First report of the parasitic crustacean rhizocephala on female *Albunea symmysta* from Indonesia

Dian Bhagawati, Agus Nuryanto, Aswi A. Rofiqoh

Faculty of Biology, Jenderal Soedirman University, Purwokerto, Central Java, Indonesia. Corresponding author: D. Bhagawati, dian.bhagawati@unsoed.ac.id

Abstract. Several studies reported rhizochepala parasitism in mole crabs (*Albunea symmysta*). However, no report about which rhizochepala species infect mole crabs from Parangkusumo beach, Yogyakarta, Indonesia, has been found. This study aims to identify the rhizochepala species that infect mole crabs in the mentioned location. Rhizochepala were identified based on morphology, micro-anatomy, and cytochrome coxudase 1 barcoding. The result showed that only female *Albunea symmysta* were infected by rhizochepala. Further examination of the parasitic rhizochepala proved that all characters were only reliable for genus level identification. All characters highly match with genus *Sacculina*. It is concluded that *A. symmsyta* from Parangkusuma beach, Yogyakata, Indonesia, was infected by *Sacculina* sp.
Key Words: gen CO1, identification, morphology.

Introduction. Rhizocephala is the most advanced parasitic metazoan. It can attack its host, mostly consisting of decapods, with a high attack rate (Brusca et al 2016). Several researchers (Høeg 1995; Høeg & Lützen 1995; Lafferty & Kuris 1996; O'Brien 1999; Thresher et al 2000; Waser et al 2016; Mouritsen et al 2018) reported that the attacks of rhizocephala have significant influences on its host population.

Studies on rhizocephala to identify members of the genus *Sacculina* based on anatomical characters and the external and internal cuticle peculiarities were carried out by Okada & Miyashita (1935) and Boshma (1953). The use of morphological characters in the taxonomic studies of rhizocephala by Høeg & Lützen (1985, 1996) and Øksnebjerg (2000) is exclusively related to externa. Spears et al (1994) have used molecular methods in the phylogenetic analysis of rhizocephala. The use of external histology in identifying rhizocephala has also been carried out by Yoshida et al (2011, 2015). Recently, several studies have combined various taxonomic characters to obtain more accurate results (Glenner et al 2003; Rybako & Høeg 2013; Lützen et al 2016; Kobayashi et al 2018).

Rhizocephala parasitism of crustaceans has been studied by Høeg et al (2019) to rearrange their relationships. Previous phylogenetic studies using molecular characters have been conducted and suggested a need for a fundamental revision of the rhizocephala. Those studies proved that rhizocephala widely vary in their development, host and control taxon, parasite morphology, and reproductive system. It was further explained that rhizocephala only consists of a few hundred species, generally with marine habitats, and significantly influence the host crustacean populations. The resulting phylogeny enabled the use of rhizocephala as a model for studying biological evolution in highly specialized and biologically thriving and diverse parasitic taxa (Glenner & Hebsgaard 2006; Glenner et al 2010, 2020; Lützen et al 2016; Høeg et al 2019).

Rhizocephala was reported to attack many crustacean species (Rees & Glenner 2014; Lutzen et al 2018; Jensen et al 2019). For example, rhizocephala was found to infect *Upogebia* spp. (Lutzen et al 2016), *Portunus sanguinolentus* (Raffi et al 2012; Yang et al 2014); mud crab *Scylla olivacea* (Kahar et al 2016); and the shrimp *Pandalina brevirostris* (Nagler et al 2017). Moreover, Elumalai et al (2014) reported that *Sacculina* spp. infect several types of commercial marine crabs, like *P. sanguinolentus*, *Portunus hastatoides*, and *Charybdis feriatus*. However, according to Chan (2004), research on rhizocephala in the Asian region is still limited, including in Indonesia.

This research aimed to identify rhizocephala infecting female *Albunea symmysta* from the coast of Parangkusumo, in the Special Region of Yogyakarta, Indonesia, based on morphology, microanatomy, and barcode marker.

Material and Method

Study sites. *A. symmysta* specimens were collected from Parangkusumo beach, Yogyakarta, Indonesia. Study sites were located at 7°59'15"S to 8°1'58"S and 110°16'52"E to 110°20'37"E (Figure 1).

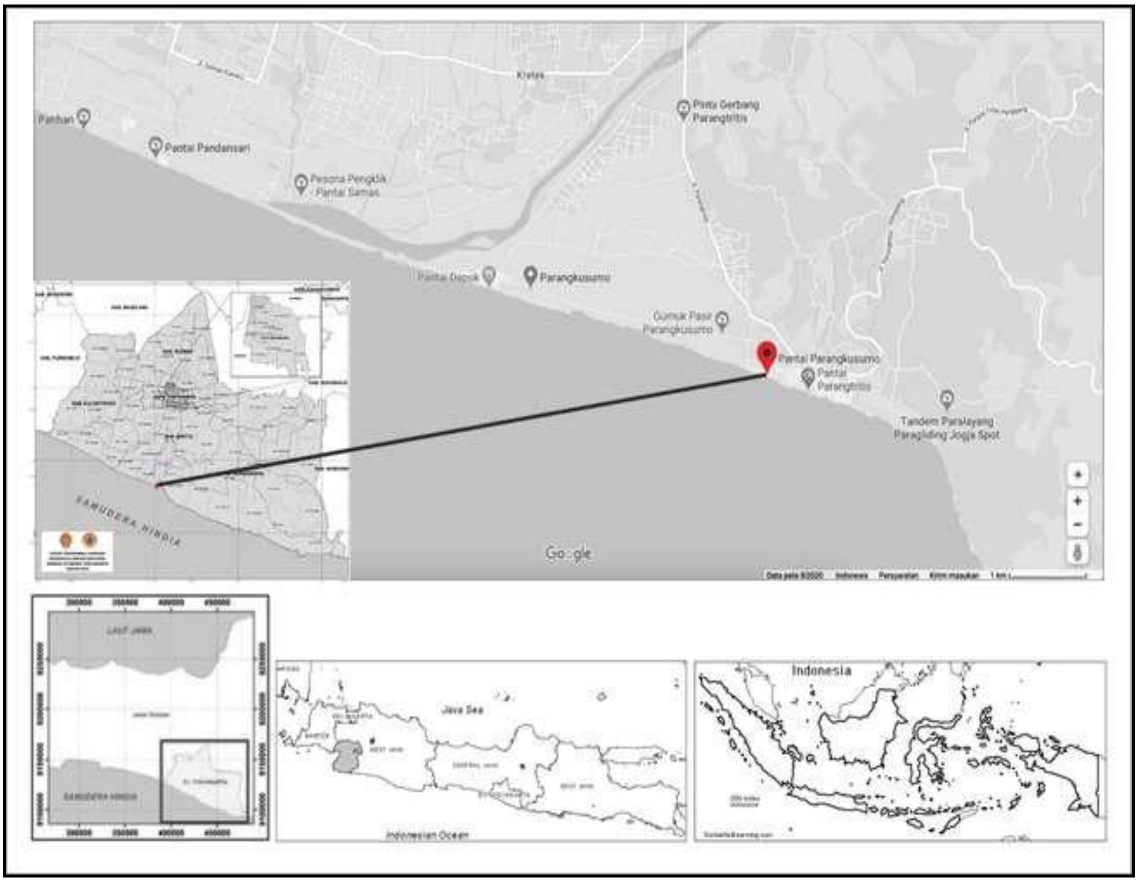

Figure 1. Study sites at Parangkusumo Beach Yogyakarta, Indonesia.

Mole crab samples were collected six times from May 2019 to March 2020. The specimens were directly, manually caught from a sloping sandy beach, on a 1 km distance along the coast. *A. symmysta* crabs were sorted according to their sex, which was determined based telson width. Females have a wider telson than males. Each female was carefully examined for the presence of parasites. The uninfected crabs were returned to the sampling site after being examined, while the infected specimens were preserved for further observation. Infection by rhizocephala was confirmed by the existence of an externa, which appears as a white sac on the host's stomach (Rees & Glenner 2014). The body of the rhizocephala was taken from the crab body using scissors and forceps, and preserved in 96% ethanol for DNA analysis. Most of the rhizochepala were preserved in 70% ethanol and 10% Neutral Buffered Formalin (NBF) solution for morphological and micro anatomic observations. The specific characteristics of the fixed and isolated externa were carefully observed under a microscope. Microanatomy examinations were carried out at the Histopathology Laboratory, Faculty of Veterinary

Medicine, Gadjah Mada University, Yogyakarta, while DNA analysis was carried out at PT Genetic Science, Indonesia.

Morphological observations. Mature or adult rizochepala live outside crab body (ectoparasite) and are called externa, while young and juveniles live inside the crab body (endoparasites). The general morphological appearance of two externa of rhizocephala that infected *A. symmysta* was examined. The parameters consisted of shape, length, and width, which were measured using a digital caliper. Other morphological characteristics were the shape and position of the mantel opening and the form of the stalk. The externa specimen was then prepared for microanatomy observations. The microanatomy preparations used paraffin and hematoxylin-eosin (HE) technique. Photomicrographs of the externa were examined under a microscope following Boschma (1933, 1953, 1955), Hoeg & Rybakov (1992), and Kobayashi et al (2018).

Molecular identification of rhizocephala. Two externa of adult rhizochepala were also subjected to molecular barcoding for taxonomic identification. The cytochrome c oxidase subunit 1 (COI) gene was used as a barcode marker. Molecular analysis was conducted at PT Genetika Science Indonesia, Jakarta. Genomic DNA of rhizochepala was extracted using Zymo Tissue and Insect DNA MiniPrep kit (Zymo Research, D6016) according to the company's protocol. The fragment of the CO1 was amplified using LCO1490 as forwards primer and HCO2198 as a reverse primer (Folmer et al 1994). The PCR amplification used KOD FX Neo (Toyobo, KFX - 201) ready mix. Amplification products were sequenced using the bi-directional sequencing technique.

Data analysis. The parasite general performance and micro-anatomy were analyzed descriptively. The COI sequences were edited manually in Bioedit 7.0.4.1. (Hall 1999). All sequences were aligned using ClustalW (Thompson et al 1994). The taxonomic status was determined based on the sample identity values compared to the sequences of conspecific references in the GenBank using the basic local alignment search tool (BLAST) method. Support to the BLAST result was obtained from phylogenetic analysis. Phylogenetic trees were reconstructed using a neighbor-joining algorithm with the Kimura-2 Parameter (K2P) evolution model in MEGA version 6.0 (Tamura et al 2013). The polarity of the phylogenetic tree was obtained from 1000 non-parametric pseudo-replicates. The parasite prevalence was also calculated using the formula of Kabata (1985).

Results and Discussion. Sixty-one specimens of mole crab were collected during the study. Sixty among 61 specimens of mole crab had more pleopods than the remaining individual, which indicated that most of the samples were female. Only one individual was an adult male. Further examination proved that ectoparasites were found on 7 out of the 60 female individuals. Thus, the prevalence was 11.48%.

Morphological assessment showed that the infected female crabs had a carapace length ranging from 2.71 to 3.23 cm (average of 2.92±0.06 cm) and a width between 1.73 to 2.33 cm (average of 2.12±0.08 cm). The careful observation proved that infected crabs did not carry eggs. It is assumed that ectoparasites might cause sterility of female mole crabs and that infected individuals could not produce eggs.

Ectoparasite attacks on mole crabs collected in Parangkusumo beach, Yogyakarta, could be recognized by the presence of externa attached to the host abdomen. A detailed and careful examination indicated that each crab was only infected by one individual ectoparasite and had a white color (Figure 2).

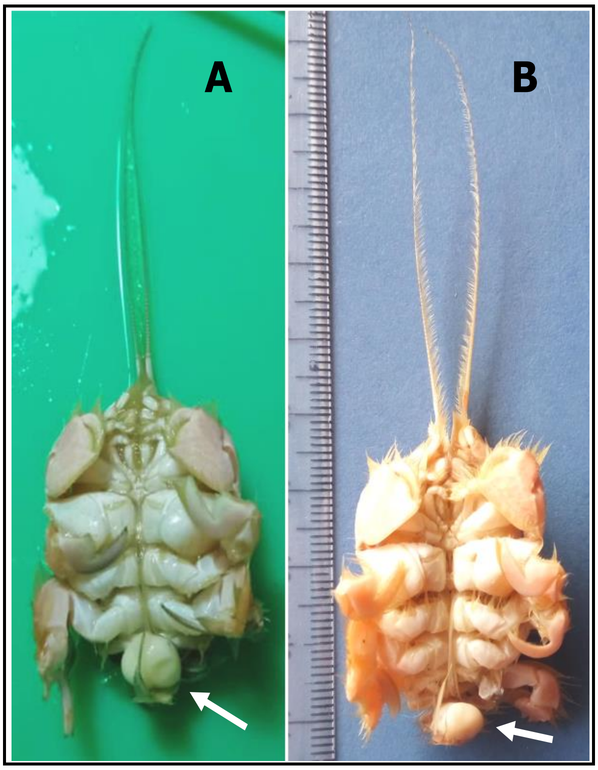

Figure 2. Externa of an adult ectoparasite on female mole crab (*Albunea symmysta*) indicated by arrow; A - fresh specimen of mole crab; B - preserved specimen of mole crab.

The outer cuticle surface of adult ectoparasites was smooth. The parasites were stalked, short tube-shaped, with a ring at the base. The externa of young ectoparasites was more rounded than that of the juveniles (Figure 3) and that of adults (Figure 4).

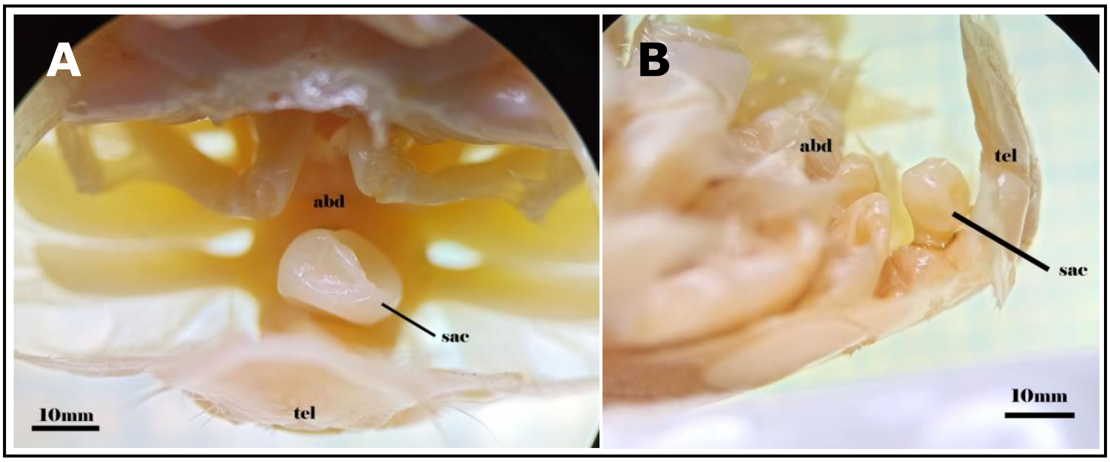

Figure 3. Externa of juvenile ectoparasites infecting female mole crabs (*Albunea symmysta*); A - dorsal view of mole crab with ectoparasite; B - lateral view of mole crab with ectoparasite; abd - abdomen; sac - saccus; tel - telson.

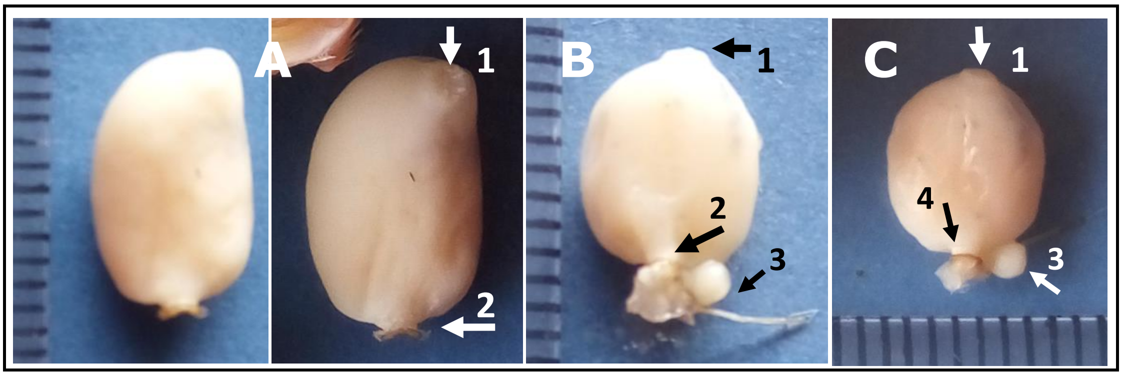

Figure 4. Externa of adult ectoparasite infecting mole crab (*Albunea symmysta*); A - asymmetry externa; B - ventral view of symmetry externa; C - dorsal view of symmetry externa; 1 - mantle aperture opening; 2 - stalk; 3 - root; 4 - ring on stalk. Bar scale: A=1 mm, B=1 mm, C= 1 mm.

The morphometric measurements showed that the externa of the observed ectoparasite had variable sizes (Table 1). Cross-section of the externa is presented in Figure 5.

Table 1

The size of externa of ectoparasites infecting *Albunea symmysta*

Individual	*Length (mm)*	*Width (mm)*	*Thickness (mm)*	*Description*
1	11	6.5	3.1	White, oval, smooth external surface, asymmetrical, circular of opening aperture mantle, visible ring on the stalk
2	8.5	6.8	3.6	White, oval, smooth outer surface, symmetrical, circular of opening aperture mantle, visible circle on the stalk
3	8.5	6,4	3.6	White, oval, smooth external surface, symmetrical, circular of opening aperture mantle, visible ring on the stalk
4	7.3	5.5	3.8	White, oval, smooth outer surface, symmetrical, circular of opening aperture mantle, visible circle on the stalk
5	7.0	5.3	3.8	White, oval, smooth external surface, symmetrical, circular of opening aperture mantle, visible ring on the stalk
6	6.1	5.0	4.0	White, rounded, smooth outer surface, symmetrical, circular of opening aperture mantle, visible ring on the stalk
7	5.2	4.8	4.3	White, rounded, smooth external surface, symmetrical, circular of opening aperture mantle, visible circle on the stalk

Basic local alignment search tool (BLAST) of the cytochrome c oxidase subunit 1 (COI) of the ectoparasite infecting mole crabs from Parangkusumo beach, Yogyakarta, showed a sequence identity of the P2 sample of 80.98% with *Sacculina* sp. sac 3 (KM087534), with the query cover of 87%. The P3 sample had a sequence identity of 80.80%, and the query covers 87% of *Sacculina* sp. sac 3 (KM087534).

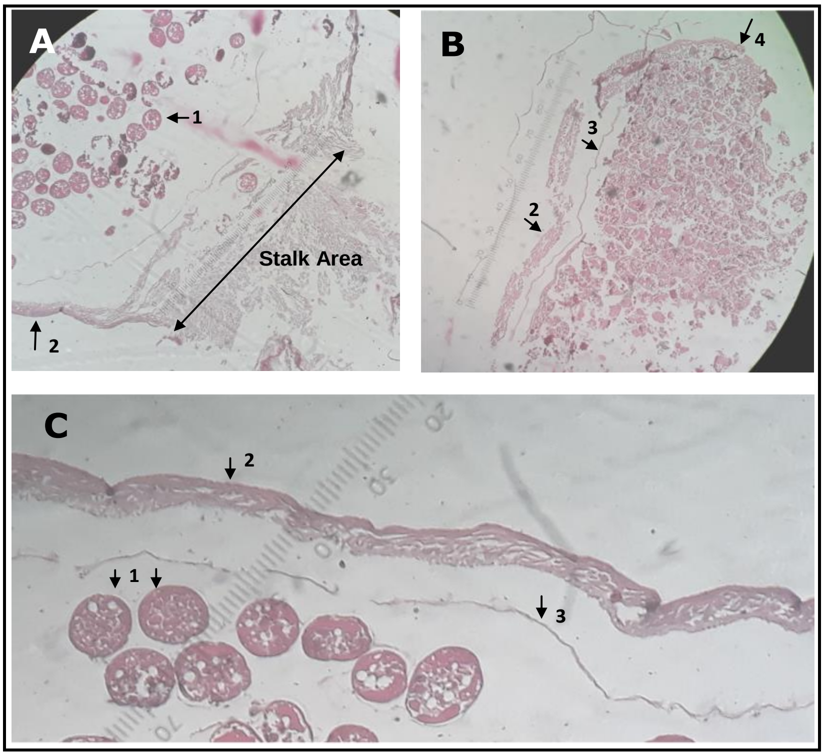

Figure 5. Cross-sections of externa of adult ectoparasite infecting mole crab (*Albunea symmysta*). 5A - cross-section of stalk areas; 4B and C - cross-section of a saccus; 1 - ovary; 2 - external mantel; 3 - internal mantel; 4 - visceral mass. Bar scales are in µm (original).

The phylogenetic tree showed that ectoparasite samples and reference species formed a monophyletic clade. The neighbor-joining (NJ) tree of the ectoparasite of mole crabs is illustrated in Figure 6.

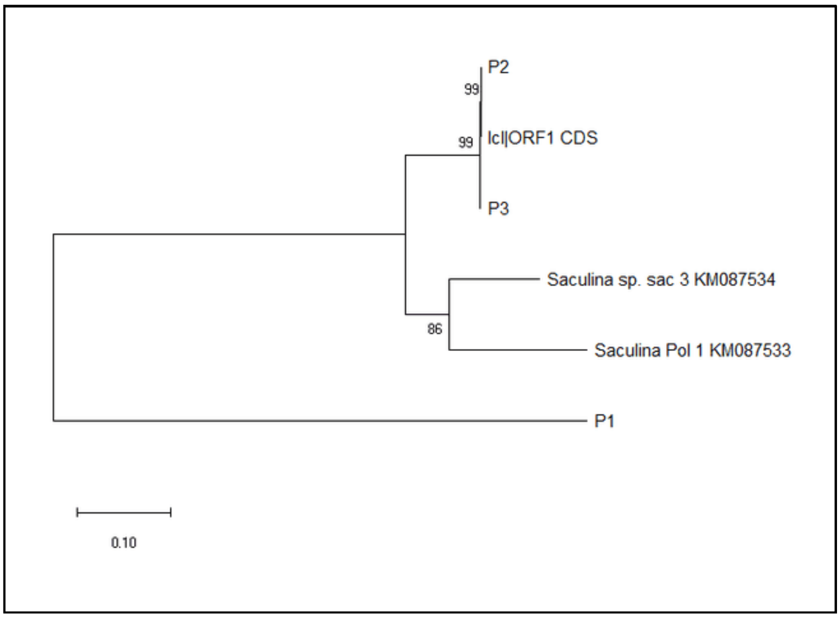

Figure 6. Neighbor joining tree of ectoparasites of mole crabs (*Albunea symmysta*).

Morphological identification of mole crabs and effect of infection. The characters of mole crab collected in Parangkusumo beach, Yogyakarta, are similar to those of *A. symmysta* previously reported by Bhagawati et al (2016) and Boyko & Harvey (1999).

Therefore, it can be determined that mole crab samples from Parangkusumo beach belong to *A. symmysta*. The rhizocephalan *Sacculina carcini* (Thompson, 1836) is a cosmopolitan parasite of many crabs species (Boschma 1972).

A detailed examination of infected *A. symmysta* from Parangkusumo beach showed reproductive disturbances. Infected individuals were unable to produce eggs. The condition is understandable because the energy that should be used for egg development is utilized by the parasite (Day 1935; Foxon 1940; Lützen 1985; Mouritsen & Jensen 2006). Previous studies proved that rhizochepala infection could alter host organisms in several ways. It has been reported that rhizochepala infection affects host behavior, energy requirement, reproductive traits, and survival (Sousa 1991; Moore 2002; Mouritsen & Poulin 2002; Lafferty & Kuris 2009; Larsen et al 2013).

Several mole crab species were found during the sampling (unpublished data). However, it was also found that only *A. symmysta* was infected by rhizocephala among mole crabs living in Parangkusumo beach, Yogyakarta. This indicated that there are hosts specific for *Sacculina* sp. It could be that *A. symmysta* has a broader carapace and telson compared to other mole crabs, such as *Hippa* spp. and *Emerita* spp. Moreover, *A. symmysta* also has a larger abdominal cavity, which makes them able to support parasite growth and develop and form saccus externa outside the host body (Boyko & Harvey 1999). The large telson of *A. symmysta* provides a protection for rizhocephala to complete their life cycle. The finding of rizhocephala in *A. symmysta* collected from Parangkusumo Beach, Yogyakarta, in this research is similar to the report of Hartnoll (1967).

Morphological and anatomical identification of the ectoparasites of mole crabs. Morphological observations showed that the ectoparasite had white externa, was oval and rounded, with a smooth surface. It had a short, tubular stalk, and the base of the stalk had a ring. The stalk was attached to the sternum between the host's abdominal joints. The position of the opening of the mantle aperture was opposite to the base of the stalk. The opening of the mantle aperture on the adult externa is visible in a circular shape. In this study, the sac (symmetry and asymmetry) form was described based on comparing the size of the sac on the right and left of the opening mantle aperture (Figure 3). The observed characters were similar to the characters of rhizochepala described by Boschma (1933, 1953, 1955), Hoeg & Rybakov (1992), and Kobayashi et al (2018).

The analysis using a micro-anatomical cross-sectional photomicrograph of the rhizocephala externa (Figure 4) in the stalk area (Figure 4A), showed an ovary, and an external mantle with a surface without burrs. Figure 4B-C presents a cross-sectional photomicrograph of the sac, showing the internal mantel and the visceral mass. The micro-anatomical feature was similar to the anatomy of *Sacculina* from the family Sacculinidae, which has been described by Boschma (1933, 1953, 1955), Hoeg & Rybakov (1992), and Kobayashi et al (2018). Therefore, in this report, the ectoparasite of mole crabs from Parangkusumo beach, Yogyakarta, was identified as *Sacculina*.

Molecular identification of ectoparasites. Ectoparasite samples could only be identified at the genus level, since they only had 80.98% identity to *Saaculina* sp. With this identity value, the ectoparasite could only be identified as *Sacculina* sp., because the identity was less than 95%. According to Lin et al (2015), specimens could be referred to as a single species if they have a minimum sequence identity of 95% to the reference species available in database. Moreover, it has been widely used that the barcoding gap for species determination in molecular identification was 5% (Candek & Kuntner 2015).

The monophyly indicated that they belong to a single genus (Figure 6). The lengths of the tree branches indicate they can only be identified up to the genus level. According to Xu et al (2015), specimens are considered a single taxon if they form a monophyletic group.

Conclusions. According to the morphological characteristics, the ectoparasite isolated from *A. symmysta* from Parangkusumo beach, Yogyakarta, Indonesia, belongs to rhizochepala. Microanatomy and molecular identification placed the ectoparasite into the *Sacculina* genus.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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Authors:
Dian Bhagawati, Faculty of Biology, University of Jenderal Soedirman, 63 Dr. Soeparno St., 53122 Purwokerto, Central Java, Indonesia, e-mail: dian.bhagawati@unsoed.ac.id
Agus Nuryanto, Faculty of Biology, University of Jenderal Soedirman, 63 Dr. Soeparno St., 53122 Purwokerto, Central Java, Indonesia, e-mail: agus.nuryanto.unsoed.ac.id
Aswi Andriasari Rofiqoh, Faculty of Biology, University of Jenderal Soedirman, 63 Dr. Soeparno St., 53122 Purwokerto, Central Java, Indonesia, e-mail: aswi.ar@unsoed.ac.id

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