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Short communication

DNA Barcoding of a Colonial Ascidian, *Botrylloides violaceus* (Ascidiacea: Stolidobrachia: Styelidae), from South Korea

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ABSTRACT

Botrylloides violaceus is native to the Northwest Pacific, including Korea. This species has many color variations in alive condition and a variety of zooid compound forms, and therefore difficult to identification in the field survey. This is the first report of *COI* DNA barcodes of *B. violaceus* from Korea. The intra-specific pairwise distance between Korean and UK populations had ranged from 1.4% to 2.6%. The inter-specific variations between *B. violaceus* and other *Botrylloides* species were 21.0–36.8%. The new DNA barcodes for Korean *B. violaceus* may be helpful in the identification of colonial ascidians, which is a difficult task when based on morphological identification.

Keywords: DNA barcoding, *COI*, colonial ascidian, Styelidae, *Botrylloides*

INTRODUCTION

The genus *Botrylloides* Milne Edwards, 1841 is one of popular colonial ascidians in the world and currently comprises 19 species (Shenkar et al., 2020). Among them, two species have been reported in South Korea: *B. magnicoecum* (Hartmeyer, 1912) and *B. violaceus* Oka, 1927 (Rho, 1977; Rho et al., 2000). *Botrylloides violaceus* is native to the Northwest Pacific, including Korea (Cohen, 2011), but has also been reported as a non-indigenous species (NIS) from western North America and Europe (Gittenberger and Moons, 2011; Simkanin et al., 2013; Viard et al., 2019). The lack of ascidian-specialized taxonomists and the consequent frequent misidentification of samples have been among the major limiting factors in the ability to detect NIS of ascidians (Izquierdo-Muñoz et al., 2009). The identification of ascidians (especially colonial ascidians) based on morphological taxonomy is a difficult task owing to many limitations, particularly of the colonial form, and this has led to misidentification of the species (Lambert, 2009; Geller et al., 2010). The efficiency of DNA barcoding was confirmed for the identification of several ascidians and resolved the limitations of identification through conventional taxonomy (Akram et al., 2017). In this study, we obtained partial sequences of cytochrome *c* oxidase subunit I (*COI*) from *B. violaceus* in South Korea, where is a part of the type locality for this species, and compared them with *COI* sequences of *B. violaceus* from UK and other *Botrylloides* species retrieved from GenBank.

The colonies were collected from artificial material (resin acrylic plate) in six harbors of South Korea and labelled each of the collected colonies: Dangjin, Gunsan, Incheon, Jukbyeon, Ulsan, and Yeosu (Fig. 1). All examined colonies were deposited in 95% ethyl alcohol solution at the Marine Biological Resource Institute of Sahmyook University. We the observed morphological characteristics using a microscope and identified as *B. violaceus* based on Rho (1977) and Tokioka (1967). Total genomic DNA was extracted from a single zooid in a colony by following the DNeasy Blood and Tissue kit protocol (Qiagen, Hilden, Germany). The partial sequences of *COI* were amplified using primer pairs as follows: LCO1490-HCO2198 (Folmer et al., 1994) and dinF-Nux1R (Brunetti et al., 2017). The polymerase chain reaction was performed on a 20.0 μL total reaction volume (AccuPower PCR PreMix & Master Mix; Bioneer, Seoul, Korea) including 1.0 μL of each primer (10 mM) and 0.5 μL of DNA template (>50 ng/μL), using the following thermal cycling profile: one cycle at 94°C for 3 min; 35 cycles of 94°C for 30 s, 50°C for 45 s, and 72°C for 60 s; and a 7 min final extension at 72°C. The pairwise

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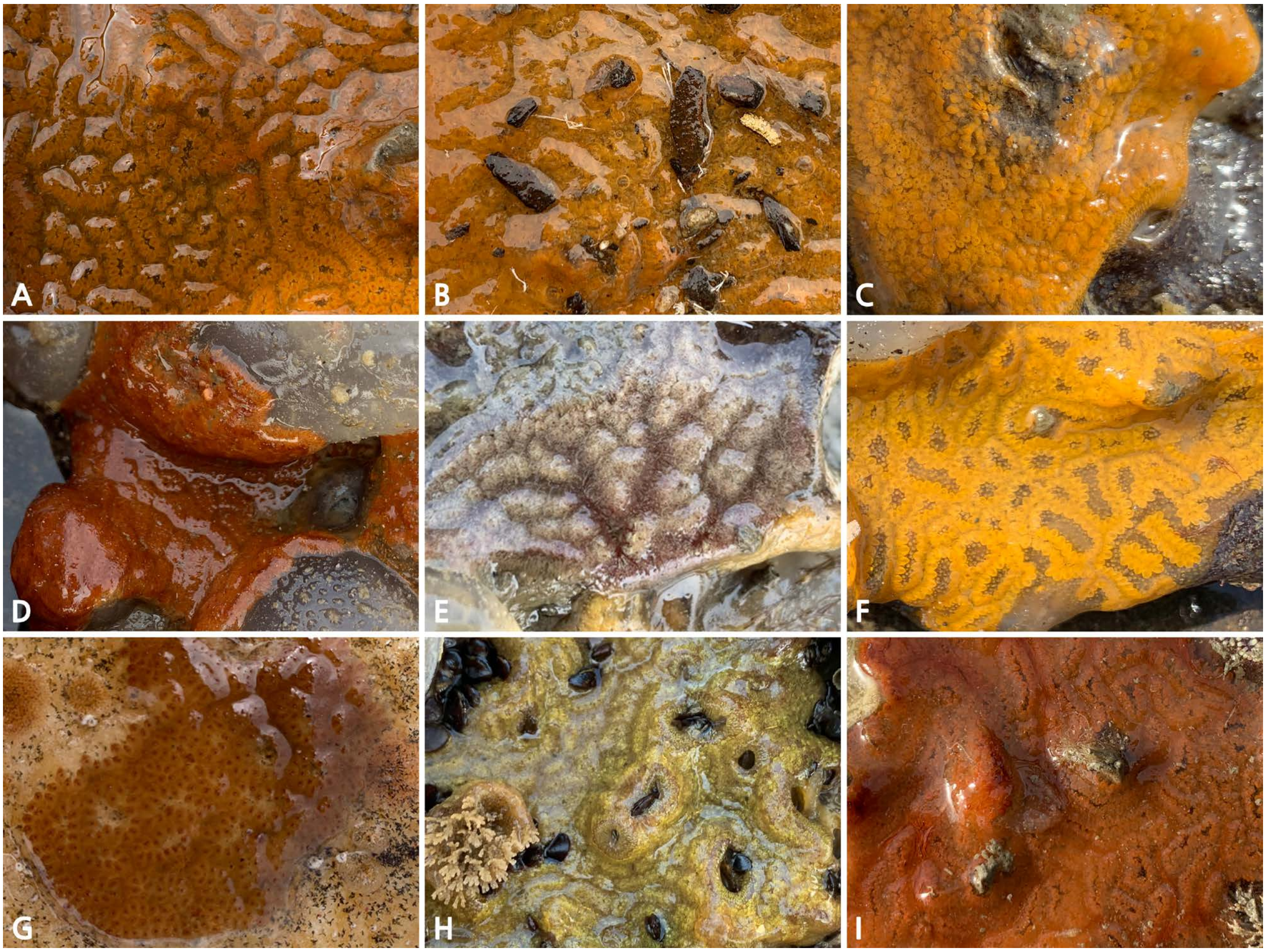

Fig. 1. Photographs of various forms of *Botrylloides violaceus* from different areas of South Korea: A, Dangjin; B, Gunsan; C–F, Incheon; G, Jukbyeon; H, Ulsan; I, Yeosu.

distance (p-distance) was calculated using the Kimura 2-parameter model (K2P) (Kimura, 1980) and neighbor-joining (NJ) tree was constructed with 1,000 bootstrapping replicates using the K2P in MEGA 7.0 (Kumar et al., 2016).

RESULTS AND DISCUSSION

Botrylloides violaceus had many variations in zooid compound form and color in alive condition, and therefore difficult to identification immediately in the field survey. In this study, we labeled each colony by color and all labeled colonies were identified as *B. violaceus* based on morphological characteristics (Table 1). We obtained newly 10 partial *COI* sequences of Korean *B. violaceus*, which were 672 bp or 858 bp (Table 1). The variations within Korean and UK populations were 0.0–2.6% and 0.2–0.9%, respectively (Table 2). And, the intra-specific variations between Korean and UK populations were 1.4–2.6%, which were slightly higher than those of other *Botrylloides* species, 0.2–0.9% for *B. diegensis*, 0.2–0.5% for *B. leachii* and 1.4% for *B. nigrum* (Table 2). The inter-specific variations between *B. violaceus* and other seven *Botrylloides* species were 21.0–36.8% (Table 2). Based on the NJ tree, although they showed various colors and zooid compound forms, all *COI* barcodes of *B. violaceus* formed a single clade separated from other *Botrylloides* species (Fig. 2). This study contributes in identifying *B. violaceus* and understanding taxonomic relationship of *Botrylloides* species.

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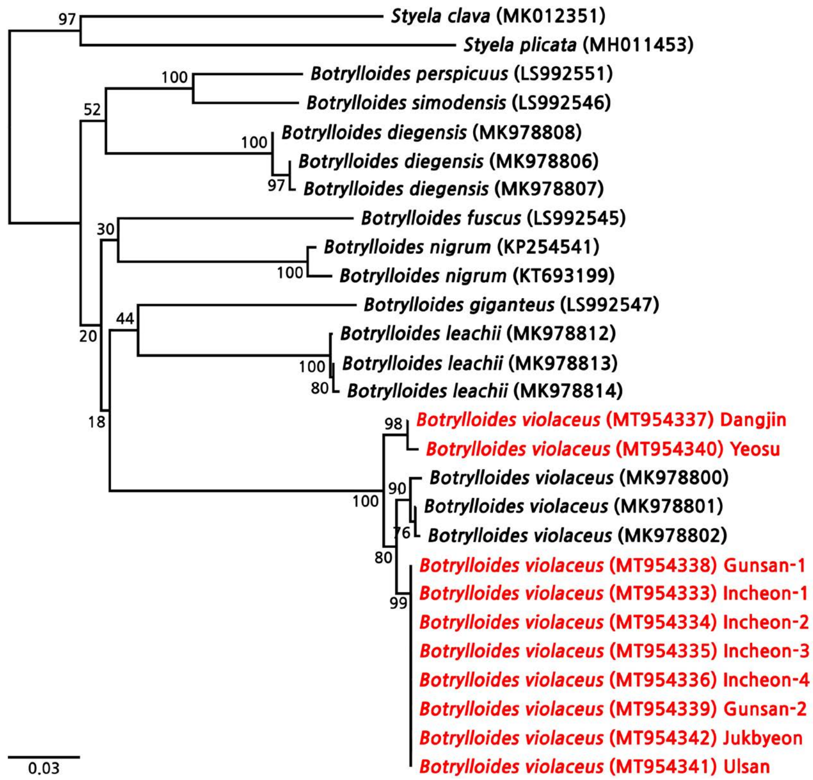

Fig. 2. Phylogenetic tree constructed using the neighbor-joining method based on the Kimura 2-parameter model, with 1,000 bootstrap replicates. Bootstrapping support values are indicated on each node.

Table 1. Sampling information, GenBank accession number, and assembled sequences length of *Botrylloides violaceus* in this study

Location	GPS	Collection date	Accession No.	Color of colony	Sequence length (bp)	Primers
(1) Incheon	37.462473N, 126.620434E	11 May 2020	MT954333 MT954334 MT954335 MT954336	Orange Reddish brown Pale purple Orange	672	LCO1490-HCO2198
(2) Dangjin	36.986826N, 126.746202E	11 May 2020	MT954337	Orange	672	LCO1490-HCO2198
(3) Gunsan	35.935197N, 126.527395E	11 May 2020	MT954338 MT954339	Orange Reddish brown	672 858	LCO1490-HCO2198 dinF-Nux1R
(4) Yeosu	34.742078N, 127.755551E	12 May 2020	MT954340	Brown	672	LCO1490-HCO2198
(5) Ulsan	35.511124N, 129.386146E	13 May 2020	MT954341	Yellow	858	dinF-Nux1R
(6) Jukbyeon	37.054902N, 129.423784E	14 May 2020	MT954342	Brown	858	dinF-Nux1R

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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Table 2. Pairwise distances (%) within eight species of *Botrylloides* from South Korea and GenBank, based on the Kimura 2-parameter model

	Species	Accession No.	Locality	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	References
1	*B. violaceus*	MT954333	Incheon, Korea																										This study
2		MT954334	Incheon, Korea	0.0																									This study
3		MT954335	Incheon, Korea	0.0	0.0																								This study
4		MT954336	Incheon, Korea	0.0	0.0	0.0																							This study
5		MT954337	Dangjin, Korea	2.1	2.1	2.1	2.1																						This study
6		MT954338	Gunsan, Korea	0.0	0.0	0.0	0.0	2.1																					This study
7		MT954339	Gunsan, Korea	0.0	0.0	0.0	0.0	2.1	0.0																				This study
8		MT954340	Yeosu, Korea	2.6	2.6	2.6	2.6	0.5	2.6	2.6																			This study
9		MT954341	Ulsan, Korea	0.0	0.0	0.0	0.0	2.1	0.0	0.0	2.6																		This study
10		MT954342	Jukbyeon, Korea	0.0	0.0	0.0	0.0	2.1	0.0	0.0	2.6	0.0																	This study
11		MK978800	UK	1.6	1.6	1.6	1.6	2.6	1.6	1.6	3.1	1.6	1.6																Viard et al. (2019)
12		MK978801	UK	1.4	1.4	1.4	1.4	2.3	1.4	1.4	2.8	1.4	1.4	0.7															Viard et al. (2019)
13		MK978802	UK	1.6	1.6	1.6	1.6	2.6	1.6	1.6	3.1	1.6	1.6	0.9	0.2														Viard et al. (2019)
14	*B. diegensis*	MK978806	UK	23.1	23.1	23.1	23.1	22.5	23.1	23.1	22.4	23.1	23.1	23.1	23.2	22.8													Viard et al. (2019)
15		MK978807	UK	23.5	23.5	23.5	23.5	22.8	23.5	23.5	22.8	23.5	23.5	23.5	23.5	23.2	0.2												Viard et al. (2019)
16		MK978808	UK	22.2	22.2	22.2	22.2	21.5	22.2	22.2	21.5	22.2	22.2	22.2	22.2	21.9	0.7	0.9											Viard et al. (2019)
17	*B. fuscus*	LS992545	Japan	23.2	23.2	23.2	23.2	22.9	23.2	23.2	23.5	23.2	23.2	25.0	24.3	24.0	19.6	19.9	19.0										Rocha et al. (2019)
18	*B. giganteus*	LS992547	Brazil	22.6	22.6	22.6	22.6	23.3	22.6	22.6	24.0	22.6	22.6	23.3	22.7	22.3	19.1	19.4	18.7	22.5									Rocha et al. (2019)
19	*B. leachii*	MK978812	UK	22.0	22.0	22.0	22.0	22.3	22.0	22.0	22.3	22.0	22.0	22.6	22.0	21.7	17.5	17.8	17.2	19.7	17.2								Viard et al. (2019)
20		MK978813	UK	22.0	22.0	22.0	22.0	22.3	22.0	22.0	22.3	22.0	22.0	22.6	22.0	21.7	17.5	17.8	17.2	19.4	17.2	0.2							Viard et al. (2019)
21		MK978814	UK	22.3	22.3	22.3	22.3	22.6	22.3	22.3	22.6	22.3	22.3	23.0	22.3	22.0	17.8	18.1	17.5	19.1	17.6	0.5	0.2						Viard et al. (2019)
22	*B. nigrum*	KP254541	USA	21.6	21.6	21.6	21.6	21.0	21.6	21.6	21.0	21.6	21.6	22.0	21.7	22.0	18.0	18.3	17.1	18.6	21.2	19.6	19.6	20.0					Leray and Knowlton (2015)
23		KT693199	India	22.9	22.9	22.9	22.9	22.3	22.9	22.9	22.3	22.9	22.9	23.3	23.0	23.3	17.4	17.7	16.5	18.3	21.5	19.6	19.6	19.9	1.4				Unpublished
24	*B. perspicuus*	LS992551	Australia	23.5	23.5	23.5	23.5	22.5	23.5	23.5	22.4	23.5	23.5	23.5	23.5	23.2	15.5	15.5	14.6	18.0	24.2	20.9	20.9	21.2	17.9	18.1			Rocha et al. (2019)
25	*B. simodensis*	LS992546	Japan	22.8	22.8	22.8	22.8	22.1	22.8	22.8	22.8	22.8	22.8	23.5	23.5	23.2	16.4	16.4	15.5	18.7	21.5	22.9	22.9	23.2	18.1	18.4	9.0		Rocha et al. (2019)

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