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Manuscript

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To: zoobiomag2004@yahoo.com

To. Dr. Ioan Valentin Petrescu-Mag
Editor-in-Chief of AACL Bioflux Journal

Sent an original manuscript entitled First report of the Parasitic Crustacean Rhizocephala on Female Albunea symmysta from Indonesia, please publish it in the AACL Bioflux Journal. Thanks.

Greetings,
Dian Bhagawati
Faculty of Biology, University of Jenderal Soedirman, Indonesia

2 attachments

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Article title:
First report of the Parasitic Crustacean Rhizocephala on Female *Albunea symmysta* from Indonesia

Name of the authors:
Dian Bhagawati, Agus Nuryanto, Aswi Andriasari Rofiqoh

Hereby I would like to submit the manuscript entitled "**First report of the Parasitic Crustacean Rhizocephala on Female *Albunea symmysta* from Indonesia**" to Aquaculture, Aquarium, Conservation & Legislation - International Journal of the Bioflux Society.

This manuscript was not submitted or published to any other journal. The authors declare that the manuscript is an original paper and contain no plagiarised text. All authors declare that they are not currently affiliated or sponsored by any organization with a direct economic interest in subject of the article. My co-authors have all contributed to this manuscript and approve of this submission.

Corresponding author

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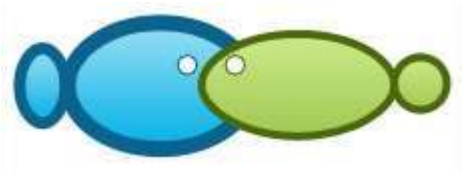
Date : 12th Oct, 2020



First report of the Parasitic Crustacean Rhizocephala on Female *Albunea symmysta* from Indonesia

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Abstrak. Several studies reported about Rhizochepala infection on mole crabs. There is no report about which Rhizochepala species infect mole crabs from Parangkusumo beach Yogyakarta Indonesia. This study provide the first report about Rhizochepala that infect mole crab from Parangkusumo beach Yogyakarta Indonesia. Rhizochepala was 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 to 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 Albune symmsyta from Parangkusuma beach Yogyakata, Indonesia was infected by Sacculina sp.

Keywords: identification, Rhizocephala, morphology, gen CO1, *Albunea symmysta*

Introduction. Rhizocephala is the most advanced parasitic Metazoan. It can attack its host, most of which is Decapoda, with a high attack rate (Brusca et al., 2017). 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 Rhizocephala attack has significant influences on its host population.

Rhizocephala's study to identify the genus Sacculina members based on anatomical characters and the external and internal cuticle's peculiarities was 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 el (2011, 2015). Recently, several studies have combined various taxonomic characters to identify and compile their phylogenetic, to obtain more accurate results (Glenner, 2003); Rybako & Høeg 2013; Lützen et al 2016; Kobayasi et al 2018).

Rhizocephala's infection of members of the crustacean group has been used by Høeg et al (2019) to rearrange their relationships. Previous phylogenetic studies using molecular characters have been conducted and end up with a fundamental revision of the Rhizocephala (Cirripedia) group. Those studies proved that Rhizocephala is varied widely 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 influences infected crustaceans' population. The resulting phylogeny will enable 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, 2019a; 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 (Lutzen et al 2016); *Portunus sanguinolentus* (HERBST 1783) (Raffi et al., 2012; Yang et al., 2014); mud crab Scylla olivacea (Kahar et al, 2016); and shrimp

Pandalina brevirostris (Nagler et al, 2017). Moreover, Elumalai et al (2014) reported that Sacculina spp infects several types of commercial marine crabs, e.g., Portunus sanquinolentus, Portunus hastatoides, and Charybdis feriatus. However, according to Chan (2004), research on Rhizocephala in the Asian region is still limited, including 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. *Albunea symmysta* (mole crab) specimens were collected from Parangkusumo beach Yogyakarta, Indonesia. Study sites were located at -7°59'15" to -8°1'58" and 110°16'52 " to 110°20'37" (Figure 1).

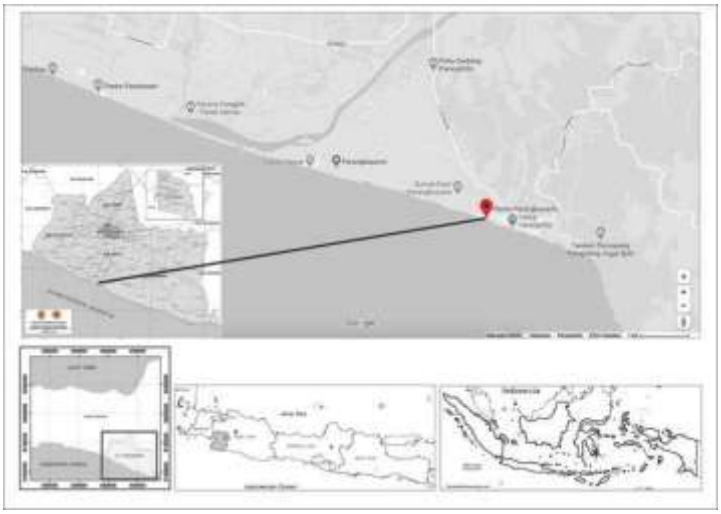

Figure 1. Study sites at Parangkusumo Beach Yogyakarta, Indonesia.

Mole crab samples were collected from May 2019 to March 2020. The specimens were directly caught accidentally using a hand from a sloping sandy beach is 1 km distance along the coast. *A. symmysta* crabs were sorted according to their sex and were carefully examined for the presence of parasites. The uninfected crabs were returned to nature after being examined, while the infected specimens were preserved for further observation. Infection by Rhizocephala is confirmed by the existence of an externa, which appears as a white sac on the host's stomach (Rees & Glenner, 2014). The body part of the obtained Rhizocephala was preserved in 96% ethanol for DNA analysis. Most of Rhizochepala body parts were preserved in 70% ethanol and 10% Neutral Buffered Formalin (NBF) solution for morphological and micro anatomic observations. The species-specific characteristics of the fixed and isolated externa are 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 observation. General morphological performance of two externa of Rhizocephala that infected A.symmysta was examined. The parameter consisted of shape, length, and width. 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 observation. Microanatomy preparation was 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 Rhizochepala. 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 Genetic 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 al1994). 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 parasites' 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 the ClustalW (Thompson et al., 1994). The taxonomic status was determined based on the sample's identity values compared to the sequence of conspecific references in 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 from Kabata (1985).

Result

Mole crab samples from Parangkusumo beach Yogyakarta have an almost rectangular carapace. It also has an ornament that resembles a monkey's face on the surface of its carapace.

Sixty-one specimens of mole crab were collected during the field trips. Sixty among 61 specimens of *mole* crab carries the eggs, which indicated that most of the samples were female. Only one individual was an adult male. Further examination proved that ectoparasite parasites infected seven out of 61 female individuals of mole crab. So, the prevalence was reached of 11.48%.

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

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

Figure 2. Externa of an adult ectoparasite on female mole crab (indicated by arrow). A is a fresh specimen of mole crab, and B is a preserved specimen of the mole crab.

The outer cuticle's surface of adult ectoparasite is smooth. Other characteristics were stalked with short tube-shaped and has a ring at the base. The externa of young individual ectoparasite is more rounded than the juveniles (Figure 3) and adults (Figure 4).

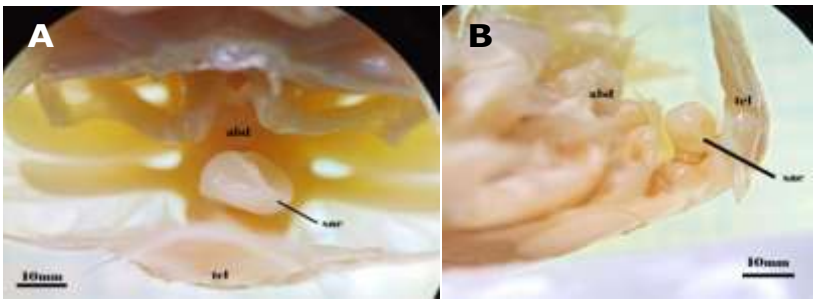

Figure 3. Externa of juvenile ectoparasite infecting female mole crab. A is the dorsal view of mole crab with ectoparasite, and B is the lateral view of mole crab with ectoparasite, abd is the abdomen, the sac is saccus, tel is telson. Scale bars: A=10 mm; B=10 mm (original)

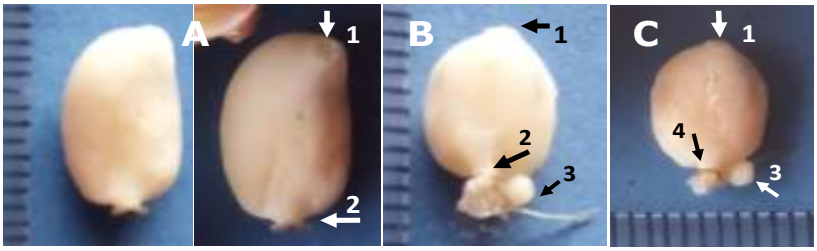

Figure 4. Externa of adult ectoparasite infecting mole crab. A is the asymmetry externa, B is a ventral view of symmetry externa, and C is the dorsal view of symmetry externa. 1 indicates mantle aperture opening, 2 indicates a stalk, 3 indicates a root, and 4 indicates a ring on stalk. Bar scale: A=1 mm, B=1 mm, C= 1 mm (original)

The morphometric measurement showed that the externa of the observed ectoparasite has variable sizes. The morphometric measure of the ectoparasite specimens is summarized in Table 1.

Table 1.

The size of externa of Rhizocephala infecting *A.symmysta*

Individu	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

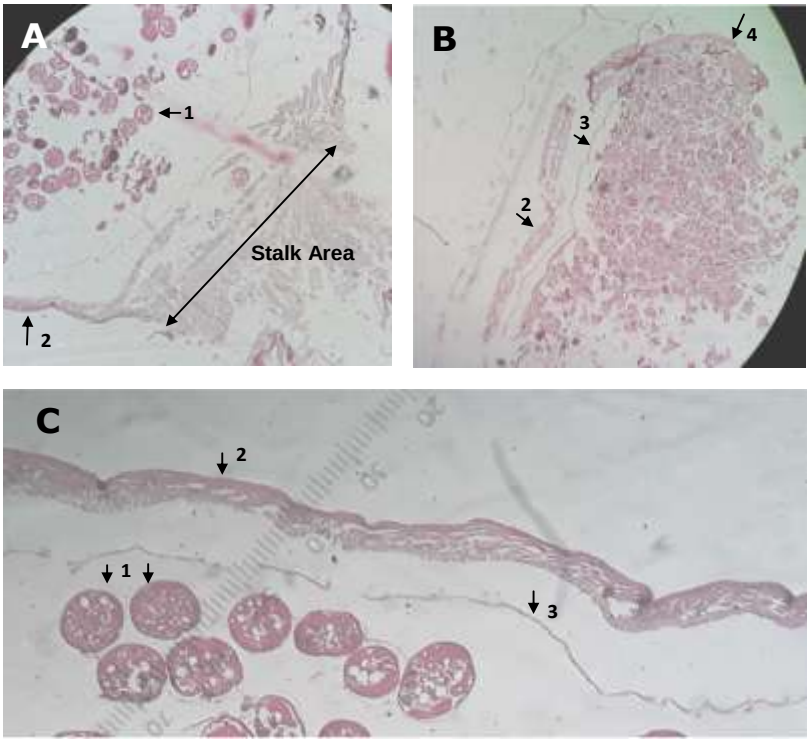

Figure 5. Cross-sections of externa of adult ectoparasite infecting mole crab. 5A is a cross-section of stalk areas, 4B-C are a cross-section of a saccus. 1 is Ovary, 2 is external mantel, 3 is internal mantel, 4 is visceral mass. Bar scales are in µm (original)

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

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

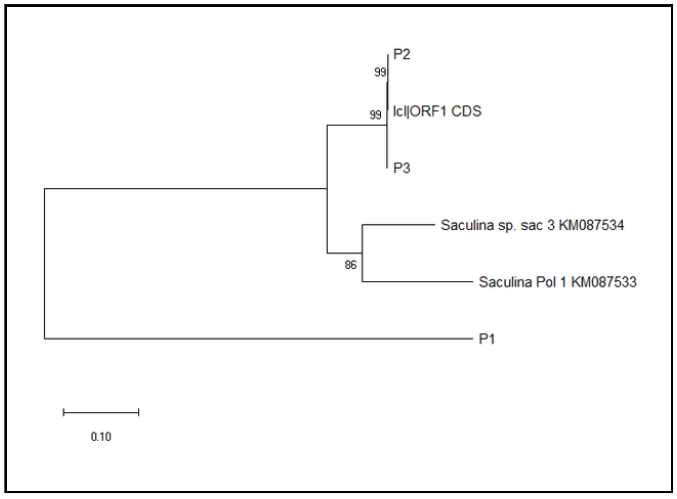

Figure 6. Neighbour Joining tree of mole crabs' ectoparasite.

Discussion

Morphological identification of mole crab and effect of infection. The characters of mole crab collected in Parangkusumo beach Yogyakarta are similar to Albunea symmysta that previously reported by Bhagawati et al. (2016) and Boyko & Harvey (1999). Therefore, it can be determined that mole crab samples from Parangkusumo beach Yogyakarta belong to *Albunea symmysta*. The rhizocephalan Sacculina THOMPSON is a cosmopolitan parasite of many crabs species (Boschma 1972).

Detail examination of infected *A.symmysta* from Parangkusumo beach showed reproductive disturbance. Infected individuals were unable to produce eggs. That condition is understandable because the parasite's energy for crab reproduction to grow and form saccus externa (Mouritsen & Jensen, 2006; Lützen, 1985; Foxon, 1940; Day, 1935). Previous studies proved that Rhizochepala infection could alter host organisms in several ways. It has been reported that Rhizochepala infection affects hosts' behavior, energy requirement, reproductive traits, and survival (Larsen et al., 2013; Lafferty & Kuris, 2009; Moore, 2002; Mouritsen & Poulin, 2002; Sousa, 1991).

It was also found that only *A.symmysta* was infected by Rhizocephala among mole crabs live in Parangkusumo beach, Yogyakarta. That condition 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, Albunea also has a larger abdominal cavity, which makes them able to support parasite growth and develop and form saccus externa outside the hosts' body. Meanwhile, Albuneas' large telson provides the best protection for saccus externa, which continuous warrant for the parasite to complete their life cycle. The finding of this research is similar to the report by Hartnoll (1967).

Morphological and anatomical identification of mole crabs' ectoparasite. Morphological observations showed that the ectoparasite has white externa, oval and rounded with a smooth surface. It has a short, tubular stalk, and the base of the stalk has a ring. The stalk is attached to the sternum between the host's abdominal joints. The position of the opening of the mantle aperture is opposite 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 character of Rhizochepala as described by Boschma (1933, 1953, 1955); Hoeg & Rybakov (1992), and Kobayashi et al (2018).

Further analysis using a micro-anatomical cross-sectional photomicrograph of the Rhizocephala externa (Figure 4.), visualizing in the stalk area (Figure 4A), there is an ovary (1), external mantle (2) with a surface without burrs. Figure 4B-C presents a cross-sectional photomicrograph of the sac, showing internal mantel (3); and visceral mass (4). The micro-anatomical feature was similar to the anatomy of Sacculina from the family Sacculinidae, which has been described by Boschma (1933, 1953, 955); Hoeg & Rybakov (1992), and Kobayashi et al (2018).Therefore, in this report, the ectoparasite of mole crab from Parangkusumo beach Yogyakarta is identified as Sacculina.

Molecular identification of ectoparasite. Ectoparasite samples could only be identified into the genus level since they only have an 80.98% identity to Saaculina sp. With that 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%. Moreover, it has been widely used that the barcoding gap for species determination in molecular identification was 50% (Candek & Kuntner 2015; Lin et al. 2015).

The monophyly indicated that they belong to a single group (genus, Figure 6). However, the tree branches' lengths 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 formed a monophyletic group.

Conclusion

According to the morphological characteristic, ectoparasite isolated from Albunea symmysta belongs to Rhizochepala. Microanatomy and molecular identification placed the ectoparasite into *Sacculina* sp.

Acknowledgment
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dian bhagawati <dian.bhagawati@unsoed.ac.id>

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Tudor Papuc <ptudor2008@yahoo.com> Fri, Oct 16, 2020 at 12:14 AM
Reply-To: Tudor Papuc <ptudor2008@yahoo.com>
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Dear Dr. Dian Bhagawati,

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Please find below the candidate of the reviewers
1. Prof. Yusli Wardiatmo, Bogor Agriculture University, ywardiatno@hotmail.com, yusli@ipb.ac.id
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Looking forward to hearing from you soon.

Thank you very much.

Best regards,
Dian Bhagawati

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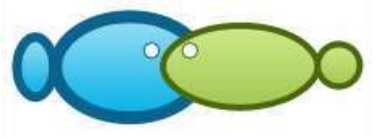

First report of the parasitic crustacean rhizocephala on female *Albunea symmysta* from Indonesia

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 infection in mole crabs. However, no report about which rhizochepala species infect mole crabs from Parangkusumo beach, Yogyakarta, Indonesia, has been found. This study provides 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: identification, Rhizocephala, morphology, gen CO1, *Albunea symmysta*

Comment [u1]: please change "infection" with "parasitism" throughout the paper, because infections refer to bacteria, viruses, and fungi; and in this case, the parasitic organism is more evolved

Comment [u2]: please add the scientific name in brackets

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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 2017). 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 2003; Rybako & Høeg 2013; Lützen et al 2016; Kobayashi et al 2018).

Comment [u5]: this is missing from references; please add it there too

Comment [u6]: this is not cited in references; but there is a reference that should be cited as Glenner et al 2003 in references, that is not cited; could this be it? please check and correct where necessary (in all places this citation appears)

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 (Cirripedia) group. 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).

Comment [u7]: this is an infraclass, while rhizocephala is a superorder; which one should have been revised? please do not use group as a taxonomic classifier, because it is not; please use the appropriate taxonomic classifier

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" to -8°1'58" and 110°16'52 " to 110°20'37" (Figure 1).

Comment [u8]: please check again the coordinates and specify the cardinal points

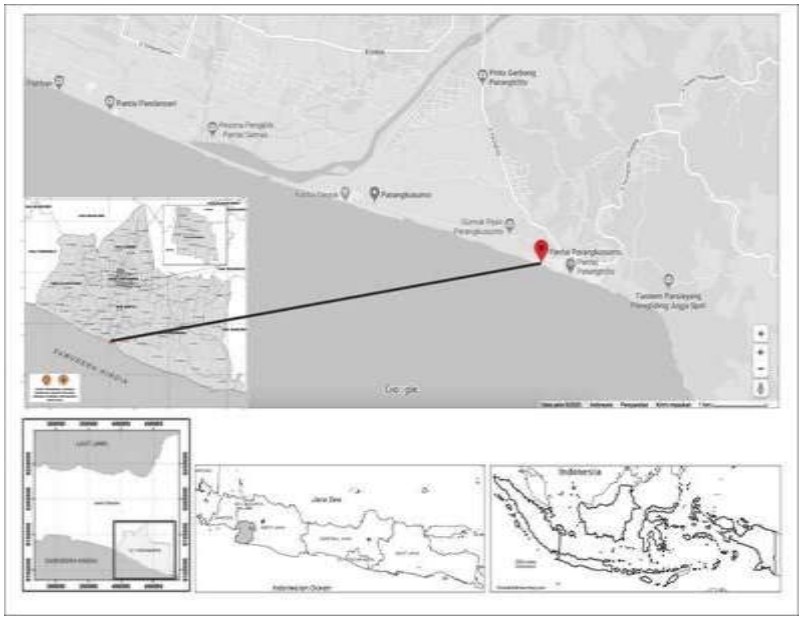

Figure 1. Study sites at Parangkusumo Beach Yogyakarta, Indonesia.

Mole crab samples were collected 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 and were 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 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.

Comment [u9]: how many trips?

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Comment [u11]: was it collected by hand? or did you used some instruments to collect the rhizocephala?

Morphological observations. The general morphological appearance of two externa of rhizocephala that infected *A. symmysta* was examined. The parameters consisted of

shape, length, and width. 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 Genetic 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. Mole crab samples from Parangkusumo beach Yogyakarta have an almost rectangular carapace. It also has an ornament that resembles a monkey's face on the surface of its carapace.

Sixty-one specimens of mole crab were collected during the study. Sixty among 61 specimens of mole crab carried eggs, 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 61 female individuals mole crabs. 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 (average of 2.92±0.06) and a width between 1.73 to 2.33 (average of 2.12±0.08). 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).

Comment [u12]: how did you make the measurements and with what instruments?

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Comment [u15]: you say above there were 60 females; please correct where necessary

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Comment [u17]: please add the measurement unit

Comment [u18]: you say above that 60 specimens out of 61 carried eggs and were females; you say 7 had parasites, and now you say that the 7 did not carry eggs, contradicting what you said before; please correct where necessary

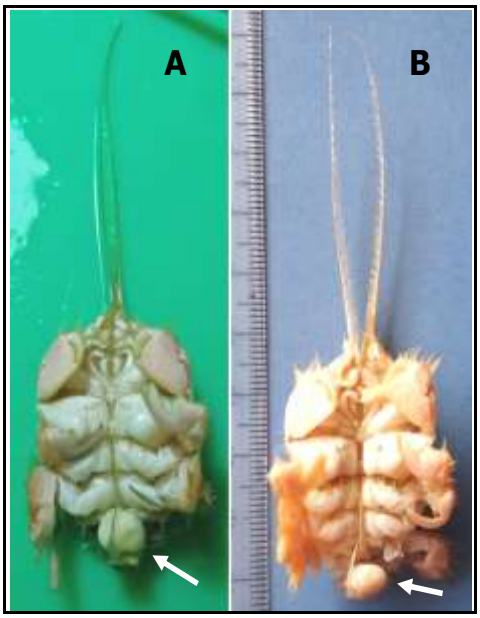

Figure 2. Externa of an adult ectoparasite on female mole crab (*Albunea symmysta*) indicated by arrow; A - fresh specimen of mole crab; B - preserved specimen of mole crab.

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).

Comment [u19]: please mention in Material and Method how you determined the age/life stage of the ectoparasites (young, juvenile, adult)

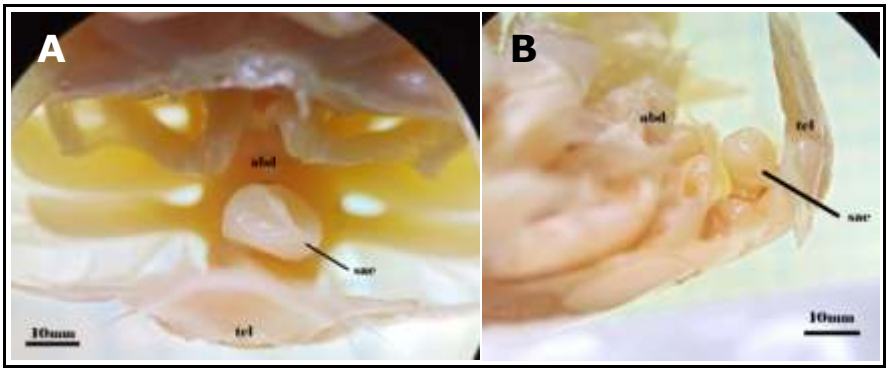

Figure 3. Externa of juvenile ectoparasites infecting female mole crabs (*Albunea symmysta*); A - dorsal view of mole crab with ectoparasite; B - lateral view of mole crab with ectoparasite; abd - abdomen; sac - saccus; tel - telson.

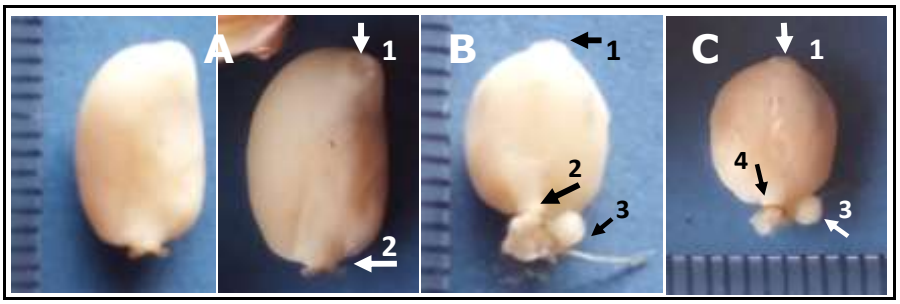

Figure 4. Externa of adult ectoparasite infecting mole crab (*Albunea symmysta*); A - asymmetry externa; B - ventral view of symmetry externa; C - dorsal view of symmetry externa; 1 - mantle aperture opening; 2 - stalk; 3 - root; 4 - ring on stalk. Bar scale: A=1 mm, B=1 mm, C= 1 mm.

The morphometric measurements showed that the externa of the observed ectoparasite had variable sizes (Table 1).

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

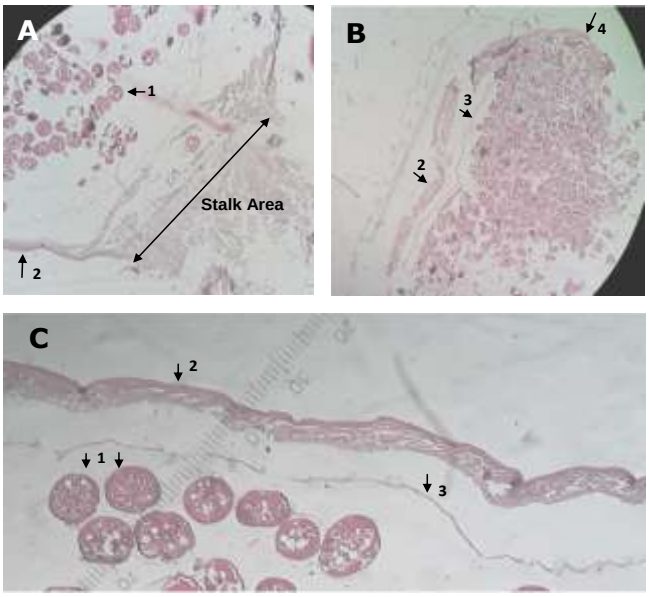

Figure 5. Cross-sections of externa of adult ectoparasite infecting mole crab (*Albunea symmysta*). 5A - cross-section of stalk areas; 4B and C - cross-section of a saccus; 1 - ovary; 2 - external mantel; 3 - internal mantel; 4 - visceral mass. Bar scales are in µm (original).

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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).

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.

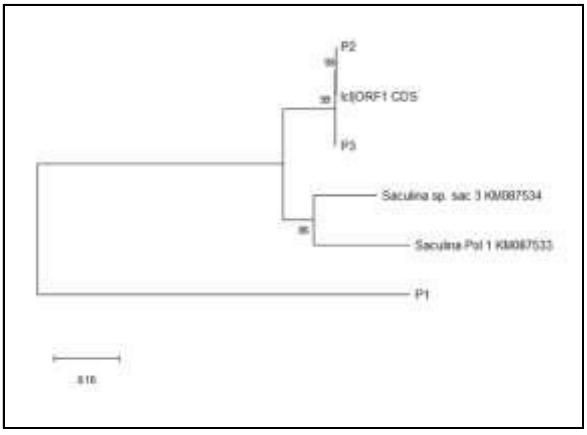

Figure 6. Neighbor joining tree of ectoparasites of mole crabs (*Albunea symmysta*).

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 THOMPSON 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 parasite's energy for crab reproduction to grow and form saccus externa (Mouritsen & Jensen 2006; Lützen 1985; Foxon 1940; Day 1935). 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 (Larsen et al 2013; Lafferty & Kuris 2009; Moore 2002; Mouritsen & Poulin 2002; Sousa 1991).

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, Albunea also has a larger abdominal cavity, which makes them able to support parasite growth and develop and form saccus externa outside the host body. The large telson of Albunea provides the best protection for saccus externa, which continuous warrant for the parasite to complete their life cycle. The finding of this research is similar to the report by 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%. Moreover, it has been widely used that the barcoding gap for species determination in molecular identification was 50% (Candek & Kuntner 2015; Lin et al 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

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rhizochepala. Microanatomy and molecular identification placed the ectoparasite into the *Sacculina* genus.

Acknowledgements. The author would like to thank the leadership of Jenderal Soedirman University, who approved the submission of fees for this research, as well as to the Chairperson of the Research and Community Service Institute, who had facilitated the implementation of this research (Contract number: T/387/UN23.18/PT.01.03/2020). Thank you to the editorial board and reviewers who have corrected, provided, and suggestions.

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

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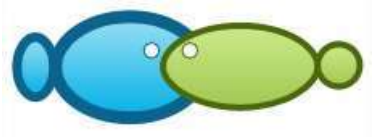

First report of the parasitic crustacean rhizocephala on female *Albunea symmysta* from Indonesia

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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 provides 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: identification, morphology, gen CO1

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*

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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).

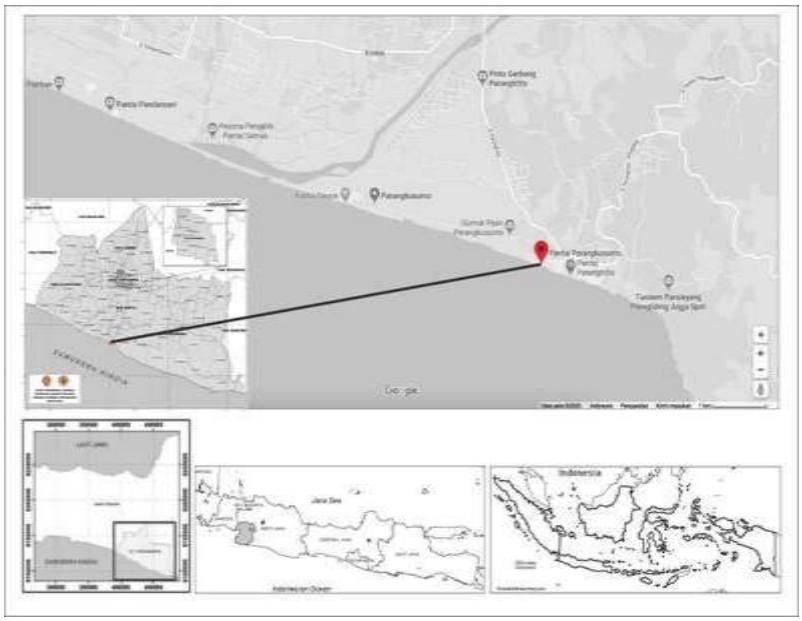

Figure 1. Study sites at Parangkusumo Beach Yogyakarta, Indonesia.

Mole crab samples were collected for 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. Female individuals have wider telson than male individuals. Each female individual 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 then 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 crabs' body (ectoparasite) and called externa, while young and juvenile live inside crabs' 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 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 Genetic 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.

Comment [u3]: this is missing from the references; please add it there too

Response: it has been added

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 has 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 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.

Comment [u4]: you say above that 60 specimens out of 61 carried eggs and were females; you say 7 had parasites, and now you say that the 7 did not carry eggs, contradicting what you said before; please correct where necessary

Response: has been corrected

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).

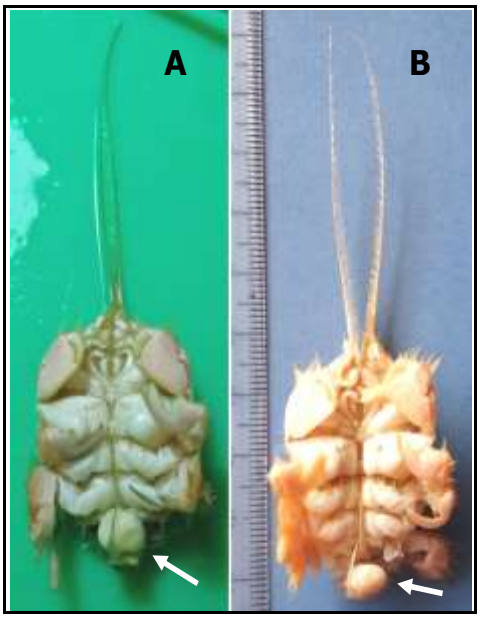

Figure 2. Externa of an adult ectoparasite on female mole crab (*Albunea symmysta*) indicated by arrow; A - fresh specimen of mole crab; B - preserved specimen of mole crab.

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).

Comment [u5]: please mention in Material and Method how you determined the age/life stage of the ectoparasites (young, juvenile, adult)

Response: has been added

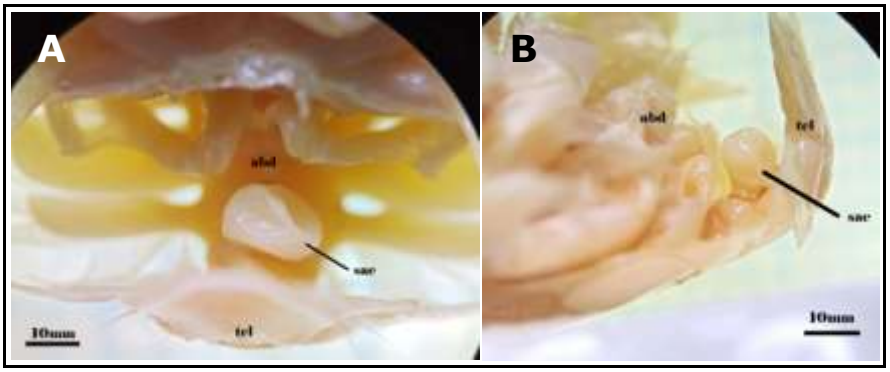

Figure 3. Externa of juvenile ectoparasites infecting female mole crabs (*Albunea symmysta*); A - dorsal view of mole crab with ectoparasite; B - lateral view of mole crab with ectoparasite; abd - abdomen; sac - saccus; tel - telson.

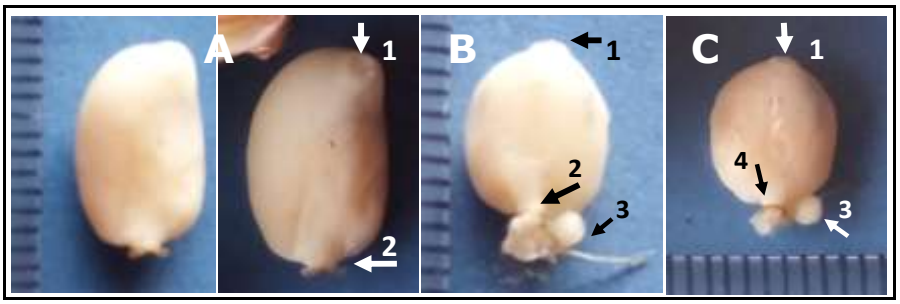

Figure 4. Externa of adult ectoparasite infecting mole crab (*Albunea symmysta*); A - asymmetry externa; B - ventral view of symmetry externa; C - dorsal view of symmetry externa; 1 - mantle aperture opening; 2 - stalk; 3 - root; 4 - ring on stalk. Bar scale: A=1 mm, B=1 mm, C= 1 mm.

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

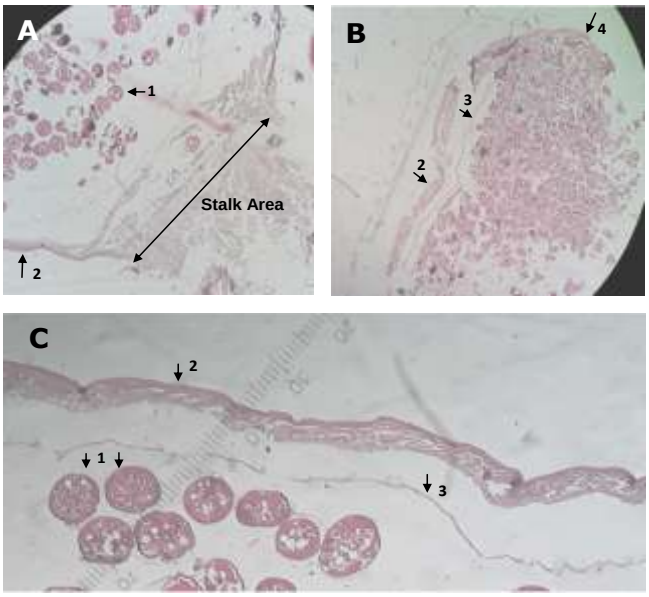

Figure 5. Cross-sections of externa of adult ectoparasite infecting mole crab (*Albunea symmysta*). 5A - cross-section of stalk areas; 4B and C - cross-section of a saccus; 1 - ovary; 2 - external mantel; 3 - internal mantel; 4 - visceral mass. Bar scales are in µm (original).

Comment [u6]: this figure is not mentioned in the text; please mention it in the text somewhere (before the actual figure)

Response: has been mentioned above Table 1.

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).

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.

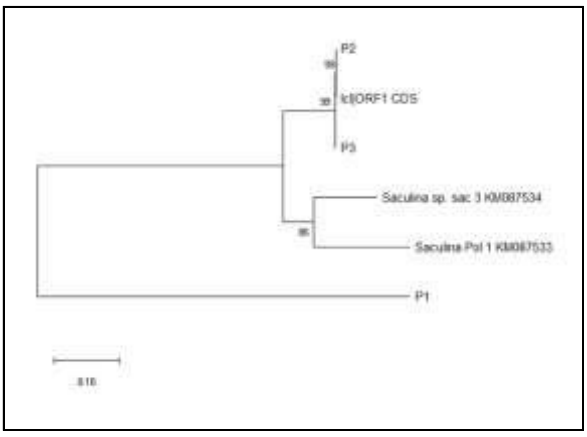

Figure 6. Neighbor joining tree of ectoparasites of mole crabs (*Albunea symmysta*).

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 energy for eggs' 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 was found during the sampling (Bhagawati et al., unpublished). 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 the best protection for rizhocephala to complete their life cycle. The finding of rizhocephala in A. symmysta collected from Parangkusumo Beash Yogyakarta in this research is similar to the report by 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.

Comment [u7]: something is incorrect here, because you say that for species level, it should be at least 95%; please check and correct where necessary (or rephrase and make it more understandable)

Response: has been corrected. 50% was a typo the correct one is 5% gaps

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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dian bhagawati <dian.bhagawati@unsoed.ac.id>

AACL Bioflux - manuscript reception confirmation

dian bhagawati <dian.bhagawati@unsoed.ac.id> Wed, Apr 21, 2021 at 8:49 AM
To: Tudor Papuc <ptudor2008@yahoo.com>

Thank you very much for your prompt reply.

Best wishes,
Dian Bhagawati
[Quoted text hidden]

dian bhagawati <dian.bhagawati@unsoed.ac.id>

AACL Bioflux - manuscript reception confirmation

dian bhagawati <dian.bhagawati@unsoed.ac.id> Wed, May 19, 2021 at 8:36 AM
To: Tudor Papuc <ptudor2008@yahoo.com>

Dear Dr. Tudor Papuc,,

Hope you are fine.
Just a short inquiry, how is the progress of our manuscript ?
I'm looking forward to hearing from you soon.
Thank you very much.

Best regards,
Dian Bhagawati
[Quoted text hidden]

dian bhagawati <dian.bhagawati@unsoed.ac.id>

AACL Bioflux - manuscript reception confirmation

Tudor Papuc <ptudor2008@yahoo.com> Fri, May 21, 2021 at 9:46 PM
Reply-To: Tudor Papuc <ptudor2008@yahoo.com>
To: dian bhagawati <dian.bhagawati@unsoed.ac.id>

You will receive the final version (probably without any more comments, just for a last check) at the start of next week.
So please expect to receive the paper by 25 May.

Best Regards,
Tudor Păpuc
Editor, Bioflux

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