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First microphological and molecular parasitological survey of *Benedenia* in humpback grouper (*Cromileptes altivelis*) of Lampung and Situbondo, Indonesia

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Abstract. *Amiin MK, Subekti S, Masithah ED, Nirmala D, Yunus M, Santanumurti MB, Rivaie AR. 2023. First microphological and molecular parasitological survey of* Benedenia *in humpback grouper (*Cromileptes altivelis*) of Lampung and Situbondo, Indonesia. Biodiversitas 24: 6858-6867.* Capsalid monogenean infections, such as *Benedenia*, affect grouper aquaculture production. *Benedenia* will cause lesions and skin damage which will cause fish prices to drop. Secondary infection from bacteria, viruses, and fungi by Benedenia triggers a slow growth rate and even mortality. *Benedenia* infection on grouper has been conducted in Indonesia, specifically in this study in Lampung and Situbondo. However, molecular identification of this parasite is still lacking. This study aims to determine the microscopic and molecular characteristics of *Benedenia* from the Hurun Bay waters of Lampung and the North Coast of the Java Sea, Situbondo. The main parameters observed in this study were DNA base sequence, morphology, and phylogenetic tree. *Benedenia* from Lampung (637 bp) and Situbondo (741 bp) in this study was confirmed by the morphological and molecular close as *Benedenia sargocentron* with nucleotides. Interestingly, the similarity was less than 98% with *B. sargocentron* with accession ID JN797597.1 (similarity of 97.15% from Lampung and 86.33% from Situbondo). It could be indicated as a new species of *Benedenia*. The prevalence of *Benedenia* in Lampung was 90%, while Situbondo showed 80% with 3 parasite/fish intensity for each location. The outcome of this study is the characteristics of *Benedenia* from Lampung and Situbondo were similar to *B. sargocentron* according to morphological and molecular identification. This is the first report of *B. sargocentron* from Lampung and Situbondo since no molecular identification has been done in those places to confirm their species. Further research by another molecular target apart from 28S rRNA should be needed to identify this parasite more accurately.

Keywords: 28S rRNA, aquaculture, *Cromileptis altivelis*, parasite

INTRODUCTION

The grouper is one of Indonesia's most valuable aquaculture commodities. Export data in 2019 show that grouper aquaculture production reached 16,641 tons (Wijayanto et al. 2023). Indonesia also ranks third in global grouper production after China and Taiwan (Megarajan et al. 2022). The grouper is a popular consumption fish for cultivation because of its high price, good taste, and fast growth, and the community well-understands the cultivation technology (Hudaidah et al. 2022). Lampung and Situbondo have high production in Indonesia, especially the humpback grouper (*Cromileptes altivelis* Valenciennes, 1828). The Ministry of Marine Affairs and Fisheries, under the Director General of Aquaculture, appointed the technical implementation unit of the Lampung Marine Aquaculture Center (BBPBL) and Situbondo Center for Brackish Water Aquaculture (BPBAP) to produce humpback grouper (Ardiyanti and Subekti 2022). Humpback grouper was one of the major Indonesian fish exports in 2017 and was exported to many countries, such as Malaysia, Japan, and the United States (Food and Agriculture Organization 2018). However, in successful aquaculture, both locations often suffer from water pollution that harms humpback grouper farmers. Many industries in Lampung and Situbondo still dump waste in the water, which harms water quality and causes mass fish deaths in farmers' cages (Subekti et al. 2021). According to the Regional Environmental Status Data of Lampung and East Java Provinces (2018), manufacturing industries in Lampung and Situbondo, such as tapioca flour, palm oil, animal feed, food and beverages, fish processing, rubber, estates, agriculture, and mining are directly responsible for environmental and marine water pollution. A report from The Lampung Province Regional Environmental Status (2018) reveals that the BOD

(Biological Oxygen Demand) value of Lampung Bay shows a high result (15.88-18.87 mg/L). This value is higher than the standard parameter of BOD based on the Minister of Environment Regulation No. 51 of 2004 concerning Sea Water Quality Standards (at 10 mg/L). Another study on water quality in Situbondo showed that the nitrates value was very high (6.92 ppm), exceeding the quality standards set of 0.12 ppm (Anrosana and Gemaputri 2017).

The impact of industrial waste also causes floating net cages to become dirty so that the residue of feed that fish do not consume and the products of fish metabolic activities (feces and urine) cannot be decomposed, which results in the appearance of various diseases, especially from parasitic worms (Anrosana and Gemaputri 2017; Subekti et al. 2021). The previous study stated that polluted waters trigger fish stress and increase their susceptibility to pathogens, especially *Benedenia* (Hassan et al. 2015; Fahmy et al. 2022). *Benedenia* is a capsalid monogenean worm parasite in fish (Nitta 2023). The fish losses due to this capsalid monogenean parasite infestation are less than other pathogenic organisms such as viruses and bacteria. However, it can be one of the predisposing factors for infection with more dangerous pathogenic organisms (Tedesco et al. 2021). *Benedenia* will cause itching, lesions, and skin damage which will cause fish prices to drop (Whittington and Chisholm 2008). *Benedenia* also triggers secondary infections such as bacteria, viruses, and fungi that enter the fish's body through wounds (Antushevich 2020). Infected fish will experience a lack of appetite, slow growth, and even mortality (Hoai 2020). A previous study stated that *Benedenia epinepheli* infections were found in groupers up to 57.1% in aquaculture and 51.4% in wild fish (Rückert et al. 2010). The parasite cases from *Benedenia* on grouper need more awareness since it will reduce grouper quality and production (Fusianto et al. 2021; Harjuni et al. 2023).

Understanding this parasite is important to prevent low production on humpback grouper aquaculture. Information regarding biology, physiology, biochemistry, and molecular will support the development of fisheries in Indonesia, especially humpback grouper aquaculture. Molecular data for parasite species differentiation is used in distinguishing species with high rates of morphological similarity. The lack of sufficient monogenean-parasite DNA sequence information has hampered using molecular data for parasite species characterization in Indonesia. Previous studies of *Benedenia* infestation in Lampung did not use molecular identification (Sumino et al. 2017). Likewise, the identification of *Benedenia* in Situbondo so far only knowing the prevalence and intensity of parasitic attacks (Wiyatno et al. 2012; Wijaya and Subekti 2019). This study focuses on the disease caused by *Benedenia* in humpback grouper in Lampung and Situbondo. Losses due to *Benedenia* infestation are not as great as losses due to infection with other pathogenic organisms such as viruses and bacteria. Still, ectoparasite infestation can be one of the predisposing factors for infection with more dangerous pathogenic organisms. Other nonlethal losses can include skin damage and slow growth, reducing selling value (Wijaya and Subekti 2019). This study is the first microscopic and molecular parasitological survey of *Benedenia* in humpback grouper (*C. altivelis*) of Lampung and Situbondo (Indonesia). This study aims to determine the morphological and molecular characteristics of *Benedenia* from Lampung and Situbondo. Morphology is the determination key by observing the body shape, while molecular is the determination key by observing the sequence of nitrogen bases in DNA (Prasad 1997; Ahmad et al. 2019). Information related to morphology and molecular can be used as a key determination in identifying living creatures; in this research, it is *Benedenia*.

MATERIALS AND METHODS

Collection site for samples and data

This research was conducted from May to October 2019. Sampling was conducted in Hurun Bay, Lampung, Indonesia (5°31'43.2"S 105°15'13.9" E), and the north coast of the Java Sea, Situbondo, East Java, Indonesia (7°41'06.2"S 113°50'32.9" E) (Figure 1). Furthermore, for parasite identification, 20 randomly grouper samples from each Lampung and Situbondo (40 fish total) with 16.5-24 cm length and 86-206 g weight (5 months old, reared for 3 months) were taken by purposive sampling with the size and weight of the fish determined by the authors. The random groupers taken for this study were those that have the characteristics of sick fish such, as slow movement, low appetite, incomplete limbs, and weak body (Abbas et al. 2023). Sample preparation, morphological identification, and Polymerase Chain Reaction (PCR) process of *Benedenia took* place in the PCR laboratory, Institute Tropical of Disease Center, Universitas Airlangga, Surabaya, East Java, Indonesia. Sequencing tests of *Benedenia* specimens were carried out at 1st Based Singapore through the Professor Nidom Foundation Laboratory Surabaya, Indonesia. Prevalence and intensity of *Benedenia* were evaluated as a previous study procedure (Mahasri et al. 2020). Prevalence is the percentage of infested fish compared to the total sample of fish examined. Meanwhile, intensity is the average total of parasites per infested fish. Prevalence can be measured using the formula as follows:

$$\text{Prevalence} = \frac{n}{N} \text{ x } 100\%$$

Where:
Prevalence : percentage of infested fish (%)
n : total of infested fish (individuals)
N : total of fish examined (individuals)
Intensity can be measured using the formula as follows:

$$\text{Intensity} = \frac{\Sigma p}{N}$$

Where:
Intensity : parasite infestation (parasites/fish)
∑p : total parasite found (parasites)
N : total of fish samples infected with parasites (fish)

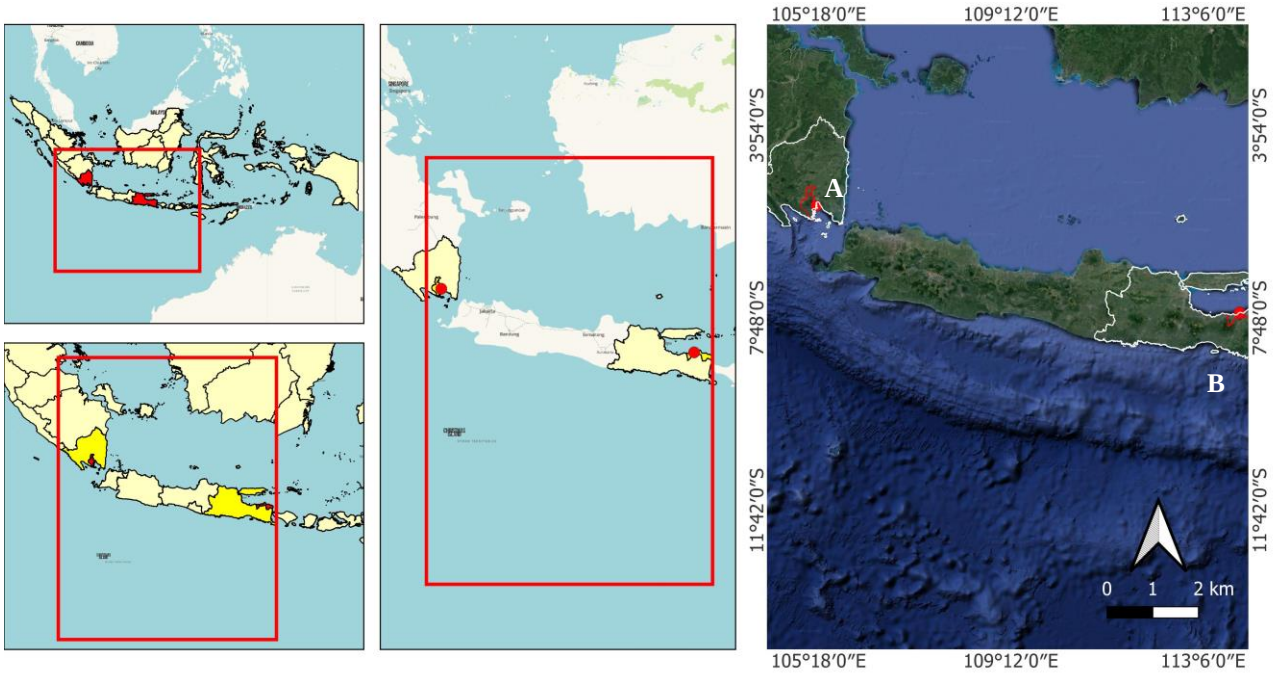

Figure 1. Sampling location A. Hurun Bay, Lampung, Indonesia; B. Situbondo, East Java, Indonesia

The results of measuring the percentage of helminth infestation found in the study were determined based on the prevalence and intensity analysis results and then categorized according to the reference of Williams and Bunkley-Williams (1996). The categories of level of prevalence and level of infestation are as follows in Tables 1 and 2.

Tools and materials

The tools used in this study were a Thermal cycler, microcentrifuge, freezer, transilluminator, micropipette, micro tube, incubator, tissue rupture, analytical balance, measuring cup, test tube, tweezers, time meter, and computer with ClustalX program. The materials used in this study were Trisol 750 µL, tissue samples, 100% ethanol 225 µL, Red Phenol Chloroform 150 µL, sodium citrate 750 µL, 70% ethanol 1.5 mL, 8mM NaOH 200 µL, Mastermix ml, 10x buff 5 µL, dNTP 5 µl, $MgSO_4$ 2 µL, forward primer 2 µL, reverse primer 2 µL, Template DNA 5 µL, DDW 28 µL, and KOD ver 2 1 µL, TBE 1x and 1% agarose, Molecular DNA marker, and Aquadestilata, sequencing mixture, Ethylenediaminetetraacetic Acid (EDTA) 125 mM, absolute ethanol, 70% ethanol, sterile 1.5 mL Eppendorf tubes, phase wrap and aluminum foil. The primer used in this study was 28S rRNA genes (De Silva et al. 2021).

Morphological identification

The dorsal, pectoral, anal, and caudal fins, as well as the body surface of groupers were scraped to get *Benedenia*. *Benedenia* were preserved with NaCl on a microtube for morphological identification. Semichoen Acetic Carmine staining was used to check its anterior attachment organ, eye spot, pharynx, penis, gland, ovary, testis, opisthaptor, accessory sclerite, anterior hamuli, and posterior hamuli (Pratama et al. 2023). *Benedenia* was identified using Kabata's identification key book (Kabata 1985). This guideline is still used since no recent update for parasite identification, even though some recent studies are using it (Johnson et al. 2019; Prastowo et al. 2023). After morphological identification, the worm samples were placed in microtubes containing sterile distilled water and stored in a freezer at -26°C for further extraction and PCR processing.

Table 1. The category of level of prevalence of helminth infestation

Level of prevalence	Notes	Prevalence (%)
Always	Very heavy	99-100
Almost always	Heavy	90-98
Usually	Medium	70-89
Very often	Very often	50-69
Generally	Moderate	30-49
Often	Often	10-29
Sometimes	Occasionally	9-1
Seldom	Light	0.1-<1
Very rarely	Very light	0.001-<0.1
Almost never	No infection	<0.001

Table 2. The category of level of infestation of helminth infestation

Level of infestation	Categories
<1	Very light
1-5	Light
6-10	Moderate
>10-50	Medium
51-100	Heavy
>100-999	Super heavy
>1,000	Superinfection

Molecular identification

This method is carried out to ensure that the parasite found was *Benedenia epinepheli*. The morphological method to determine the fish parasite use is the traditional method that is still practiced (Eszterbauer et al. 2006). Unfortunately, this method is subjective (depending on taxonomic information and the taxonomist experience) (Will and Rubinoff 2004). It will affect the accuracy of the identification activity. One method that can be used to identify fish more accurately is molecular DNA barcoding. DNA Barcoding is an identification method for living organisms, including fish parasites, using specific genes (Bouguerche et al. 2021). This study was an observational exploratory study by sampling at a direct location with a molecular approach. The sample material used in this study was capsalid monogeneans worm preserved in a sterile distilled water solution from infected grouper. The procedures were DNA extraction from capsalid monogenean worms, DNA isolation through PCR, DNA purification, and sequencing. The results of PCR amplification were purified using a DNA purification kit (Qiagen) and sequencing of the 28S rRNA gene as used in the previous study (De Silva et al. 2021). The cycle PCR setting was according to a previous study with conditions of 35 cycles of 95°C for 15 minutes (predenaturation), 35 cycles of 94°C for 30 seconds (denaturation), 55°C for 30 seconds (annealing), 72°C for 30 seconds (first extension), and 72°C for 5 minutes (final extension) (Subekti et al. 2021). The mixture containing 10x buffer PCR 1x, dNTP mix 0.2 mM, Primer F, and R 0,8 mM each, Taq Polymerase 0.02 U/µL, 2µL DNA template, then added milliQ to provide a reaction volume of 20 µL. For control, a master mix (GoTaq® Green Master Mix, Wisconsin) without DNA was used. Visualization was performed after electrophoresis using 1% agarose for 25 minutes and staining with Ethidium Bromide for 10-20 minutes for 40-60 minutes.

Furthermore, visualization was carried out on the UV-Transiluminator, and documentation was carried out. Pure DNA from capsalid monogenean Lampung and Situbondo was carried out at 1st Base Laboratories Singapore (ABI 3730 DNA Sequencer) using the same primers during PCR amplification. The result was compared with Genebank for phylogenetic tree analysis.

Data analysis

The relationship between *Benedenia* was established using the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) based on 28S rRNA sequences. The phylogenetic tree was created using Molecular Evolutionary Genetics Analysis (MEGA) 11 according to the Neighbor-joining (NJ) method to assess the robustness of the inferred relationships for *Benedenia* spp. worms. Molecular data of 13 other monogeneans for phylogenetic trees were obtained from GeneBank with the highest similarity and different species to our samples, and their relationship was then compared. The research data are presented as tables and figures and then described descriptively.

RESULTS AND DISCUSSION

Prevalence and Intensity

The result showed that 54 helminths were suspected to be *Benedenia* from Lampung, while 48 helminths of suspected *Benedenia* were suspected from Situbondo Sea, Indonesia (Table 3). The prevalence of *Benedenia* from Lampung was 90%, while the results from Situbondo were 80%. The prevalence results indicated that the *Benedenia* pathogenicity level in Lampung was categorized as having a heavy level (90-98%) of pathogenicity, while Situbondo was categorized as having a medium level (70-89%) of pathogenicity. This high prevalence can be caused by the waters there being polluted by manufacturing industries, such as tapioca flour, palm oil, animal feed, food and beverages, fish processing, rubber, estates, agriculture, and mining, which are directly responsible for the environment and marine water pollution (Regional Environmental Status Data of Lampung and East Java Provinces 2018). Especially Lampung, which has a higher prevalence value than Situbondo; this is because there are many residential areas in Hurun Bay, Lampung, so anthropogenic activity also increases marine pollution in addition to manufacturing industries (Estigade et al. 2019).

Previous studies stated that marine pollution causes fish to become stressed and weakened, making *Benedenia* attacks more intensive (Hassan et al. 2015). According to Novriadi et al. (2014), supporting factors for *Benedenia* are also related to salinity, low oxygen, or high ammonia. The prevalence of *Benedenia* in groupers in Situbondo previously reached 53.33% in 2012 and 80% in 2019 (Wiyatno et al. 2012; Wijaya and Subekti 2019). The prevalence value in this study was higher than in 2012 but the same as in 2019 for the Situbondo area. Previous research in Lampung showed that the prevalence value of *Benedenia* was less than 40% in 2010 and 32% in 2017 (Rückert et al. 2010; Sumino et al. 2017). The prevalence value in this study is greater than in the previous report. The intensity of *Benedenia* from Lampung and Situbondo was 3 parasites/fish and was categorized as low (Mahasri et al. 2020). These results indicated that *Benedenia* in both locations infested an average of 3 fish in each floating net cage. Previous studies of *Benedenia* detection in Situbondo did not contain intensity results, only prevalence (Wiyatno et al. 2012; Wijaya and Subekti 2019).

So, this study complements the prevalence results reported in previous studies. For Lampung, the intensity of *Benedenia* was less than 5 parasites/fish in 2010 and 3.33 parasites/fish in 2017 (Rückert et al. 2010; Sumino et al. 2017). The intensity value in this research is less than thst of the 2017 report. The lower number of intensities may be due to *Benedenia* in this study were from humpback grouper (*C. altivelis*). Previous studies related to *Benedenia* in Lampung were carried out on *Epinephelus fuscoguttatus* Forsskål, 1775 or brown-marbled grouper (Rückert et al. 2010) and *Epinephelus* sp. (Sumino et al. 2017). This study finding was the first *Benedenia* from humpback grouper in Lampung.

Table 3. Parasite *Benedenia* was found on humpback grouper (*C. altivelis*) in this study

Location	Sample	Length (cm)	Weight (g)	Found parasite	Capsalid monogenean parasite (parasite/fish)
Lampung	1	24	170	*Benedenia*	2
	2	23	165	-	0
	3	24	149	-	0
	4	19.5	86	*Benedenia*	3
	5	25	166	*Benedenia*	4
	6	23.5	147	*Benedenia*	2
	7	23.5	206	*Benedenia*	2
	8	20	99	*Benedenia*	2
	9	21	139	*Benedenia*	6
	10	22	135	*Benedenia*	4
	11	24	120	*Benedenia*	2
	12	19	100	*Benedenia*	3
	13	17	60	*Benedenia*	2
	14	24	178	*Benedenia*	2
	15	21	120	*Benedenia*	4
	16	22	136	*Benedenia*	5
	17	23	121	*Benedenia*	2
	18	20	154	*Benedenia*	3
	19	19.5	95	*Benedenia*	4
	20	24	194	*Benedenia*	2
∑ parasite					54
Prevalence (%)					90
Intensity (parasite/fish)					3
Situbondo	1	22	139	*Benedenia*	3
	2	19	91	*Benedenia*	3
	3	19	87	*Benedenia*	4
	4	17.5	67	-	0
	5	18	57	-	0
	6	19	113	-	0
	7	17.5	66	*Benedenia*	3
	8	20	87	*Benedenia*	2
	9	18.5	92	*Benedenia*	3
	10	20	96	*Benedenia*	2
	11	23	153	*Benedenia*	3
	12	20.5	102	*Benedenia*	3
	13	22.5	143	*Benedenia*	2
	14	19.5	92	*Benedenia*	3
	15	23	148	*Benedenia*	3
	16	21	105	-	0
	17	22	127	*Benedenia*	4
	18	21	105	*Benedenia*	3
	19	16.5	50	*Benedenia*	3
	20	19	77	*Benedenia*	4
∑ parasite					48
Prevalence (%)					80
Intensity (parasite/fish)					3

DNA identification

After native examination and morphometric measurements of *Benedenia* from Lampung and Situbondo, the next step was DNA identification. The results of this study showed that a fragment of 637 bp of *Benedenia* from Lampung was successfully amplified. The percentage of bases in the ITS 28S rRNA gene sequence of *Benedenia* from Lampung waters were adenine 28.41%, guanine 24.01%, thymine 29.51%, and cytosine 18.05%. The results of DNA barcoding were then compared with the existing database at NCBI. A comparison of the parasite sample from Lampung with *Benedenia* registered in NCBI is shown in Table 4. The nearest relationship of this sample was *Benedenia sargocentron* from China (JN797597.1), with 96.15% similarity and 77% coverage. This result indicated that *Benedenia* found in Lampung was *B. sargocentron*, although the query coverage of this sample was quite low at 77%.

This study also showed that a fragment of 741 bp of *Benedenia* from Situbondo was successfully amplified. This sample had a percentage of adenine base of 28.20%, guanine base of 24.15%, thymine base of 27.93%, and cytosine base of 19.70%. A comparison of parasite samples from Situbondo with *Benedenia* registered in NCBI is shown in Table 5. The nearest relationship of this sample was *B. sargocentron* from China (EU707803.1), with 86.33% similarity and 91% coverage. The phylogenetic tree of *Benedenia* from Hurun Bay Lampung and the north coast of the Java Sea Situbondo was presented in Figure 2.

The advantage of DNA barcoding is its very high accuracy compared to the traditional morphological method (Nehal et al. 2021). The molecular identification results showed that this study's samples were close to *B. sargocentron*. This is because the two samples are similar to *B. sargocentron* with accession ID JN797597.1. However, the similarity was less than 98% (96.15% from Lampung and 86.33% from Situbondo); it could be indicated as a new type of *Benedenia*. Furthermore, using the NJ method, the phylogenetic tree result (Figure 4) showed that our samples were in the same clade as other *Benedenia*. NJ method was used to reconstruct the phylogenetic tree according to evolutionary distance and, generally, showed better results than others (Lima et al. 2022).

The phylogenetic tree also indicated that *Benedenia* from Lampung and Situbondo have the same clade as *Benedenia hoshinaii* (LC718905.1) and *B. sargocentron* (JN797597.1). This research is the first molecular identification for *Benedenia* in Lampung and Situbondo, and the parasite found in the study can be classified as a new species. The 28S rRNA gene was successfully used to identify *Benedenia* species, which are pathogenic to marine fish and cause epizootic events. Epizootic is a rapidly spreading disease that is temporary or quickly grows in vast numbers within a wide area (Dang et al. 2011). The 28S rRNA gene can explain unique biological features such as accessory sclerites, taxonomy, host association, and geographical distribution of *B. epinepheli* (Subekti et al. 2021). Further research needs to be carried out using the 16S rRNA, 18S rRNA, or COI gene, which are also useful for molecularly detecting this parasite (Sepúlveda and González 2014; Turgay et al. 2019).

Morphometric identification

Semichoen Acetic Carmine staining used morphological identification to ensure the *Benedenia* species (Figures 3 and 4). The details of the morphological length of *Benedenia* are presented in Table 6. According to Kabata's identification key book, the results showed that the parasite in Lampung and Situbondo was *Benedenia*. The morphology of *Benedenia* from Lampung found in this study was 2.55±1.54 mm long with a width of 1.09±0.58 mm, while from Situbondo, it was 5.15±2.93 mm long with a width of 2.00±1.09 mm.

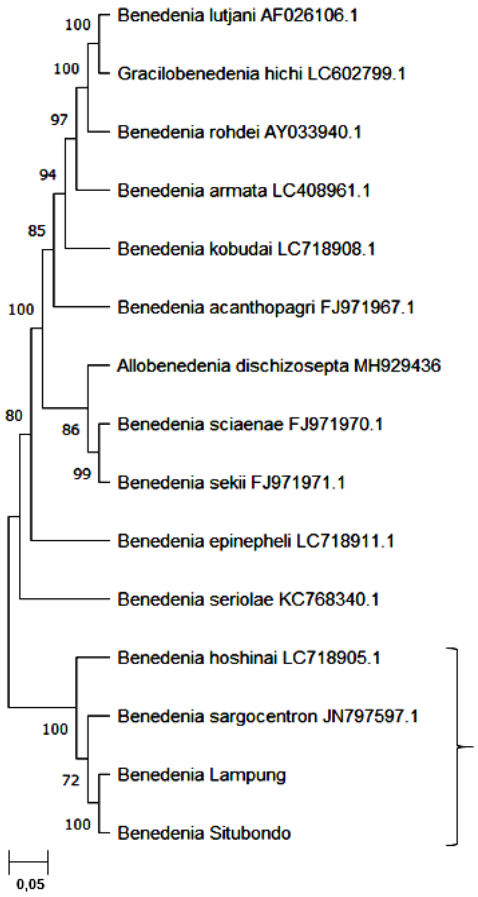

Figure 2. Phylogenetic tree of *Benedenia* from Lampung and Situbondo

Benedenia had two pairs of eye spots on the anterior, with anterior eye spots smaller than the posterior. In the body's posterior part, an opisthaptor was equipped with hooks. The opisthaptor was oval, not insulated, shaped like a plate, and equipped with three pairs of large and small hooks with as many as 14 pieces on the edge of the opisthaptor. Anterior hamuli (anchor) are strongly curved at their point. The posterior hamuli, with a much smaller point, was located almost at the posterior end of the haptor and overlapped the anterior hamuli. It had sucker pairs at the anterior end of the body and was ovoid. Two pairs of eye spots were found behind its anterior suckers. Its pharynx was rounded with several incisions. Two ovoid testes were detected in the exact center of the body. Two efferent vases emerge on the testes' inner side and soon unite to form the vas deferens. The vas deferens advance anteriorly through the left side of the ovary, vitelline reservoir, and receptaculum seminis and finally lead to the base of the cirrus without forming vesicula seminalis. The ovary was located in front of the testis. This can be observed from its oval and flattened shape (Dewi et al. 2018).

Moreover, *B. epinephelii* also had a pair of antennae at the front and a haptor at the back (Rumondang et al. 2022). *B. epinepheli* is a skin worm parasite from the Platyhelminthes phylum, Trematoda Monogenea class, and the genus *Benedenia* (Jithendran et al. 2005). This parasite attacks fish's gills and skin (Turgay et al. 2019). This parasite is reported to attack humpback groupers in Indonesia (Sudewa et al. 2020; Subekti et al. 2021). This parasite even attacks other grouper species, such as *Epinephelus* spp., *Epinephelus coioides* Hamilton, 1822, and even hybrid grouper (*Epinephelus fuscoguttatus* x *Epinephelus lanceolatus*) (Lestari et al. 2020; Fusianto et al. 2021; Rumondang 2022).

Table 4. Species identification of *Benedenia* from Lampung and its similarity

Species	Host	Location	Accession ID	Query coverage (%)	Percent identity (%)
				***Benedenia*, Indonesia: Lampung**	
B. sargocentron	*Sebastes schlegeli*	China	JN797597.1	77	96.15
B. hoshinai	*Oplegnathus fasciatus*	Japan: Hiroshima	LC718905.1	77	93.93
B. seriolae	*Seriola quinqueradiata*	Japan	KC768342.1	69	76.61
B. epinepheli	*Epinephelus akaara*	Japan: Hiroshima	LC718911.1	72	74.63
B. armata	*Lethrinus haematopterus*	Japan: Nagasaki, Danjo Islands	LC408961.1	21	89.21
B. kobudai	*Semicossyphus reticulatus*	Japan: Ehime	LC718908.1	20	90.08
B. lutjani	*Lutjanus carponotatus*	Australia: Heron Island	AF026106.1	20	89.31
B. acanthopagri	*Sparidentex hasta*	Kuwait	FJ971967.1	17	89.57
B. rohdei	*Lutjanus carponotatus*	Australia	FJ971969.1	17	89.57
B. sciaenae	*Argyrosomus japonicus*	Australia	FJ971970.1	15	92.93
B. sekii	*Chrysophrys auratus*	Australia	FJ971971.1	15	92.93
Allobenedenia dischizosepta	*Acanthistius patachonicus*	AArgentina	MH929436	17	92.98
Gracilobenedeniahichi	*Heteropriacanthus carolinus*	Japan	LC602799.1	20	89.31

Note: Source: NCBI GeneBank

Table 5. Species identification of *Benedenia* from Situbondo and its similarity

Species	Host	Location	Accession ID	Query coverage (%)	Percent identity (%)
				Benedenia, Indonesia: Situbondo	
B. sargocentron	*Sebastes schlegeli*	China	JN797597.1	91	86.33
B. hoshinai	*Oplegnathus fasciatus*	Japan: Hiroshima	LC718905.1	93	85.40
B. epinepheli	*Semicossyphus reticulatus*	Japan: Hiroshima	LC718907.1	56	78.66
B. seriolae	*Seriola quinqueradiata*	Japan	KC768342.1	91	71.86
B. lutjani	*Lutjanus carponotatus*	Australia: Heron Island	AF026106.1	56	78.65
B. kobudai	*Semicossyphus reticulatus*	Japan: Ehime	LC718908.1	56	77.08
B. rohdei	*Lutjanus carponotatus*	Australia	FJ971969.1	53	77.45
B. armata	*Lethrinus haematopterus*	Japan: Nagasaki, Danjo Islands	LC408961.1	57	78.26
B. acanthopagri	*Sparidentex hasta*	Kuwait	FJ971967.1	53	77.17
B. sciaenae	*Argyrosomus japonicus*	Australia	FJ971970.1	53	75.34
B. sekii	*Chrysophrys auratus*	Australia	FJ971971.1	53	75.34
Allobenedenia dischizosepta	*Acanthistius patachonicus*	Argentina	MH929436	55	76.04
Gracilobenedeniahichi	*Heteropriacanthus carolinus*	Japan	LC602799.1	56	78.12

Notes: Source: NCBI GeneBank

Table 6. Morphometric value of *Benedenia* from this study

Parameter	Measurement (mm)	
	Benedenia Lampung (Mean ± SD)	Benedenia Situbondo (Mean ± SD)
Body length	2.55 ± 1.54	5.15 ± 2.93
Body width	1.09 ± 0.58	2.00 ± 1.09
Anterior attachment organs	L = 0.20 ± 0.14 x W = 0.19 ± 0,12*	L = 0.18 ± 0.11 x W = 0.23 ± 0.11*
Pharynx	L = 0.51 ± 0.31 x W = 0.21 ± 0,12*	L = 0.61 ± 0.42 x W = 0.40 ± 0.27*
Testis	L = 0.22 ± 0.14 x W = 0.26 ± 0,14*	L = 0.41 ± 0.21 x W = 0.43 ± 0.22*
Ovary	0.18 ± 0.08	0.18 ± 0.09
Opisthaptor	0.64 ± 0.38	1.35 ± 0.85
Accessory sclerites	0.09 ± 0.06	0.28 ± 0.13
Anterior hamuli (anchor)	0.64 ± 0.38	0.33 ± 0.17
Posterior hamuli	0.05 ± 0.03	0.14 ± 0.09

Notes: *Length (L) and width (W) measurements

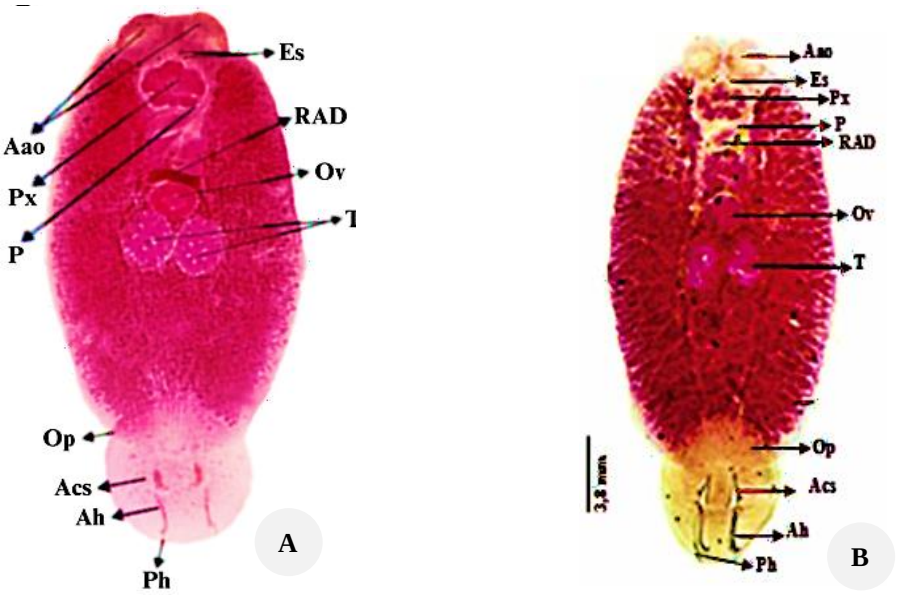

Figure 3. Semichoen Acetic Carmine staining of: A) *Benedenia* from Lampung; B) *Benedenia* from Situbondo. Notes: Staining image based on 40x magnification binocular microscope; Aao: Anterior attachment organ; Es: Eye spot; Px: Pharynx; P: Penis; RAD: Gland; Ov: Ovary; T: Testis; Op: Opisthaptor; Asc: Accessory sclerite; Ah: Anterior hamuli; Ph: Posterior hamuli

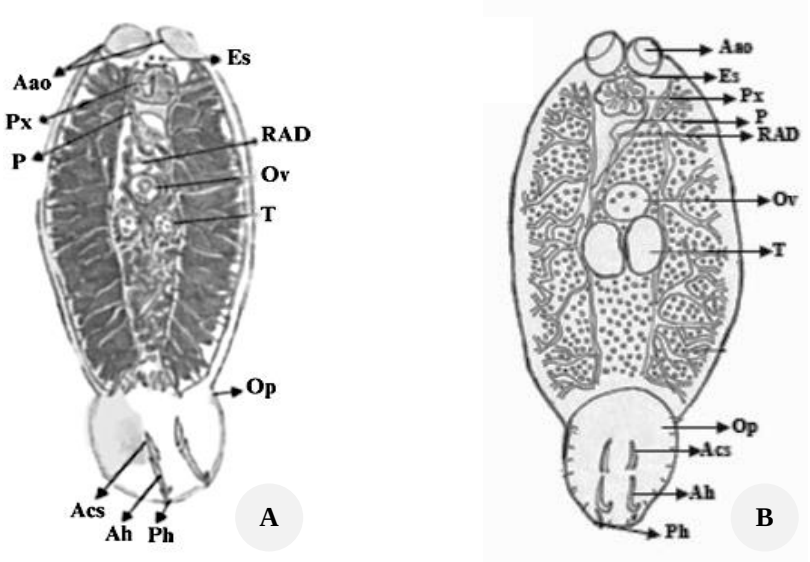

Figure 4. A) *Benedenia* from Lampung; B) *Benedenia* from Situbondo. Notes: Image with a binocular microscope equipped with a Lucida camera; Aao: Anterior attachment organ; Es: Eye spot; Px: Pharynx; P: Penis; RAD: Gland; Ov: Ovary; T: Testis; Op: Opisthaptor; Asc: Accessory sclerite; Ah: Anterior hamuli; Ph: Posterior hamuli

Benedenia is quite dangerous because it is often reported with secondary diseases such as viruses and bacteria that cause mortality (Turgay et al. 2019; Fusianto 2021). This is because *B. epinepheli* attaches to the skin's surface and gills, causing wounds/ulcers in fish (Effendi et al. 2023). These wounds are an entry point for viruses, bacteria, and fungi to attack the fish's immune system easily because there is no mucus or skin as initial protection (Junior et al. 2021; Garabawi et al. 2022).

In conclusion, 54 helminths of suspected to be *Benedenia* (90% of prevalence and 3 parasites/fish of intensity) were found from Lampung, while 48 helminths of suspected *Benedenia* (80% of prevalence and 3 parasites/fish of intensity) were found from Situbondo. *Benedenia* species from Lampung and Situbondo were identified as new species of *Benedenia*. The sample from Lampung was close to *B. hoshinaii* (LC718905.1) with 93.93% similarity, while the sample from Situbondo was close to *B. sargocentron* (JN797597.1) with 86.33% of similarity. The different similarities were probably caused by the contrast geographic between Lampung and Situbondo. It could be indicated as a new species of *Benedenia* since the *B. hoshinai* and *B. sargocentron* were found in Japan and surrounding (China, Korea) and never reported in Indonesia. Further research by another molecular target should be needed to identify this parasite more accurately.

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