Morphological and molecular description of *Argulus indicus* Weber, 1892 (Crustacea: Branchiura) found from striped snakehead fish (*Channa striata*) in Lake Towuti, Indonesia

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Abstract. *Argulus indicus* is an ectoparasite that causes an adverse effect on fish. This study aims to identify molecularly and provide additional description on the morphology of *Argulus indicus* infecting striped snakehead fish from Indonesia. Striped snakehead fish were caught from Lake Towuti using fish traps. The occurrence of *Argulus indicus* on the fish was observed visually on the whole body, including head, gills, body, and fins. *Argulus indicus* specimens were collected and preserved in 70% ethanol. Parasites were cleared in 10% potassium hydroxide (KOH) before examination of the parasite. The specimen was drawn under a microscope equipped with a lucida camera for detail drawing of the parasite specific organs and under a stereo microscope to observe the entire body surface of the parasite. Genetic analysis was performed with the PCR technique using 18S rRNA sequence data. The sequences were analyzed using MEGA Ver. X software and a phylogenetic tree was constructed by Maximum likelihood method. Additional illustration on the morphology of *A. indicus* presented here are shape of mouth tube, cephalic part, the number of ribs and shape of sclerite. In *A. indicus* male, copulatory organs are present in the second to fourth swimming legs. Morphological characteristics and phylogenetic tree analysis showed that the present samples are identified as *Argulus indicus.* They were all in the same clade, separated from other species of *Argulus*, indicating that the present samples have different lineages with other species of *Argulus,* but they have common ancestry.

Key Words: *Argulus*, fish diseases, morphology, phylogenic tree, small ribosomal RNA.

Introduction. *Argulus* spp. is a cosmopolitan parasite that can infect various species of fish in different water bodies. This parasite is known to infect freshwater fish (Alsarakibi et al 2014; Öztürk 2010; Sahoo et al 2012) and marine fish (Bottari et al 2017; Schram et al 2005). *Argulus* spp. infections are known to cause decrease in fish weight, disruption of physiological processes, and stress on fish (Sahoo et al 2012). Other studies also show that *Argulus* spp. can affect fish appearance (Taylor et al 2006). *Argulus* spp. can determine a higher mortality rate, caused by secondary infections of spring viraemia of carp virus (Ahne 1985). *Argulus* spp. also may act as a disease vector of other parasite, bacteria and fungi (Alom et al 2019).

Spread of *Argulus* spp. and cases of argulosis in fish in natural water and aquaculture facilities have been widely reported from various countries (Neethling & Avenant-Oldewage 2016), including Indonesia. There are at least 155 valid species of *Argulus* spp. have been described from marine fish and freshwater fish worldwide (Walter T. C. & Boxshall G. 2020). However, *Argulus* spp. species that have been reported in Indonesia comprised of three common species, *Argulus folicaeus, Argulus japonicus,* and *Argulus coregoni* (Neethling & Avenant-Oldewage 2016)*.* Most of the reports of *Argulus* spp. infection cases in Indonesia do not specify the responsible species. On the other hand, our parasitology survey in Lake Towuti found a species of *Argulus* sp. which shows different morphology than those frequently reported from cultured fish in Indonesia, which is quite similar with *Argulus indicus* that was first recorded in West Java, Indonesia in 1892, from the local catfish (*Clarias* sp.) as its primary host (Weber 1892). Since the first discovery, there has been no report of *A. indicus* infection in Indonesia. Lake Towuti is one of the largest lakes in Sulawesi Island, Indonesia. This lake has a high diversity of aquatic fauna, including various types of endemic and introduced fish (Nasution et al 2015). *Channa striata* is one of the fish that inhabit Lake Towuti and is frequently caught by local fishermen. Striped snakehead is the primary demand by local community for consumption and broodstock supply for aquaculture activity. The aim of this study was to describe morphology and molecular characteristics of *Argulus* sp. found in Lake Towuti as *Argulus indicus*. This report provides the extent of knowledge about the variety of *Argulus* spp. in Indonesia. Especially in the re-emergence and additional note on the morphology of *A. indicus* since its first report in 1892.

Material and Method

Sample collection. *Argulus* specimens observed in this study (n=15, selected from the total of 2252 specimens collected) were obtained from infected *Channa striata* (n=391), in a survey of parasite infection of fish in Lake Towuti, conducted for one year, from February 2018 to January 2019. Striped snakehead fish (*Channa striata* Bloch 1793) were collected from the inlet, middle, and outlet sites of Lake Towuti using fish traps. The occurrence of *Argulus* sp. was observed visually over the whole body, including head, gills, main body, and fins of the fish. Each of the *Argulus* found was collected and preserved in 70% ethanol for PCR analysis and morphological observation. Visual observation for morphological characteristics of the parasite was conducted in wet clearing conditions using 10% potassium hydroxide (KOH) (Kennedy 1979). Morphological parameters were determined using a stereo microscope (Stereo Discovery.V12 with cold light source CL 4500, Zeiss). All morphological parameters were expressed in millimetre (mm). Sketch of the specimens was performed under the lucida microscope (Olympus BX 50) for the body part and under a stereo microscope (Nikon SMZ 800) for the whole body. Description was based on 4 males and 11 females specimen referring to Meehan (1940) and Weber (1892).

Materials examination. The observation materials used for measurements and descriptions were the selected specimens from our collected samples. These were used to describe the specimens of *Argulus indicus*. The specimens are identified in the following manner: Indicus Towuti (INTW) 6871 (male), INTW 6872 (female), INTW 6873 (female), INTW 6874 (female), INTW 6875 (female), INTW6877 (female), INTW 6878 (female), INTW 3230 (male), INTW 3231 (female), INTW 3232 (female), INTW 3233 (female), INTW 3234 (female), INTW 3235 (male), INTW 3236 (male), INTW 3237(female).

Argulus indicus diagnosis. *Argulus indicus* has an ovate carapace, reaching the last pair of legs. The abdomen in females is about one-fourth of the entire body length, more extensive than the length. Slightly split in the posterior part. Both parts have round points. Both sexes have four swimming legs and at the fourth leg an elongated oval lobe that looks like a pair of legs extending out. The lobe is parallel to the pair of swimming legs and is longer than half of it. An edge of the lobe was equipped with setae. In the antennae, spines are triangle-shaped, with the tip extending between the first and the second antennae. Flagellum has three parts on the tip and two parts on the stem, which in the first branch has a strong spine. The mouth tube and stylet are widened to the second pair of the maxilla. The basal plate portion of the maxilla is widened and comprised of three hooks. The small end consists of two curved hooks with the tip that looks like a finger. Two hard hooks are located between the tip of the second maxilla and the first swimming leg. The first and second swimming legs have flagella, while the second and third have copulation organs in male specimens.

DNA extraction and amplification with PCR. Three *Argulus* sp. were individually extracted following the manufacturer's protocol by DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Genomic DNA of an individual *Argulus* sp. was placed in a 2 ml microtube and grinded using micro pestle. Each sample was added 180 µL of ATL solution (Tissue Lysis Buffer) and 20 µL of proteinase K. The micro tubes were vortexed and incubated at 56° C overnight. The extracted DNA was then amplified in 18S sRNA region using forward primer ERIB 1 (5´-ACC TGG TTG ATC CTG CCA G-3´) and reverse primer ERIB 10 (5´-CTT CCG CAG GTT CAC CTA CGG-3´) (Patra et al 2016). PCR reaction was performed in 20 µl master mix solutions (1 µl of each primer, 10 µl of The Qiagen Taq DNA polymerase, 7 µl of Nucleus Free Water, and 1 µl of sample DNA). Amplification of DNA was carried out using Labcycler Thermal SensoQuest (Germany) and set accordingly by initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 51°C for 30 sec, extension at 72°C for 60 sec and final extension at 72°C for 5 min (Mondal et al 2014). The PCR Products were visualized on 1.5 % agarose. PCR products which showed clear band on gel analysis were prepared for sequencing. Samples were sent to a commercial company (1^{st} BASE Asia) through PT Genetika Science Indonesia for DNA sequencing.

DNA alignments and phylogenetic tree analysis. The nucleotide sequences received from the commercial company were analyzed using BioEdit and MEGA X software. The DNA sequences of the samples in this study which had nucleotide length of about 1800 bp., were aligned with other *Argulus* species available in the NCBI GenBank databases (NCBI 2020). The present sample of *Argulus indicus* which has accession number of MT538302, MT538303, and MT538304, respectively, were aligned with other *Argulus* species (*Argulus* sp.: JN558648, *Argulus coregoni*: JQ740820, *Argulus nobilis*: MH458748, *Argulus foliaceus*: JQ740819, *Argulus japonicus*: JN558647; *Argulus rhipidiophorus*: KF747862, *Argulus siamensis*: KM597747, and *Argulus pugettensis*: MH915670). *Eurycyde curvata* (DQ389897) was used as an outgroup. The phylogenetic tree analysis was constructed in MEGA X software (Kumar et al 2018) using the method of Maximum Likelihood with 1000 bootstrap replicates (Tamura & Nei 1993). Genetic distance was measured using the evolutionary Pairwise distance by Tamura and Nei (1993).

Results

Morphological description of Argulus indicus. The results in the study indicate that *A. indicus* has a widely rounded carapace, covering the fourth swimming leg in females (Figure 1). In contrast, the male has an ovate carapace, and it is partially covering the lobus natatory in males (Figure 2). In females, the Carapace length is 2.31 – 7.14 mm and 4.93 – 6.35 mm in males. The maximum carapace width is 2.11 – 7.17 mm in females and 4.70 – 6.02 mm in males. The cephalic area is broadly triangular with two median curved ribs. The base of ribs in the posterior is extended to the front of the eye. Those are branching towards the outer margin and near to the anterior margin. Diameters of compound eyes is 185 – 261 µm in females and 186 – 258 µm in males. In females, abdomen is wider than long, acorn-shaped, posterior lobes broadly rounded and short and slightly split in the back. Spermatheca is a tiny rounded and abdominal short sinus. Brown flecked in all the body. The natatory lobes appear to coalesce with the fourth thoracic segment, boot-shaped with cilia on the outside. Abdomen length is 0.60 – 1.64 mm in females and 1.45 – 1.73 mm in males. Maximum width is 0.86 – 2.28 mm in females and 1.46-2.05 mm in males.

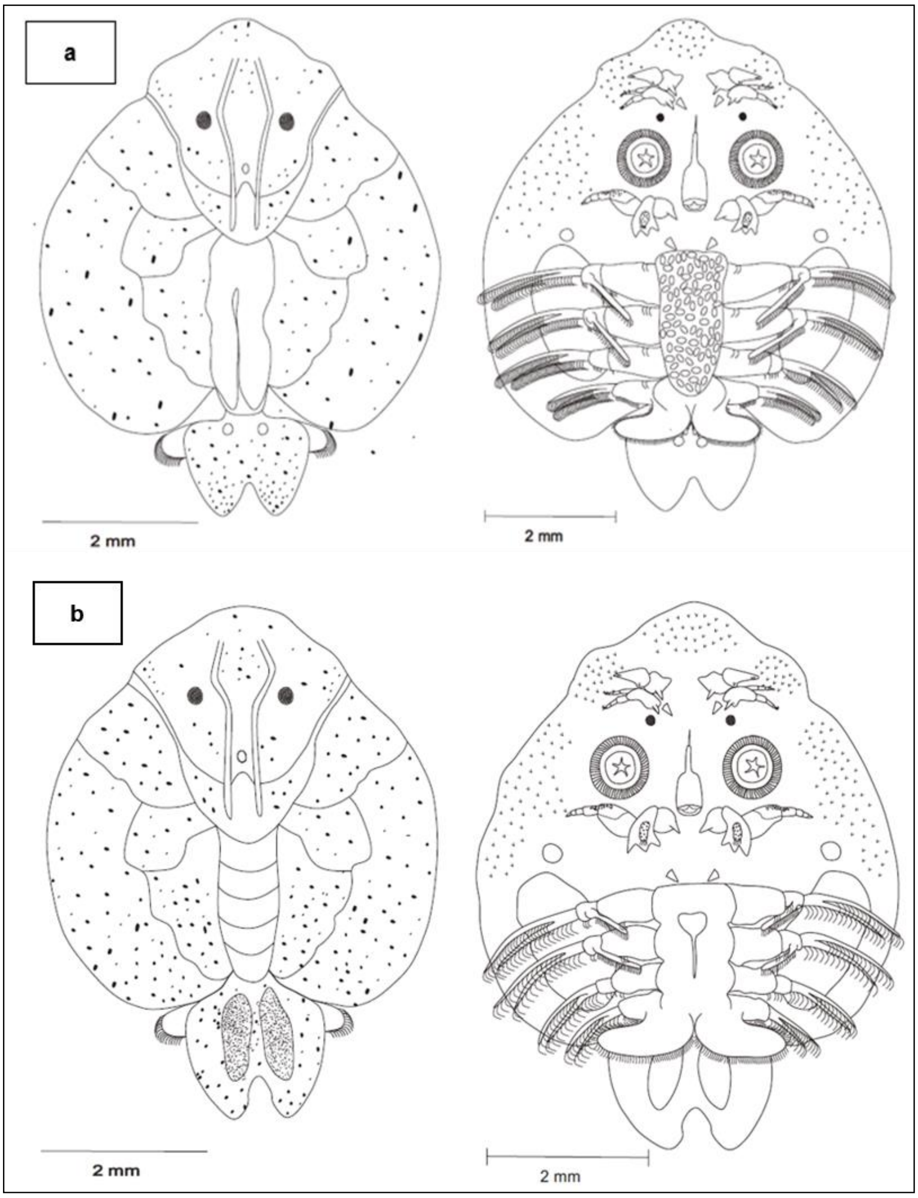

Figure 1. Dorsal-ventral view of *Argulus indicus* from Lake Towuti (a. female, b. male).

The abdomen in males is wide and slightly elongated to form like a Brazil nut. Lobus natatory is boot-shaped with cilia. The rounded testicles are elongated, not reaching the posterior lobe. Abdominal sinuses are longer in males than in females. The respiratory area divided into two parts, the anterior part; small, round, and the posterior section; extensive, elongated, round shaped. The first antennae is slender with hooks and two spines on the anterior margin, elongated with a pointed tip. Flagellum on the first antennae is short and long on the second antennae.

First maxilla or suction cups diameter is 0.40 – 1.23 mm in females and 0.74 – 1.12 mm in males. Sucker has 67 – 76 supporting ribs, each rib consisting of three elongated sclerites. Mouth tube is ovate-shaped, with two spines near the posterior end, accompanied by a stylet on the anterior part that can elongate and shorten (Figure 2. a). Second maxilla is slender with radiant scale, elongated round basal plate, with eleven pectinate scales and five hairs on the front. There are three sturdy thorns at the edges of basal plate that are pointed and reddish-brown. The tip of the maxilla is in the form of hooks, and finger-like sharp protrusions. Flagella present on the first and second pair of legs. Second to fourth swimming legs equipped with copulatory accessories in males. Testis are located between first and second thoracic segments, extending slightly to third thoracic segment. Eggs are placed in the thoracic 1-4 segment that are brownish and round in females.

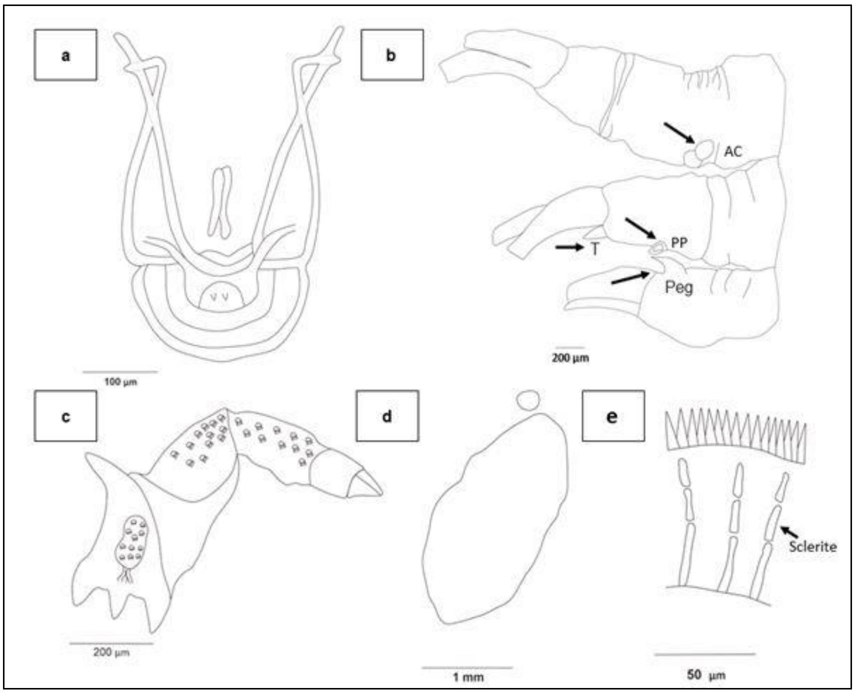

Figure 2. a = mouth-tube; b = copulatory accessories in males (AC, accessory cushion in second leg; PP, posterior projection; T, tubulate in third leg; Peg in fourth leg); c = maxilla; d = respiratory area in the second to fourth legs; e = ribs (sclerite) supporting sucker.

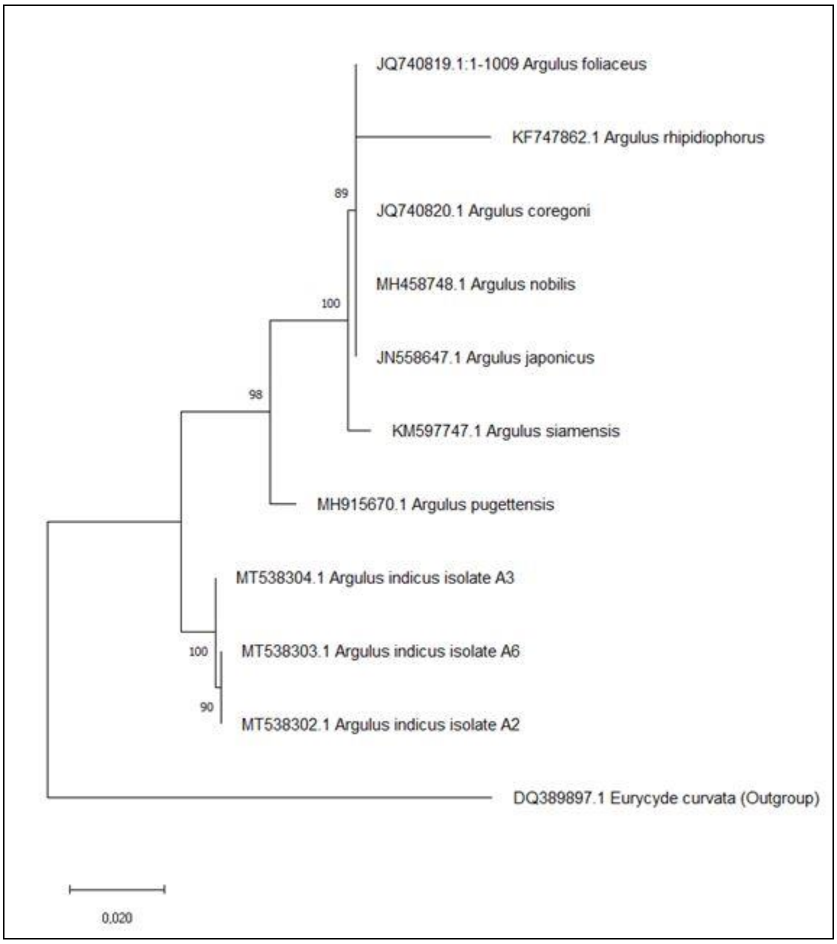

Figure 3. Phylogenetic tree of the present *Argulus* samples and other *Argulus* species, using the Maximum Likelihood Method with 1000 bootstrap replicates.

Table 1

Homogeneity of *Argulus* sequences and related species in GenBank database

Argulus Species	*Homogeneity (%)*			*Accession number*
	A. indicus A2 (MT538302.1)	*A. indicus A3 (MT538304.1)*	*A. indicus A6 (MT538303.1)*	
Argulus nobilis	96.42	96.99	96.42	MH458748.1
Argulus pugettensis	96.73	96.85	96.73	MH915670.1
Argulus sp	96.88	92.29	96.88	DQ531766.1
Argulus coregoni	96.98	97.13	96.98	JQ740820.1
Argulus foliaceus	96.88	97.04	96.88	JQ740819.1
Argulus japonicus	96.80	96.96	96.80	JN558647.1
Argulus siamensis	97.29	97.37	97.29	KM597749.1
Argulus rhipiodopirus	96.86	97.03	96.86	KF747862.1
Eusycyde curvata	89.52	89.64	89.52	DQ389897.1

Table 2

The Evolutionary Pairwise Distance of sequences

Accession Number	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
MT538303.1										
MT538302.1	0.000									
MT538304.1	0.001	0.001								
DQ389897.1	0.129	0.129	0.128							
JN558647.1	0.038	0.038	0.036	0.111						
JQ740819.1	0.029	0.029	0.029	0.111	0.000					
JQ740820.1	0.038	0.038	0.036	0.112	0.000	0.000				
KF747862.1	0.052	0.052	0.050	0.124	0.029	0.004	0.029			
KM597747.1	0.033	0.033	0.031	0.103	0.004	0.006	0.005	0.031		
MH458748.1	0.046	0.046	0.045	0.157	0.000	0.000	0.000	0.029	0.0056	
MH915670.1	0.031	0.031	0.030	0.122	0.011	0.012	0.011	0.040	0.0155	0.0094

***Molecular analysis of sequences**.* The samples nucleotide sequences were submitted to the GenBank database with the accession number of MT538302, MT538303, and MT53830, respectively. Blast analysis (Table 1) showed 96-97% homology among the *Argulus* sequences in GenBank Database. Result of the phylogenetic tree (Figure 3) showed separate clade between *Argulus* species and the outgroup. The present samples showed different cluster with the following: *A. japonicus*, *A. foliaceus* and *A. coregoni*, frequently recorded from freshwater fish in Indonesia. The first cluster represents the common ancestor of *Argulus* species whereas the second cluster explains a different ancestor. The present *Argulus* sp. sample had distinct branches to other species of *Argulus*, which indicate the distance lineages among *Argulus* species. The evolutionary pairwise distances of *Argulus* species (Table 2) exhibit variated from 0.000 to 0.152 in the *Argulus* species and 0.129 in outgroup, respectively. The result reveals that morphologic and phylogenetic tree of the present *Argulus* specimens were identified as *Argulus indicus.*

Discussion. The morphological characteristics of *Argulus* sp. specimens in this study were confirmed to be the same species as *A. indicus*. However, as it has been illustrated by the previous studies, the morphological characteristics of this parasite have not been clearly explained. The morphology of *A. indicus* was first described by Weber (1892) with the descriptions limited on female specimens organs such as carapace, abdomen, antennae, tube-mouth, maxilla, and swimming legs. Van Kampen (1909) completed Weber's illustration with several lines of information and two images. The first one is from the dorsal appearance and another is from the ventral surface of an adult female. Wilson (1927) described and explained the information about the respiratory and cephalic areas of *A. indicus*. In 1944, the first male of *A. indicus* was described by Wilson (1927). Other studies, Ramakrishna (1952) reported a short clarify of the body color of *A. indicus* and the size of the cephalic area in young and adult males and female specimens. However, there was no further description related to the morphology and characteristic of *A. indicus* since 1952.

The *A. indicus* female was first reported by Weber in 1892 which stated that the female carapace is ovate, reaching the last pair leg. While Wilson (1927) showed that the female carapace is nearly orbicular. Moreover, Meehan (1940) reported that female carapace is broadly rounded. The present finding showed different result from Weber (1892) and Wilson (1944) description. However, this result was like Meehan (1940) result, which stated that female carapace is widely rounded. The first information related to *A. indicus* male was mentioned by Wilson (1944) that the male carapace is ovate and considerably narrowed in anterior, which is like in this study. The carapace was ovate and partially covering the lobus natatory. In general, the carapace is widely rounded in females and ovate in males. The carapace is wider than longer in females and longer than wider in males. Abdomen part was widened in females and elongated in males. Testes in the abdomen was elongated shape while the female spermatheca was small, rounded in shape. In the present study, abdomen was widened in females and elongated in males. These findings were similar to the descriptions of Weber (1892), Meehan (1940), and Wilson (1944) but distinct of Wilson's (1927) explanation on abdomen shape. Wilson (1927) reported that the abdomen is heart-shaped in females and acorn-shaped in males. Whereas this study showed that abdomen had acorn-shaped in females and the Brazil nut-shaped in males. Similar to our findings of the descriptions of Wilson (1944) was that the pair of testes were elongated elliptical and the female spermatheca was small, rounded in shape. The sucker of *A. indicus* was not described by Weber (1892). This section was described by Wilson (1927) which found that the sucker consists of five rods supporting apparatus. While Meehan (1940) mentioned that the sucker has a moderate size and consists of three rods that are located inside the supporting ribs. In 1944, Wilson revised the description of the sucker and stated that the sucker composed of three rods supporting ribs, one of them has an enlarged proximal end. In this study, the sucker was relatively large with 67-75 supporting ribs, and had three elongated sclerites inside. This result was like the number of sclerite found in Meehan (1940) and Wilson (1944) descriptions. Moreover, in this study, additional information on the total of the supporting ribs is provided.

A. indicus has four swimming legs with flagella in the first and second legs in the thoracic part. Egg depository is in the middle of the female thoraces. While the copulatory accessory of a male is in the second to the fourth swimming leg. Weber 1892 reported siphon and sting in *A. indicus* with limited explanation. In this study, we found that the mouth tube was elongated ovate; two spines in labial, and stylet above it. The maxilla was rather slender, the basal plate was broadly triangular, and the three spines were on its posterior margin that were short and taper. Those were like the descriptions of Weber (1892), Meehan (1940), and Wilson (1944). Furthermore, our study presented more information about pectinate scales both in the maxilla and basal plate. The body color of *A. indicus* was reported by Meehan (1940). The whole body of *A. indicus* has dark spots that spread throughout the body. While Ramakrishna (1952) mentioned the color of *A. indicus* is dark green with dots on the dorsal surface of carapace. Dissimilar to the previous studies, we found that the color of the dorsal surface in *A. indicus* was a brown spot that spread from the cephalic area to the abdomen.

The phylogenetic tree is the history of evolutionary relationship among organism at genera or species level (Yang & Rannala 2012). Their reconstruction is based on the degree of similarity in morphological, molecular and observed characters (Hasegawa 1999). Molecular analysis in *Argulus* genera was first performed by Abele et al (1989) using 18S rRNA to explain the divergence of evolutionary time and the common ancestor of *Argulus* and Pentastomida. Ribosomal RNA is the target for observing the phylogenetic relationship among organisms. The ribosomes had rRNA and proteins which all organisms had the small ribosomal sub unit including single RNA species (Patwardhan et al 2014). In our research, samples were amplified by 18S rRNA and successfully determined the target nucleotide at 1800 bp. Phylogenetic tree was gathered in one cluster of *Argulus* species and indicated the most recent common ancestor in different lineages. Based on the morphological characteristics and genetically grouping analysis, the present sample is identified as *Argulus indicus*. Moreover, the evolutionary pairwise distance shown the value from 0.000 to 0.017 of *Argulus* species, indicating the low genetic distance among them. According to Hasegawa (1999), distance value can describe the organism polarization. If the distance value is close to 1, it means the organism is classified as apomorphic. In contrast, if the value is close to 0, the organism can be classified as plesiomorphic. These explain that *A. indicus* has distant lineages to other *Argulus* species but is a closer relationship. The length of the branches indicates the evolutionary time of the species, which represents the difference in evolutionary time between each species in the topological tree (Patwardhan et al 2014).

Conclusion. The morphological data of *Argulus indicus* added in this description are the ovate-shape of mouth tube, a widened triangle-shaped of cephalic part, total number of 67-76 supporting ribs, three elongated sclerites on the sucker, and copulatory organ which is located at the second to fourth swimming legs in male. Those data were added to complete the undescribed part of the previous description of this species. Sexual dimorphism of *A. indicus* is shown by the size and shape of the carapace and abdomen. A male's carapace is ovate, and its length more than its width, while the female has a rounded carapace, and it is wider than it is long. The abdomen part is widened in females and elongated in males. Based on morphologic observation and molecular analysis, the present *Argulus* sp. sample is deduced as *A. indicus.*

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