

A newly recorded brittle star, *Amphiura* (*Amphiura*) *digitula* (H.L. Clark, 1911) (Ophiuroidea: Amphilepidida: Amphiuridae), from Geoje Island, Korea

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We describe a newly recorded brittle star to South Korea, *Amphiura* (*Amphiura*) *digitula* (H.L. Clark, 1911), that was collected from Geoje Island, at a depth of 47 m. The species is characterized by a small disk, covered by numerous fine scales, small radial shields that are wider than long, a small stumpy hook at the distal end of the radial shield, two tooth papilla, two adoral shield spines, 2nd adoral shield spine longer than other, tapered dramatically toward dull tip, five arms with four proximal arm spines, and two tentacle scales. We also obtained a 657 bp sequence from *COI* gene and the amplified sequence matched the general DNA barcoding region. The NJ and ML phylogenetic analyses revealed *A. (A.) digitula* as monophyletic in the *Amphiura* clade. This species is clearly distinguished from other *Amphiura* species by morphological characteristics and the mitochondrial *COI* sequence, and thus represents the sixth *Amphiura* species reported to occur in Korea.

Keywords: Echinodermata, mitochondrial *COI*, morphology, ophiuroid, taxonomy

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DOI:10.12651/JSR.2020.9.3.273

INTRODUCTION

Amphiura Forbes, 1843 is one of the larger and more diverse genera in Amphiuridae Ljungman, 1867 (Stöhr *et al.*, 2020). It is generally characterized by a disk covered in fine, flattened imbricating scales, a lack of disk papillae, and arms very long and slender (Clark, 1970; Shin, 2012). Previous studies reported five *Amphiura* species in Korea (Yi and Irimura, 1987; Shin, 2012), including *A. koreae* Duncan, 1879, *A. iridoides* Matsumoto, 1917, *A. (Fellaria) sinicola* Matsumoto, 1941, *A. (F.) vadicola* Matsumoto, 1915, and *A. (Ophiopeltis) aestuarii* Matsumoto, 1915. These species inhabit soft substrates, such as clay, sandy clay, or sand, and are primarily distributed in the Yellow Sea, the Korean Strait, and around Jeju Island.

DNA barcoding is the analysis of sequence variation in a 658 bp region of the mitochondrial cytochrome c oxidase subunit 1 gene (*COI*) to determine specimen identification or to discover new species (Hebert *et al.*, 2003). The *COI* marker has also been used for population genetics studies of Ophiuroidea and other echinoderm groups (Laakmann *et al.*, 2016; Layton *et al.*, 2016; Boissin *et al.*, 2017; Knott *et al.*, 2018; Pawson, 2018).

An integrative approach to taxonomy has emerged as a powerful and necessary means for assessing species boundaries and diversity (Puillandre *et al.*, 2012). Molecular genetic approaches have enabled more detailed descriptions of cryptic and sibling species compared to morphology-based approaches (Laakmann *et al.*, 2016). The aim of this study was to redescribe the brittle star, *A. (A.) digitula*, by analyzing the morphological characteristics with scanning electron microscopy (SEM) and sequencing the *COI* gene for DNA barcoding.

MATERIALS AND METHODS

Samples were collected from the waters around Geoje Island, located along the southern coast of South Korea. The samples were collected via the Smith-McIntyre grab method, carefully removed from the collected substrate, and then immediately preserved in ethyl alcohol (>95%). The sorted samples were deposited in the National Institute of Biological Resources (Incheon, Korea). The

samples for observation were lightly bleached following Stöhr *et al.* (2012), dried following Lee and Shin (2019), and examined by scanning electron microscopy (SEM; JSM-microscopes 6510; JEOL, Tokyo, Japan).

Total genomic DNA was extracted from arm tissue, following the DNeasy kit protocol (Qiagen, Hilden, Germany), and partial *COI* sequences were amplified using an echinoderm-specific primer pair: LCOech1aF1 (Layton *et al.*, 2016) and HCO2198 (Folmer *et al.*, 1994). Polymerase chain reactions (PCR) were executed with 25.0 µL reaction volumes (20.0 µL AccuPower® PCR PreMix & Master Mix (Bioneer, Seoul, Korea), 1.5 µL of each primer (10 mM), and 2.0 µL of DNA template (> 50 ng/µL)) using a thermocycling profile of one cycle at 94°C for 1 min, 35 cycles of 94°C for 45 sec, 52°C for 45 sec, and 72°C for 1 min, and a 7 min extension at 72°C. Amplicon quality was assessed by gel electrophoresis on 1.0% agarose gel and via a NanoDrop 1000 (Thermo Scientific, Waltham, MA, USA). The amplified products were sequenced with ABI Big Dye Terminator kits (Applied Biosystems, Foster City, CA, USA) and an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA).

For the phylogenetic analyses, we used 21 sequences of *Amphiura* (*Amphiura*) species and two species of *Ophiura* (*O. albida* and *O. luetkeni*), two asteroids (*Aphelasterias japonica* and *Asterias amurensis*), and two crinoids (*Florometra serratissima* and *Phanogenia gracilis*), which served as the outgroups for all analyses (Table 1). The phylogenetic analyses for each dataset were conducted via two methods: neighbor joining (NJ) in MEGA7 (Kumar *et al.*, 2016) with the Kimura 2-parameter (K2P) (Kimura, 1980) and maximum likelihood (ML) in RAxML 8.2 (Stamatakis, 2014). The best-fit nucleotide substitution model for ML was estimated with jModelTest 2.1.1 (Guindon and Gascuel, 2003; Darriba *et al.*, 2012), and the best-fit model was GTR + I + G. ML analyses were performed with rapid bootstrapping and searches for the best-scoring ML tree algorithm, with 1000 bootstrap replicates. The pairwise distance estimates of interspecific sequence divergence were calculated using the K2P distance model (Kimura, 1980).

TAXONOMIC ACCOUNT

Superorder Ophintegrida O'Hara, Hugall, Thuy, Stöhr and Martynov, 2017

Order Amphilepidida O'Hara, Hugall, Thuy, Stöhr and Martynov, 2017

Suborder Gnathophiurina Matsumoto, 1915

Superfamily Amphiuroidea Ljungman, 1867

Family Amphiuridae Ljungman, 1867

Genus *Amphiura* Forbes, 1843

Key to species of the genus *Amphiura* from Korea

1. One or two tentacle scales present 2
– Tentacle scales absent 3
2. Three proximal arm spines. No projection at distal end of radial shield *A. koreae*
– Four proximal arm spines. Projection situated at distal end of radial shield with stumpy hooks at outermost *A. (Amphiura) digitula*
3. Dorsal arm plates rudimentary or very small at free arm joints. Arms very long, more than 20 times longer than disk diameter *A. (Fellaria) vadicola*
– Dorsal arm plates small or large at free arm joints. Arms very long, 17 to 18 times longer than disk diameter 4
4. Six or seven arm spines present at free arm joints. Dorsal arm plate small *A. (Fellaria) sinicola*
– Four or five arm spines present at free arm joints. Dorsal arm plate very large *A. (Ophiopeltis) aestuarii*

Amphiura (Amphiura) digitula

(H.L. Clark, 1911) (Fig. 1)

뽕족니턱뱀거미불가사리 (신칭)

Amphiodia digitula H.L. Clark, 1911: 162.

Amphiura leptopholida H.L. Clark, 1915: 226.

Amphiura digitula Matsumoto, 1917: 199; D'yakonov, 1954: 71; A.M. Clark, 1965: 49.

Diamphiodia digitula Fell, 1962: 14.

Amphiura (Amphiura) digitula: Stöhr *et al.*, 2020: 243028.

Material examined. Four specimens, Geoje Island (34° 44'41"N, 128°40'01"E), 27 August 2017, Lee T., 47 m depth by grab (NIBRIV0000863921, NIBRIV0000863922, NIBRIV0000863926, NIBRIV0000863927).

Description. Disk small (diameter: 5.2–7.3 mm), slightly concave at interradii and covered by numerous fine scales (Fig. 1A). Arms long (length: 25.0–35.1 mm), approximately 4.8 times longer than disk diameter. Disk scales circular form, overlapping, with indistinguishable primary scales (Fig. 1A). Radial shield small (Fig. 1A), longer than wide (Fig. 1B), separated by disk scales proximally but connected distally (Fig. 1B). Small projection on distal end of radial shield, stumpy hook situated at outermost of projection, hook tip divided into three or four (Fig. 1B). Oral shield ovoid form, slightly longer than wide, more angular distally than proximally (Fig. 1C, D). Madreporite shield more rounded than others (Fig. 1C). Tooth papilla one and two adoral shield spines on an adoral shield (Fig. 1D). The 2nd adoral shield spine longer than other, tapered dramatically toward dull tip (Fig. 1D). Dorsal arm plate rounded triangular, proximal end more angular than distal end (Fig. 1E). Lateral arm plate wider than long (Fig. 1E). Arm spines four.

Table 1. Species, GenBank accession numbers, and lengths of the *COI* sequences used in this study.

Classification	GenBank accession No.	Length (bp)	References
Class Ophiuroidea			
Order Amphilepidida			
Family Amphuridae			
<i>Amphiura (Amphiura) angularis</i>	KU895037	1410	Hugall <i>et al.</i> , 2016
<i>Amphiura (Amphiura) bidentata</i>	KU895046	1425	"
<i>Amphiura (Amphiura) constricta</i>	KU895029	1431	"
<i>Amphiura (Amphiura) diacritica</i>	KU895030	1431	"
<i>Amphiura (Amphiura) digitula</i>	MT323224	657	This study
<i>Amphiura (Amphiura) duncani</i>	KU895038	1431	Hugall <i>et al.</i> , 2016
<i>Amphiura (Amphiura) elandiformis</i>	KU895027	1410	"
<i>Amphiura (Amphiura) lanceolata</i>	KU895016	1425	"
<i>Amphiura (Amphiura) magellanica</i>	KU895042	1431	"
<i>Amphiura (Amphiura) maxima</i>	KU895024	1431	"
<i>Amphiura (Amphiura) microsoma</i>	KU895039	1425	"
<i>Amphiura (Amphiura) poecila</i>	KU895035	1410	"
<i>Amphiura (Amphiura) ptena</i>	KU895034	1431	"
<i>Amphiura (Amphiura) rosea</i>	KU895019	1431	"
<i>Amphiura (Amphiura) septemspinosa</i>	KU895032	1431	"
<i>Amphiura (Amphiura) spinipes</i>	KU895041	1431	"
<i>Amphiura (Amphiura) stictacantha</i>	KU895036	1410	"
<i>Amphiura (Amphiura) trisacantha</i>	KU895033	1410	"
<i>Amphiura (Amphiura) tutanekai</i>	KU895043	1431	"
<i>Amphiura (Amphiura) uncinata</i>	KU895026	1410	"
<i>Amphiura (Amphiura) velox</i>	KU895031	1404	"
Outgroups			
Order Ophiurida			
Family Ophiuridae			
<i>Ophiura albida</i>	AM404180	1605	Perseke <i>et al.</i> , 2008
<i>Ophiura luetkenii</i>	AY184223	1605	Scouras <i>et al.</i> , 2004
Class Asteroidea			
Order Forcipulatida			
Family Asteriidae			
<i>Aphelasterias japonica</i>	NC_025766	1552	Tang <i>et al.</i> , 2014
<i>Asterias amurensis</i>	NC_006665	1552	Matsubara <i>et al.</i> , 2005
Class Crinoidea			
Order Comatulida			
Family Antedonidae			
<i>Florometra serratissima</i>	NC_001878	1554	Scouras and Smith, 2001
<i>Phanogenia gracilis</i>	NC_007690	1554	Scouras and Smith, 2006

Ventral arm plate longer than wide, pentagonal with dull angles, and proximal end relatively more angular (Fig. 1F). Two tentacle shields in single tentacle pore, not fully covered (Fig. 1F).

Distribution. Korea (Korea Strait), Japan (Enoshima, Sadoga Island, Sagami Bay).

Habitat. Clay or sandy clay substrates.

Color. In live specimens, disk is gray and arms are pale brown.

Remarks. This species has several distinguishing morphological characteristics, including a long, tapered oral papilla and small, hooked stumpy spine on the distal

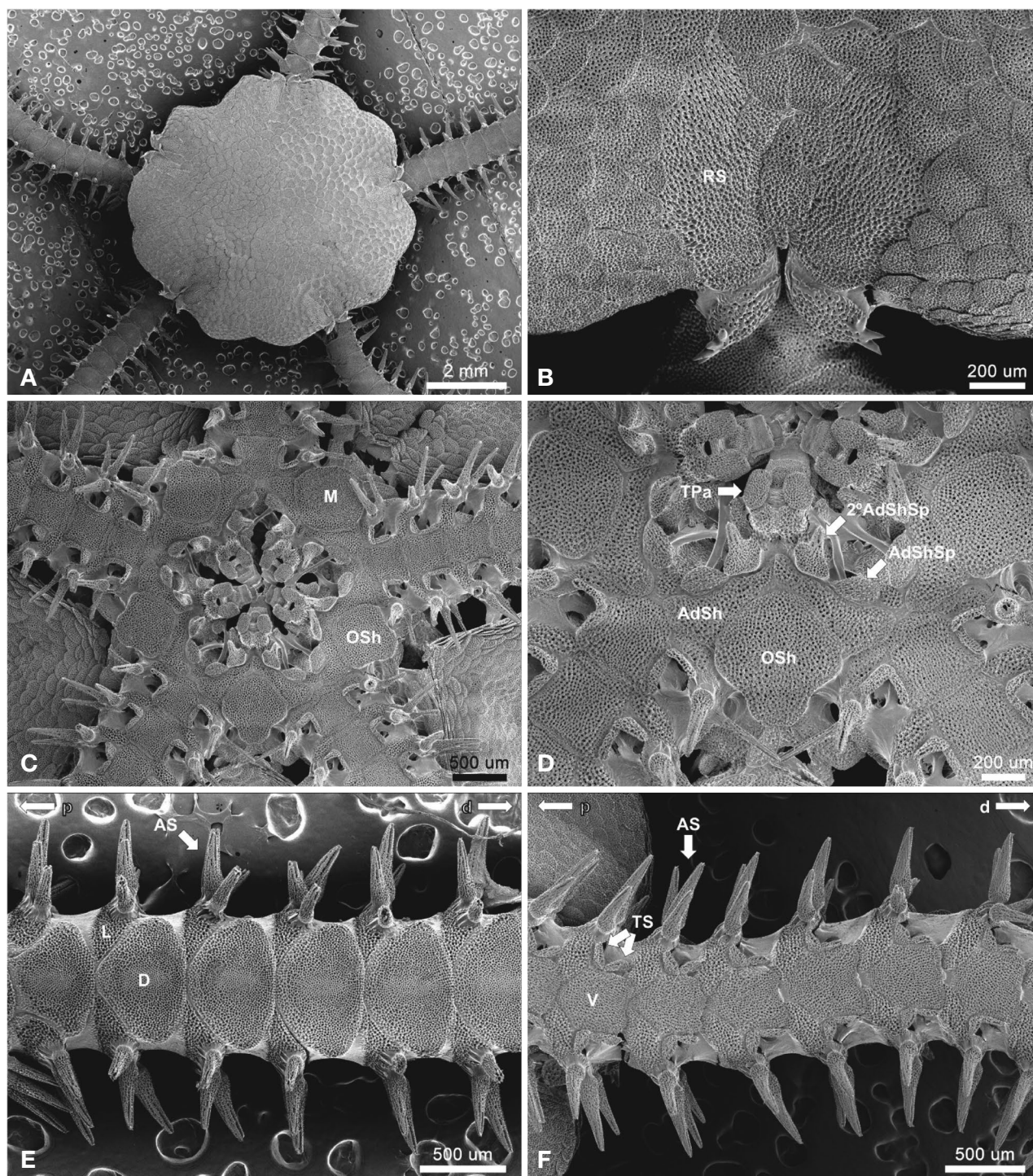


Fig. 1. Morphological characteristics of *Amphiura (Amphiura) digitula* (H.L. Clark). (A) dorsal side of disk; (B) radial shields; (C) ventral side of disk; (D) oral frame; (E) dorsal side of proximal arm; (F) ventral side of proximal arm. AS, arm spines; AdSh, adoral shield; AdShSp, adoral shield spine; D, dorsal arm plate; d, distal; L, lateral arm plate; M, madreporite; OP, oral papilla; OSh, oral shield; p, proximal; RS, radial shield; TPa, tooth papilla; TS, tentacle scale; V, ventral arm plate.

part of the radial shield. As such, the species is easily distinguished from other *Amphiura* species in Korea. Matsumoto (1917) reported *A. (A.) digitula* specimens

with four oral papillae, but A.M. Clark (1965) described that *A. leptopholida*, with three oral papillae, had almost morphological characteristics nearly identical to *A. dig-*

Table 2. Pairwise distances of 21 *Amphiura* species and other echinoderms (two ophiuroids, two asteroids, and two crinoids) as the outgroups, performed by a K2P model with 1000 bootstrapping method. The unit of distance values is a percentage (%).

Species name	GenBank accession No.	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	26	27
1 <i>Amphiura</i> (A.) <i>digitula</i>	MT323224																											
2 <i>Amphiura</i> (A.) <i>angularis</i>	KU895037	26.6																										
3 <i>Amphiura</i> (A.) <i>bidentata</i>	KU895046	23.3	29.4																									
4 <i>Amphiura</i> (A.) <i>constricta</i>	KU895029	25.7	21.8	26.6																								
5 <i>Amphiura</i> (A.) <i>diacritica</i>	KU895030	21.2	25.3	24.5	21.6																							
6 <i>Amphiura</i> (A.) <i>duncani</i>	KU895038	22.8	25.4	22.2	25.5	22.2																						
7 <i>Amphiura</i> (A.) <i>elandiformis</i>	KU895027	20.4	23.8	24.2	25.2	23.0	22.4																					
8 <i>Amphiura</i> (A.) <i>lanceolata</i>	KU895016	24.9	28.6	24.9	24.7	24.5	25.0	24.1																				
9 <i>Amphiura</i> (A.) <i>magellanica</i>	KU895042	24.1	27.0	25.7	26.3	25.0	24.5	24.6	24.3																			
10 <i>Amphiura</i> (A.) <i>maxima</i>	KU895024	26.2	24.9	23.7	26.1	24.6	19.0	22.2	22.3	23.3																		
11 <i>Amphiura</i> (A.) <i>microsoma</i>	KU895039	23.4	27.4	24.9	23.7	22.8	17.8	22.2	23.2	24.1	21.9																	
12 <i>Amphiura</i> (A.) <i>poecila</i>	KU895035	24.3	24.5	25.7	21.0	22.4	21.8	23.9	22.0	24.9	26.5	22.2																
13 <i>Amphiura</i> (A.) <i>plena</i>	KU895034	24.7	25.5	28.0	21.7	22.5	23.7	25.0	23.5	24.3	26.2	21.2	21.2															
14 <i>Amphiura</i> (A.) <i>rosea</i>	KU895019	22.0	24.8	25.9	23.5	24.5	25.1	21.8	21.0	23.7	25.8	22.8	24.9	22.2														
15 <i>Amphiura</i> (A.) <i>septemspinosa</i>	KU895032	25.3	24.8	27.8	22.1	21.0	24.9	24.3	29.7	25.0	25.4	23.9	24.2	25.8	26.4													
16 <i>Amphiura</i> (A.) <i>spinipes</i>	KU895041	24.3	30.0	24.0	27.8	24.1	22.9	23.0	29.0	25.3	26.2	25.2	26.2	29.1	25.5	28.1												
17 <i>Amphiura</i> (A.) <i>stictacantha</i>	KU895036	23.0	26.2	29.2	25.0	24.6	25.7	22.8	27.1	26.9	25.8	25.0	17.0	20.3	22.9	27.1	27.8											
18 <i>Amphiura</i> (A.) <i>trisacantha</i>	KU895033	24.3	22.7	26.9	22.9	20.8	25.3	24.7	24.1	23.9	27.1	26.5	21.4	24.6	25.4	25.7	25.6	23.2										
19 <i>Amphiura</i> (A.) <i>tutankai</i>	KU895043	26.2	25.7	26.0	25.2	24.7	23.4	24.3	25.8	25.6	25.6	23.4	23.6	26.2	26.4	25.8	25.9	26.0	25.7									
20 <i>Amphiura</i> (A.) <i>uncinata</i>	KU895026	26.2	25.8	25.7	25.7	23.7	22.9	22.2	26.0	21.8	22.3	23.7	24.3	23.3	24.1	26.5	25.3	24.0	24.3	26.4								
21 <i>Amphiura</i> (A.) <i>velox</i>	KU895031	23.4	26.8	27.8	21.2	23.4	26.9	24.9	25.9	25.9	26.7	22.4	22.8	23.4	26.8	21.6	26.7	21.2	22.6	27.0	22.7							
22 <i>Ophiura albida</i>	AM404180	28.6	28.8	29.0	28.5	28.3	28.3	27.5	26.2	25.2	28.4	29.3	25.1	26.4	24.7	29.6	24.9	27.6	26.4	26.7	26.8	31.4						
23 <i>Ophiura lutea</i>	AY184223	26.6	28.7	29.5	29.8	25.4	26.3	27.3	28.1	24.3	26.6	27.8	26.7	25.6	25.8	28.2	30.5	24.7	26.7	30.1	23.7	31.2	21.3					
24 <i>Apelasterias japonica</i>	NC_025766	33.7	35.5	34.0	36.0	32.4	31.9	30.8	31.2	30.0	33.7	30.5	33.5	33.0	32.2	33.6	30.9	34.3	32.4	33.4	32.0	31.9	33.8	30.8				
25 <i>Asterias amurensis</i>	NC_006665	32.0	35.5	33.8	30.9	34.8	31.0	33.3	31.4	30.7	32.3	31.0	29.2	28.8	28.5	33.3	33.8	32.1	30.6	36.7	34.0	33.2	31.4	31.0	18.9			
26 <i>Florumetra serratissima</i>	NC_001878	38.6	39.1	40.5	33.9	39.4	40.8	38.8	35.0	31.4	37.3	39.0	33.7	32.9	36.1	38.1	40.4	34.8	35.8	38.2	38.4	38.5	28.4	33.4	32.9	25.3		
27 <i>Phanogenia gracilis</i>	NC_007690	39.9	35.7	40.5	34.0	37.6	38.2	38.4	35.6	33.1	38.6	39.1	33.9	31.5	36.5	38.5	39.0	34.3	34.6	38.1	39.6	37.1	27.5	32.9	31.9	26.2	12.9	

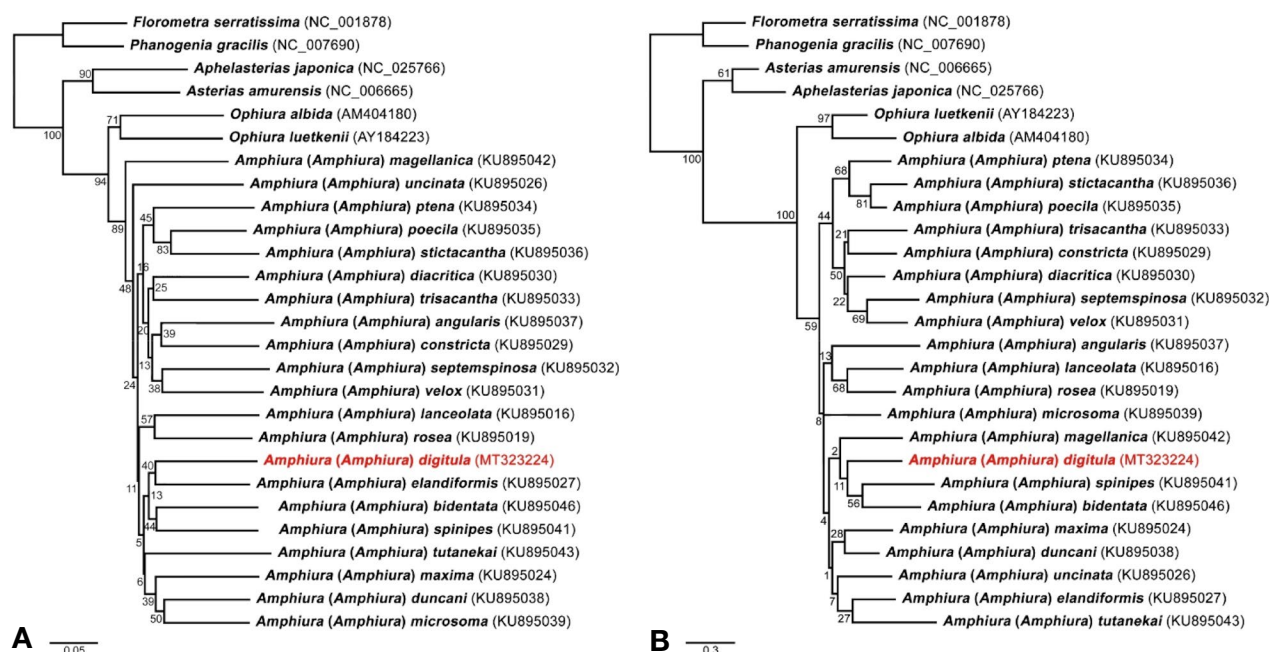


Fig. 2. Neighbor joining (NJ) and maximum likelihood (ML) trees based on 657 bp of the mitochondrial *COI* gene from 21 *Amphiura* species, including *A. (A.) digitula*. Bootstrap support values indicated on each node. (A) Phylogenetic tree of neighbor joining based on the kimura 2-parameter distance model, with 1000 non-parametric bootstrap replicates; (B) phylogenetic tree of maximum likelihood based on the GTR + I + G model, with 1000 bootstrap replicates.

itula. So, *A. leptopholida* synonymized *A. digitula*, even though the number of the oral papilla differs. All of the Korean specimens of *A. digitula* were identical to the description of *A. digitula* forma *leptopholida* in Clark, 1965, so we classified these samples as *A. digitula*.

We also obtained a partial sequence of *COI* from one *A. (A.) digitula* specimen in this study. The amplified sequence was 657 bp and matched the general DNA barcoding region. The NJ and ML phylogenetic analyses revealed that species of the *Amphiura* group was monophyletic, but supported by a low bootstrap value (Fig. 2). But, the group of *Amphiura* shown high interspecific pairwise distance (p-distance) values between each species in this study, and which was higher than the mean of interspecific p-distance in previous echinoderms DNA barcoding studies (Layton *et al.*, 2016; Boissin *et al.*, 2017). The interspecific p-distance between *A. (A.) digitula* and the other 20 *Amphiura* species ranged from 20.4% (*A. (A.) elandiformis*) to 26.6% (*A. (A.) angularis*), with a mean of 24.5% (Table 2). This is slightly higher than the mean in ophiuroids (18.9%) reported by Boissin *et al.* (2017) but corresponds to the mean and average value of interspecific *Amphiura*: mean = 24.1% and range = 17.0–30.0% (Table 2). The pairwise distance of *COI* presented the identification clearly between each species of the *Amphiura*, even though not shown the clear phylogenetic relationships within in NJ and ML trees. This study used morphological and molecular

analyses to identify the recently collected specimens as *A. (A.) digitula*, a newly recorded species in the Korea marine fauna.

ACKNOWLEDGEMENTS

This study was a part of the project titled ‘Improvement of management strategies on marine disturbing and harmful organisms (No. 20190518)’ funded by the Ministry of Oceans and Fisheries and a grant from the National Institute of Biological Resources (NIBR), the Ministry of Environment, Korea (Grant No. NIBR202002110). We are also grateful to the Marine Research Center, National Research Institute, Korea National Park Service (KNPS), for allowing us to join the project.

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Submitted: April 17, 2020

Revised: May 8, 2020

Accepted: May 11, 2020