi-enclosed brackish water bay), Shizuoka Prefecture, collected sometime between 2005–2015 (Okamoto et al., 2016). Studies undertaken on the species at the Tsuruoka City Kamo Aquarium by Murakami (2016) and Yamamori et al. (2017) were likely based on cultures of medusae from Iwaki, Fukushima Prefecture, established in 2005. In our opinion, these medusae were likely all conspecific with *T. nigroannulata* rather than

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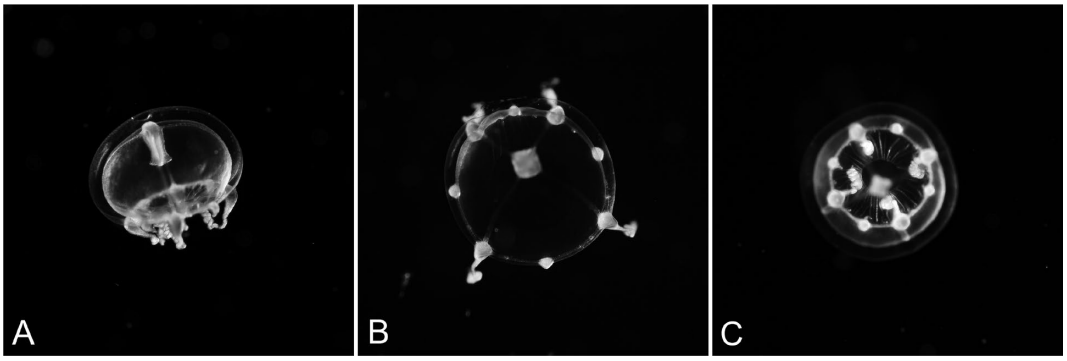


Fig. 9. *Tima nigroannulata*, newly liberated medusae, from hydroids raised at the Enoshima Aquarium from a medusa collected at Miyazaki, April 2020. **(A)** lateral view. **(B)** aboral view. **(C)** oral view (photographs by G. Yamamoto).

the northeast Atlantic *T. formosa*, and the records of them have been included with question in the synonymy list above. In this study, medusae of *T. nigroannulata* are reported from Sendai, Miyagi Prefecture; Iwaki, Fukushima Prefecture; Fujisawa, Kanagawa Prefecture; and Miyazaki, Miyazaki Prefecture (Table 1). Excluded from the synonymy list are records of *T. formosa* from China (e.g., Kao et al., 1958; Chow and Huang, 1958; Zhang, 1982; Xu and He, 1996; Xu et al., 2014; Zheng et al., 2014; Zuo et al., 2016). Based on the phylogenetic tree (Fig. 2), these records were not based on medusae of *T. nigroannulata*.

Medusae of *Tima* Eschscholtz, 1829 are a group of particularly large hydromedusae, accounting for part of their public appeal at aquaria. Those of *T. nigroannulata* available for study ranged from 2.0–4.6 cm in umbrella diameter, although most were juveniles below 3.0 cm. The species likely attains a considerably larger size, with specimens from Oshoro identified as *T. formosa* by Uchida (1925) being 6.0 cm in diameter. In other species of the genus, *T. flavilabris* reaches a diameter of about 8.0 cm (Kramp, 1961), *T. bairdii* a diameter of about 6.5 cm (Kramp, 1961; Cornelius, 1995), *T. formosa* a diameter of 10.0 cm (Mayer, 1910; Petersen, 1962), and *T. saghalinensis* a diameter of 10.0 cm (Kramp, 1961).

Bioluminescence is known to occur in *T. bairdii* (Johnston, 1833: 321; Evans and Ashworth, 1909: 305), and it may occur in others of the genus. No evidence yet indicates that *T. nigroannulata* is bioluminescent (Aya Adachi and Gaku Yamamoto, personal observations), but under black light, bases of the tentacles were shown to emit fluorescence (Fig. 10).

The cnidome of both medusa and hydroid stages of *T. nigroannulata* comprised heteronemes, provisionally identified here as microbasic mastigophores (Fig. 6A–I). No discharged nematocysts were observed in examined material, even after treatment with sodium hypochlorite. Tentacles of the medusa were densely armed with these nematocysts. Their capsules were distinguished as three size classes (small, medium, large), although they approached being a continuum in their size variations. Those of the oral arms, and those of the hydranths, were quite small and much more uniform in length. Nematocysts of the adult medusae appeared to be slightly larger than those of juveniles.

The hydroid stages of *Tima* are poorly known. Besides the hydroid of *T. nigroannulata*, reported herein, that of only one other species has been described. Alexander Agassiz (1865: p. 115, fig. 172) provided a brief description and an illustration of a hydroid raised for 6 months from planulae of the medusa *T. formosa* in Massachusetts, USA. Its hydranth was slender with a reduced hydrotheca, and it bore a distal whorl of about 14 long, slender tentacles. An intertentacular web was present basally. Colonies of the species grew in small tufts on the side of a glass culture vessel. The account by A. Agassiz (1865) generally corresponds with the hydroid of *T. nigroannulata*.

In the shape of their hydranths, in possessing an intertentacular web, and in having hydrothecae reduced to a basal collar, hydroids of *T. nigroannulata* resemble those of several other eirenid species, including *Eirene viridula*

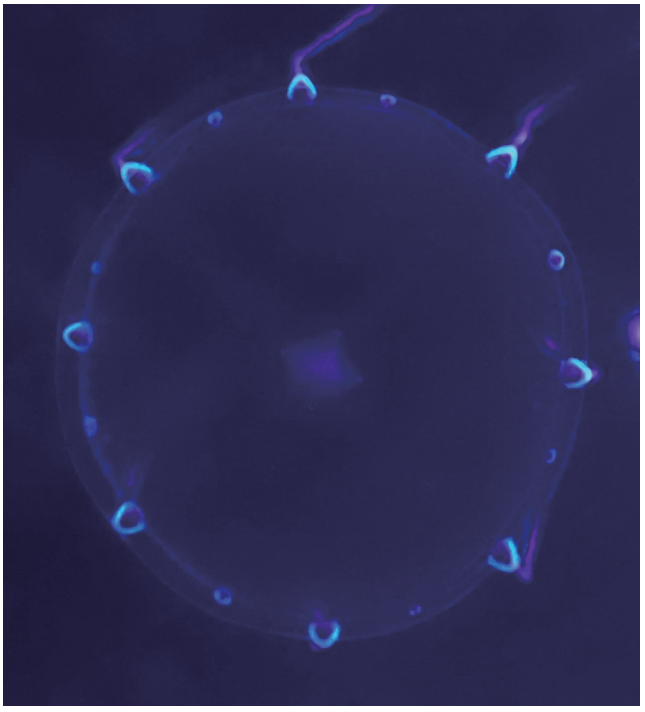


Fig. 10. *Tima nigroannulata*, juvenile medusa (5 mm in diameter), showing fluorescence of tentacle bases illuminated by black light. Specimen liberated from a hydroid raised at the Enoshima Aquarium from a medusa collected at Miyazaki, April 2020 (photograph by G. Yamamoto).

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(Péron and Lesueur, 1810), *Eirene lactea* (Mayer, 1900), *Eutima gracilis* (Forbes and Goodsir, 1853) and *Eutima gegenbauri* (Haeckel, 1864) (see Russell, 1970: pp. 259, 260; Brinckmann-Voss, 1973; Hündgen, 1978; Cornelius, 1995: pp. 227, 229). In these characters, eirenid hydroids thus appear to possess a distinctive morphology.

It is of nomenclatural and systematic significance that the hydroid *Campanulina tenuis* Van Beneden, 1847 is of the same type as those noted above (see Van Beneden, 1847: p. 457, fig. 6). That hydroid is the type species of the genus *Campanulina* Van Beneden, 1847 (not *Campanulina* sensu Allman, 1864 and Hincks, 1869), the type genus of the family Campanulinidae Hincks, 1869. Indeed, the family Campanulinidae, when based on its true type genus, is taxonomically distinct from the long-misdiagnosed and polyphyletic collective group currently assigned that name. That misclassified assemblage is currently a cache of diverse operculate species, many of uncertain identity, rather than being based on a hydroid with a reduced hydrotheca like those of the so-called eirenids (Rees, 1939; Calder, 1991). Nevertheless, current usage of the family name Eirenidae has been maintained here even though Campanulinidae would have been nomenclaturally correct. As knowledge of these hydrozoans advances, rectifications in the taxonomy of the group may be expected.

DNA analysis

The cytochrome *c* oxidase (COI) sequence fragments were aligned by eye and using Clustal X version 2.0 (Larkin et al., 2007). The NCBI GenBank Blastn Suite was used for initial taxonomic confirmation. We tested maximum likelihood fit of 24 nucleotide substitution models for this aligned dataset, and found that GTR + I (general time reversable + invariant sites) was the best fit. Therefore, this model was used to reconstruct phylogenetic trees. Tree topologies remained consistent, with three well-supported ingroup clades regardless of the phylogenetic algorithm used (Fig. 2). Bootstrap support for the three main ingroup clades was strong, ranging from 98% to 100%, depending on the optimality criterion used.

The maximum likelihood molecular phylogenetic reconstruction presented used a single cubozoan outgroup, *Alatina alata* (Reynaud, 1830), and resulted in three ingroup clades, representing three distinct lineages, each with bootstrap support of 100% (Fig. 2). Of the resulting 613 nucleotides per sequence, excluding the outgroup, there were 149 variable positions, 145 of which were parsimony informative and four of which were singleton sites. Including the outgroup, there were 214 variable sites. The overall mean genetic distance was 0.16 or 16%.

Tree topology provides strong evidence for the presence of three distinct, genetically cohesive evolutionary lineages. Clade 1 is comprised of three closely related COI sequences from three individuals sampled along the coast of Japan. These three sequences represent two haplotypes, TNJ2 and TNJ3 from Fukushima, which are identical to one another and differ from TNJ1 from Sendai by six base pairs (less than 1%), forming a strongly supported sister group to Clade 2 (95% bootstrap support). Clade 2 is made up of two sequences from *Tima bairdii*, TBS1 and TBS2, collected in Norway and Sweden, representing two haplotypes that differ by a single basepair. Clade 3 includes four sequences (TFC1–TFC4) that comprise a single haplotype, from samples collected off of coastal China, identified as ?*T. formosa* (Fig. 2; Table 2). The four specimens comprising Clade 3 form the basal ingroup clade; COI sequences were obtained from GenBank (Table 2). Sequences for *T. nigroannulata* were submitted to GenBank to obtain accession numbers (Table 2).

Biogeography

The Japanese Archipelago as we know it today is about 15–20 million years old, extends some 3500 km in length, and is 170 km from the continental mainland of Asia (Barnes, 2003; Neall and Trewick, 2008). The coastline of west-central Japan is influenced by cool waters from the Sea of Japan, ranging from 11–15°C during winter and spring (Fujikura et al., 2010). By contrast, surface water temperatures on the east-central Pacific side of the country are warmer depending on the path of the north-flowing Kuroshio Current, ranging from 13–28°C during winter and spring (Fujikura et al., 2010). Cool water temperatures in the Sea of Japan, and the deep-water basin separating Japan and the continental Asian mainland, may limit the natural dispersal of *T. nigroannulata* beyond its current geographic distribution.

Biogeographic distributions of the two fully documented species of *Tima* reported from the North Pacific Ocean are distinct. *Tima saghalinensis* is a predominantly offshore boreal species, restricted to Russian waters off Sakhalin Island and the Sea of Okhotsk. It has typically been collected at latitudes between 47–59°N, and in water temperatures of 3–15°C (Zavolokin, 2010). *Tima nigroannulata* is a temperate coastal species occurring on the Pacific coast of Japan. Specimens have been collected at four sites from Sendai to Miyazaki, between latitudes 31–37°N, and at a coastal surface water temperature in nature at Enoshima of 15°C. If the record of *Tima formosa* by Uchida (1925) was based on this species, its range extends northwards to southern Hokkaido. Medusae of the species have been maintained in a healthy reproductive state, at 20°C, in cultures at both the Enoshima and Kamo aquaria.

ACKNOWLEDGMENTS

This paper is dedicated to the memory of Professor Tohru Uchida (1897–1981) for his pioneering work on gelatinous plankton in Japanese waters. He was the first to report this species from Japan, as *Tima formosa*, and provided early taxonomic accounts of it. We are grateful to Takako Tamaya and Yoshiki Kawagoe for collecting wild specimens of medusae studied here. Thanks are also due to Seiichi Mizutani at Aquamarine Fukushima for specimens of the hydroid stages of the species. Gratitude is expressed to Dr. Hiroshi Kajihara for thoroughly editing the manuscript. Rae Ding Calder provided translations of some papers in Chinese containing information on species of *Tima* from the Far East. DC acknowledges, with thanks, financial support from the Natural Sciences and Engineering Research Council of Canada and the United States National Science Foundation program Partnerships for Enhancing Expertise in Taxonomy (PEET). GC acknowledges the support of Ocean Research Explorations, along with that of the Holland Laboratory and the Hawaiian Biodiversity and Research Laboratory at Hawaii Pacific University. This is Ocean Research Explorations publication number 006.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

DRC wrote the section on systematics, prepared line drawings and photomicrographs, organized the References, edited the manuscript, and approved the final draft. GLC conceived the project, coordinated different parts of it, obtained specimens, authored or reviewed drafts of the paper including a section on biogeography, and approved the final draft. SI, AA, and GY provided specimens and images for study, and approved the final draft. AH generated molecular data. BSH prepared the map, aligned and edited DNA sequences, performed analyses on the molecular results, and approved the final draft.

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