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16S MITOCONDRIAL GENE SEQUENCE ANALYSIS OF SOME *Turritopsis* (HYDROZOA, OCEANIDAE) FROM JAPAN AND ABROAD

By

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Abstract

16S mitocondrial gene sequence analysis using eight individuals prepared in the present study from three countries (Israel, Italy, Japan) revealed and confirmed that there are five species in *Turritopsis* in the world, and among which three species (*Turritopsis rubra, T.* sp. and *T. dohrnii*) are distributed in Japan and one of them (*T.* sp.) is endemic and distributed in central Japan. Among the species distributed in the Pacific Ocean, *T.* sp. is a sister species of the Chinese species (*T. lata*) that might be another endemic one, while *T. rubra*, that has a brooding habit, is more remote species than the Atlantic and the Mediterranean species (*T. nutricula* and *T. dohrnii*). The present analysis suggests that *T. dohrnii* is found in Israel in addition to Okinawa Island, Japan, possibly as another introduction example.

Introduction

Two molecular species of *Turritopsis*, that is well-known as an immortal jellyfish (Hasegawa *et al*. 2016), is distributed in southern Japan, one from Wakayama and Kagoshima Prefectures as *T.* sp. that is assignable to a new species in the future and probably endemic to Japan, and the other from Okinawa Island as *T. dohrnii* that is thought to be an introduced species from the Mediterranean (Miglietta *et al.* 2007; Miglietta & Lessios 2009; Kubota 2015). In the present paper, *Turritopsis* specimens were collected from three countries such as Italy, Israel and Japan, and cultured for up to five years in the laboratory of the Seto Marine Biological Laboratory, Kyoto University in Japan. However, their morphological discrimination is very difficult due to mutual very similar morphology (Kubota 2005; 2015; Kubota & Niina 2014), therefore to determine their systematic position the present molecular analysis was

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carried out, and some phylogenetic considerations are done with emendations.

Materials and methods

All the materials newly used were collected and cultured by Shin Kubota (#: Fig. 1, in Italy and Israel obtained during the international Workshop). Present materials analyzed are young medusae, polyps, rejuvenated young polyps from immature medusae collected and cultured as follows: 1 female polyp zooid rejuvenated 12 or 13 times originated from an immature medusa collected from Okinawa Island, Japan in May, 2009 (cf. Kubota 2011; as Okinawa_2 for this material, see Table 1 and Fig. 1); 1 rejuvenated (twice) polyp zooid cultured in the laboratory from a medusa found in a vessel in cultured vessel for Shirahama colony in Tanabe Bay, Wakayama Prefecture, Japan in August, 2010 (Shirahama, but emended as below and actually from Okinawa Island: Fig. 1, Okinawa_1); 1 polyp zooid cultured in the laboratory after metamorphosed by a planula larva born from medusae collected in Tanabe Bay, Wakayama Prefecture, Japan in August, 2015 (Shirahama_15-8); 1 immature medusa collected from Tanabe Bay, Wakayama Prefecture, Japan in July, 2016 (Shirahama_16-7); 1 rejuvenated polyp zooid cultured in the laboratory originated from immature medusa collected in Tomari Port, Okinawa Prefecture, Japan in April, 2016 (Okinawa_16-4); 1 polyp zooid cultured in the laboratory collected in Eilat, Israel in December 2013 (Israel); 1 colony collected in Porto Cesareo, Italy in September, 2010 (Kubota & Gravili 2011) and cultured in Shirahama, Japan. From this colony, that grows very well, spreading all over the surface of the rearing vessel, the first release of their medusae took five years after collection (Kubota 2015) (Porto Cesareo, Italy); 1 rejuvenated polyp zooid cultured in the laboratory originated from a polyp collected in Ischia Island, Italy in June, 2015 (Italia_15-6).

All the polyps, that attached to 60 cc polystyrene vessel (60 mm in diameter; 20 mm high), were maintaind in steady water flow of a natural seawater (c. 32 psu) in a running system, fed with the newly hatched *Artemia* nauplii in the laboratory of the Seto Marine Biological Laboratory, Shirahama, Wakayama Prefecture, Japan, removing algae and other macroscopic organisms that grew on the bottom of the rearing vessels by frequent cleaning by a wooden stick with a pointed tip. All the materials used for molecular analysis are preserved in 95% ethanol after starvation and registered in GenBank under accession numbers* (Table 1: LC36118-361125).

Genomic DNA was extracted from the 95% ethanol preserved tissue of specimens using the QuickGene DNA tissue kits (FUJI FILM, Tokyo, Japan) according to the manual instruction. The target gene (mitochondrial 16S) was amplified using primers SHA and SHB (Cunningham & Buss 1993). The PCR was performed using a thermal cycler (PC-808; ASTEC, Fukuoka, Japan) in a reaction mixture (25 μL) containing 1.0 μL template DNA; 0.2 mM of each dNTP; 1× PCR buffer; 1.5 mM Mg^{2+}; 1.0 U KOD-Plus-ver.2 (TOYOBO, Osaka, Japan),

Table 1. List of species in *Turritopsis* analyzed in phylogenetic trees (Fig. 1) with corresponding sequence names in Genbank, resulting clade, collection localities, and Genbank accession numbers (*: present materials used).

Sequence Names in Genbank	Resulting clade	Collection site	16S GenBank accession Number
Turritopsis dohrnii	*Turritopsis dohrnii*	Panama, Bocas del Toro	EU624355
Turritopsis dohrnii	*Turritopsis dohrnii*	Japan, Okinawa Island	EU624360
Turritopsis dohrnii	*Turritopsis dohrnii*	USA, Florida, Fort Pierce	EU624353
Turritopsis dohrnii	*Turritopsis dohrnii*	Italy, Apulia	EU624363
Turritopsis dohrnii	*Turritopsis dohrnii*	Italy, Apulia	EU624364
Turritopsis dohrnii	*Turritopsis dohrnii*	Italy, Apulia	EU624365
Turritopsis dohrnii	*Turritopsis dohrnii*	Japan, Okinawa Island, Okinawa	EU624366
Turritopsis dohrnii	*Turritopsis dohrnii*	Japan, Okinawa Island, Okinawa	EU624368
Turritopsis dohrnii	*Turritopsis dohrnii*	Japan, Okinawa Island, Okinawa_1	LC361118*
Turritopsis dohrnii	*Turritopsis dohrnii*	Japan, Okinawa Island, Tomari Port, Okinawa_2	LC361122*
Turritopsis dohrnii	*Turritopsis dohrnii*	Japan, Okinawa Island, Tomari Port, Okinawa 16-4	LC361123*
Turritopsis dohrnii	*Turritopsis dohrnii*	Italy, Porto Cesareo off Naples	LC361120*
Turritopsis dohrnii	*Turritopsis dohrnii*	Italy, Ischia Island off Naples 15-6	LC361121*
Turritopsis dohrnii	*Turritopsis dohrnii*	Israel, Eilat, Aquba Bay	LC361119*
Turritopsis nutricula	*Turritopsis nutricula*	USA, MA, Woods Hole	EU624348
Turritopsis nutricula	*Turritopsis nutricula*	USA, MA, Woods Hole	EU624349
Turritopsis sp.	*Turritopsis* sp.	Japan, Kagoshima, Kyushu	EU624375
Turritopsis sp.	*Turritopsis* sp.	Japan, Kagoshima, Kyushu	EU624376
Turritopsis sp.	*Turritopsis* sp.	Japan, Kagoshima, Kyushu	EU624377
Turritopsis sp.	*Turritopsis* sp.	Japan, Tanabe Bay	EU624378
Turritopsis sp.	*Turritopsis* sp.	Japan, Tanabe Bay, Shirahama, Wakayama 16-7	LC361124*
Turritopsis sp.	*Turritopsis* sp.	Japan, Tanabe Bay, Shirahama, Wakayama 15-8	LC361125*
Turritopsis rubra	*Turritopsis rubra*	Australia, Tasmania, Hobart	AM183134
Turritopsis rubra	*Turritopsis rubra*	New Zealand, Wellington Harbour	EU624380
Turritopsis rubra	*Turritopsis rubra*	New Zealand, Hauraki Gulf	EU624382
Turritopsis rubra	*Turritopsis rubra*	New Zealand, Hauraki Gulf	EU624383
Turritopsis rubra	*Turritopsis rubra*	Japan, Fukushima Prefecture	EU624384
Turritopsis rubra	*Turritopsis rubra*	Japan, Fukushima Prefecture	EU624386
Turritopsis lata	*Turritopsis lata*	China, Xiamen	JX965914
Turritopsis lata	*Turritopsis lata*	China, Xiamen	KF962530
Turritopsis lata	*Turritopsis lata*	China, Xiamen	KF962531
Bougainvillia triestina		Croatia	KJ660344
Rathkea octopunctata		Norway	AM411415

which has intensive 3′ → 5′ exonuclease activity; and 1.0 μM of each primer. The PCR cycling conditions were as follows: initial denaturation at 94°C for 3 min, followed by 30 cycles at 94°C for 15 s, 52°C for 30 s, and 68°C for 40 s. PCR amplification was verified by 1.5%

agarose gel electrophoresis. The PCR products were transformed into DH5α cells (Promega, Madison, WI, USA) after ligation into the pGEM T-Easy Vector (Promega). The plasmid DNAs were purified after color selection. DNA sequences were determined using Dynamic ET terminator cycle sequencing kit (GE Healthcare, Little Chalfont, UK) in combination with M13 Reverse and U19 primers and analyzed on a DNA sequencer (ABI3730, Applied Biosystems, Foster City, CA, USA).

The mitochondrial 16S region, excluding the primer regions, of *Turritopsis* was aligned with the sequences of other *Turritopsis* species obtained from GenBank using the ClustalX package (Thompson *et al.* 1997) and edited manually (459 bp). The mitochondrial 16S region was aligned with 8 isolates of 25 other *Turritopsis* species or individuals with *Bougainvillia triestina* and *Rathkea octopunctata* as outgroup sequences. These sequences were selected at random and were aligned with the representative sequences in each OTU obtained in the present study using the Clustal W algorithm in Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 (Kumar *et al.* 2016), using default settings. The target sequences, all of which were susceptible to amplification using the primer pair, were edited manually. Primer regions were removed during the editing process. An optimal base substitution model was calculated using the default settings of Modeltest in MEGA, based on which T92 + G was chosen as the best-fit model. All sites, including indels were used for model selection and phylogenetic tree construction. A maximum likelihood (ML) tree was generated using the default settings in MEGA. Bootstrapping with 100 replications was used to estimate the reliability of the phylogenetic tree generated in this way.

Results and Discussion

The present molecular tree (Fig. 1) acccords with the former tree (Miglietta *et al.* 2007; Miglietta & Lessios 2009), separating five molecular species, and among which three species are distributed in Japan, i.e. *Turritopsis rubra, T.* sp., and *T. dohrnii.* The noticeable group is ditributed in China as *T. lata* as a sister group of *T.* sp., that is distributed in central Japan, excluding one specimen grouped together with *T. rubra* and another specimen cultured in Shirahama (Okinawa_1: Fig. 1) that grouped with *T. dohrnii.* The latter problematic case is explainable here and emended its locality as Okinawa Island since this medusa is misidetified as *T.* sp. This medusa was found in the culture dish in the Shiarahma colony in a culture tank as a shrinked medusan body due to dilution of seawater by typhoon effect, and thought to be its medusa released from this colony (Kubota & Niina 2014).

Among the species distributed in the Pacific Ocean, *T.* sp. is, as mentioned above, a sister species of the Chinese species (*T. lat*a) that may be another endemic species in China. On the other hand, *T. rubra* is distributed in northern Japan (Kubota 2005) and bred planulae on the manubrium (Kubota *et al.* 2005), and is more remote and/or ancestral species than the Atlantic

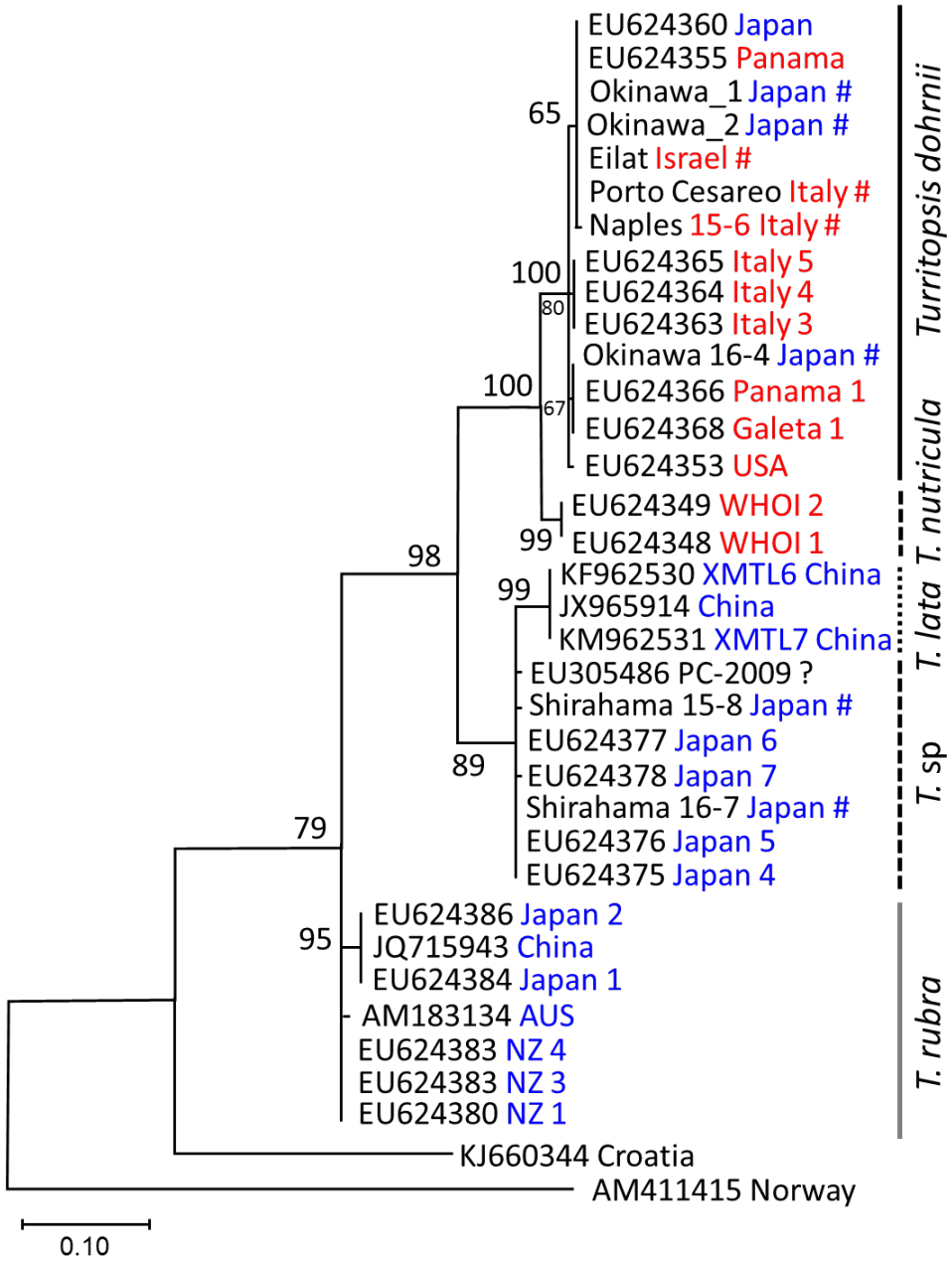

Fig. 1. Maximum likelihood 16S mitocondrial phylogenetic tree of *Turritopsis* in the world. Samples from the Pacific Ocean (blue) or from the Mediterranean Sea and the Atlantic Ocean (red). #: The present materials prepared after collection and cultured.

and the Mediterranean Sea species (*T. nutricula* and *T. dohrnii*) that are closely related with each other than others (Fig. 1).

The present analysis suggests that *T. dohrnii* is found in Israel in addition to Okinawa Island, Japan (Fig. 1), showing another introduction example. Further survey in many materials from various localities is needful to strengthen the present considerations, together with more detailed morphological studies on mature medusa of all species to discriminate species.

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