BIODIVERSITAS
Volume 19, Number 5, September 2018
Pages: 1798-1804

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d190529

Short Communication: Identification of marine leech and assessment of its prevalence and intensity on cultured hybrid groupers (*Epinephelus* sp.)

MURWANTOKO[1,♥], SRI LAKSONO CONDRO NEGORO[1], ALIM ISNANSETYO[1], ZAFRAN[2]

[1]Department of Fisheries, Faculty of Agriculture Universitas Gadjah Mada, Jl. Flora Bulaksumur, Sleman 55281, Yogyakarta, Indonesia.
♥email: murwantoko@ugm.ac.id

[2]Gondol Institute for Mariculture Research and Fisheries Extention. Penyabangan, Gerokgak, Buleleng 81155, Bali, Indonesia.

Manuscript received: 23 November 2017. Revision accepted: 7 September 2018.

Abstract. *Murwantoko, Negoro SLC, Isnansetyo A, Zafran. 2018. Short Communication: Identification of marine leech and determination of its prevalence and intensity on cultured hybrid groupers (*Epinephelus *sp.). Biodiversitas 19: 1798-1804.* Grouper is an important fish species due to its high price both in domestic and international markets. Several hybrid groupers have been produced and can be accepted in market. A major production constraint in grouper culture is mortality due to diseases. Leech is an ectoparasite for groupers which may cause significant loss. The aims of this study were to identify and to assess the prevalence and intensity of leech on hybrid grouper cultured in sea cages at Buleleng waters. Morphological identification was conducted using fresh and stained specimens while molecular identification was conducted using nucleotides sequence of mitochondrial cytochrome oxidase subunit I (COI). The presence of leech was observed by unaided observation of 14 populations of hybrid grouper. Morphological identification showed that the leech belonged to *Zeylanicobdella arugamensis.* This result was also supported by analysis of COI sequence that showed 100% homology with *Z. arugamensis* (accession number KY 441721.1) and 90% homology with *Aestabdella abditovesicu*lata (accession number DQ414300.1). Hybrid groupers at sea cages were infected by leeches with prevalence and intensity, respectively, of 100% and 21.2 leeches fish^{-1}. The prevalence and intensity were varied depending on the farm and population. Cantik grouper was more susceptible to leech infection than cantang grouper. The bigger fish tended to have higher leech prevalence and intensity.

Keywords: Cytochrome oxidase, hybrid grouper, identification, leech, *Zeylanicobdella arugamensis*

INTRODUCTION

Several species of grouper have been cultured in Indonesia and become important fish commodities due to its high price in both domestic and international markets. Several types of hybrid grouper have been developed to increase the quality of fish. Cantang grouper is produced as a result of crossbreed between female tiger grouper (*Epinephelus fuscoguttatus*) and male giant grouper (*Epinephelus lanceolatus*). The crossbreed between the female tiger grouper and the male brown-marbled grouper (*Epinephelus microdon*) was named cantik grouper. A crossbreed between mouse grouper (*Cromileptes altivelis*) and giant grouper was named kustang grouper (Ismi et al. 2013). Cantang grouper culture has developed well from the rearing of fry to consumption size (Ismi 2012), and fast grows in cages (Sutarmat et al. 2013). Cantik grouper could increase production and showed better quality than brown-marbled and tiger groupers (Ismi et al. 2013).

The emergence of diseases is one of the main problems in the aquaculture. Emerging disease of epizootics frequently causes substantial, often explosive, losses among populations of fish, resulting in significant economic losses in commercial aquaculture and threats to valuable stocks of wild aquatic animals (Walker et al. 2010). Koesharyani et al. (2001) have compiled the viral, bacterial, parasitic, and noninfectious diseases in grouper. The hirudinea infection on grouper is one problem for parasitic diseases. Hirudinea has four orders, namely Acanthobdellia, Gnathobdellia, Pharyngobdellida, and Rhynchobdellida. The Rhynchobdellida Order has three families, i.e., Glossiphonidae, Ozobranchidae, and Piscicolidae. The Piscicolidae family is characterized by having a symmetrical, flattened cylinder body, an anterior suction and a posterior suction. Their habitats are freshwater and seawater, swimming by extending their body (Sawyer 1986). Species of leeches the family Pisciolidae are often parasitic seawater fish such as *Pterobdella amara*, *Aestabdella leiostomi*, *Piscicola* spp., and *Zeylanicobdela arugamensis* (Chandra 1991).

Marine leeches are an essential threat to the aquaculture industry (Ravi and Yahaya 2017). Heavily infested fish with leeches often have chronic anemia (Noga 2000). Grouper having infected leeches on its skin will rub the body on objects around it causing injuries and a large ulcer on the skin or in the mouth. Those conditions can cause secondary infection (Noga 2000; Johny and Roza 2006). Fishes mortality usually occurs within a 3-day period following infestation due to secondary infections with pathogenic bacteria such as *Vibrio alginolyticus* (Ravi and Yahaya 2017). Leeches infection also often transmits microbes and hemoparasites during feeding (Noga 2000). Marine leeches *Z. arugamensis* have been reported to have the ability to transmit the hemogregarine and trypanosomes

simultaneously between fish (Hayes et al. 2006).

Grouper culture using floating net cages in Pegametan Buleleng waters has been started in 2003. The number of sea cages in these waters is increasing due to the potential and reasonable price of grouper fish and high export demand. An outbreak of leeches was reported in grouper farm on August 2016. In this study, we identified the leech based on morphological and molecular approaches and determined the prevalence and intensity of leeches on hybrid groupers.

MATERIALS AND METHODS

Leeches sampling

Seven farms were selected to represent grouper culture in Pegametan Bay, Buleleng waters in September-October 2016. The position of farms were: Farm A at 8°07'10.7"S 114°36'47.1"E, Farm B at 8°07'03.0"S 114°36'42.2"E, Farm C. at 8°07'17.6"S 114°37'04.9"E, Farm D. at 8°07'47.3"S 114°36'06.9"E, Farm E. at 8°07'27.9"S 114°35'58.5"E, Farm F. at 8°07'40.5"S 114°36'09.3"E and Farm G at 8°07'40.9"S 114°35'44.6"E. All fish populations on the selected farm were sampled for the study. We defined population as fishes in a cage which have the same species, the same age and the same source of hatchery when stocked into the cage.

Leech observation

Thirty-six fishes were randomly sampled from each population to meet detection with a minimum prevalence rate of 10% with a 95% confidence level. For one population, fishes were sampled from three cages with twelve fishes in each cage. Fishes were collected from cages using scope net, then kept in a bucket. The species, length, and weight of fishes were recorded. The presence of leeches was observed with unaided eyes from all surface body of fish. The number of parasites was counted, and infected organs were recorded. The prevalence was calculated as the proportion of infected fishes among all the fishes in population. The intensity was calculated as the number of leeches found in the infected fish. For morphological identification, the leeches were collected alive and kept in containers with seawater for further identification. For molecular identification, ten parasites were fixed in 5 ml tubes containing 70% ethanol for further analysis.

Morphological identification

Morphological identification was performed using five fresh samples and five acetocarmine stained samples. Parasites were stained basically from Roberts et al. (2012) with 1% acetocarmine, and then destained using 1% HCl in 70% ethanol. The observations were conducted under a microscope and documented. Identification of species based on morphology and anatomy followed the guidelines of Sawyer et al. (1982) and Chandra (1983).

Molecular identification

The genomic DNA was isolated based on TNES method (Murwantoko et al. 2008). Approximately 50 mg of leech was ground in up 400 µl TNES on the microtube and added with three µl of Proteinase K (Roche) and incubated for 2.5 hours at 37 °C. After incubation, the mixture was centrifuged at 13500 x g for 5 minutes with Sorval Legend Micro 17 Microcentrifuge (Thermo scientific). The supernatant was collected and extracted with 300 µl of PCIAA solution (Phenol Chloroform Isoamyl Alcohol). After centrifugation at 13500 x g for 1 min, the aqueous phase was collected and added with 30 µl 5 M NaCl (1/10 volume of supernatant), and 600 µl cold absolute ethanol (2x volume of supernatant) then incubated for 24 hours in the refrigerator. The mixture was centrifuged at 13500 x g for 5 minutes, the supernatant was discarded, and the pellet was washed with 500 µl of ethanol 70%. After drying, the pellet was resuspended in 100 µl of TE containing 0.5 µl of RNAse.

The molecular identification was conducted based on mitochondrial cytochrome oxidase subunit I (COI) gene. LCO 1490 (GGT CAA ATA ATA AAG ATA TTG G) as the forward primer and HCO 2198 (TAA ACT TCA GGG TGA CCA AAA AAT CA) as the reverse primer (Lobo et al. 2013) were used. Amplification was performed in T100TM Thermalcycler (Biorad) with initial denaturation program at 95 ºC for 3 minutes, followed by 30 cycles of denaturation at 95 ºC for 30 seconds, annealing at 55 ºC for 30 seconds, extension at 72 ºC for 1 minute and the final extension at 72 ºC for 5 minutes. PCR product was electrophoresed in 1% agarose (Nacalai) in TAE solution with Fluorosave DNA stain (1st Base) using Mupid_2Plus electrophoresis tank (Advance). After electrophoresis, the gel was observed under UVP Transilluminator (Pasificimage Electronic). The PCR products were then sequenced through the sequencing service company. Aligned sequences were also subjected to nucleotide BLAST (Basic Local Alignment Search Tool) search to know the identity. Cluster tree was constructed under unweighted pair group method with arithmetic mean (UPGMA) using MEGA 7 software (Komar et al. 2016).

RESULTS AND DISCUSSION

Grouper culture

The culture of grouper using floating net cages in Pegametan bay has been started since 2003. In 2016 there were 24 farms with approximately 4000 cages as recorded by Association of Coastal Fish Farmer of Buleleng. The number of cages in each farm varied between 40 to 500 cages. The size of each cage ranged from 2 m x 2 m, 3 m x 3 m and 3 m x 6 m. A cage of 3 m x 3 m size was stocked with 500-600 fishes of 11-15 cm length, and cage of 3 m x 6 m was stocked with 700-800 fishes of 11-15 cm length.

The most commonly cultured grouper commodities were cantik hybrid grouper (*Epinephelus* sp) and cantang hybrid grouper (*Epinephelus* sp). Tiger grouper (*Epinephelus fuscoguttatus*), mouse grouper (*Cromileptes altivelis*), orange spotted grouper (*Plectropomus leopardus*), malabar grouper (*Epinephelus malabaricus*), and brown-marbled grouper (*Epinephelus microdon*) were

cultured in limited number. Based on information from the farms, the leech started to infect groupers with low intensity on few cages in April 2016. In August 2016, when there was a high tide, the leech infection spread to many floating net cages in the waters. Therefore, the sampling conducted around September to October was in condition with relatively high leech infection.

Fish samples

The grouper samples were taken from seven different farmers with total sample of 14 populations. The samples were composed by 9 populations of cantik grouper and 5 populations of cantang grouper. Based on the size, samples can be categorized on small, medium and big with 7, 5 and 2 populations, respectively (Table 1).

Location of leech infection

Leeches were easily observed and founded in mouth, eyes, operculum, skin, dorsal fin, anal fin, pectoral fins and tail (Figure 1). This parasite attaches to its host using its sucker and takes its host blood causing the leeches to become diverse in color as black and brown.

Table 1. Grouper samples from Pegametan cages

Farm	Pop.	Species	Length (cm)	Weight (g)	Category
A	A1	Cantik	15.9 +1.3	62.6 + 14.1	Small
	A2	Cantang	20.2 + 1.7	235.7 + 27.4	Medium
B	B1	Cantik	16.6 + 1.5	72.2 + 17.2	Small
C	C1	Cantik	17.1 + 0.7	75.1 + 7.4	Small
	C2	Cantik	29.9 + 1.4	434.5 + 78.1	Big
D	D1	Cantang	21.2 + 1.9	268.0 + 38.4	Medium
	D2	Cantik	14.1 + 0.9	46.2 + 9.7	Small
E	E1	Cantang	14.4 + 9.6	50.9 + 10.3	Small
	E2	Cantik	21.0 + 14.7	304.5 + 19.5	Medium
F	F1	Cantik	32.2 + 3.0	461.6 + 103.8	Big
	F2	Cantik	13.9 + 1.2	43.6 + 9.7	Small
G	G1	Cantang	14.5 + 1.1	51.1 + 10.5	Small
	G2	Cantik	21.3 + 1.1	252.7 + 20.4	Medium
	G3	Cantang	20.4 + 1.5	258.7 + 33.5	Medium

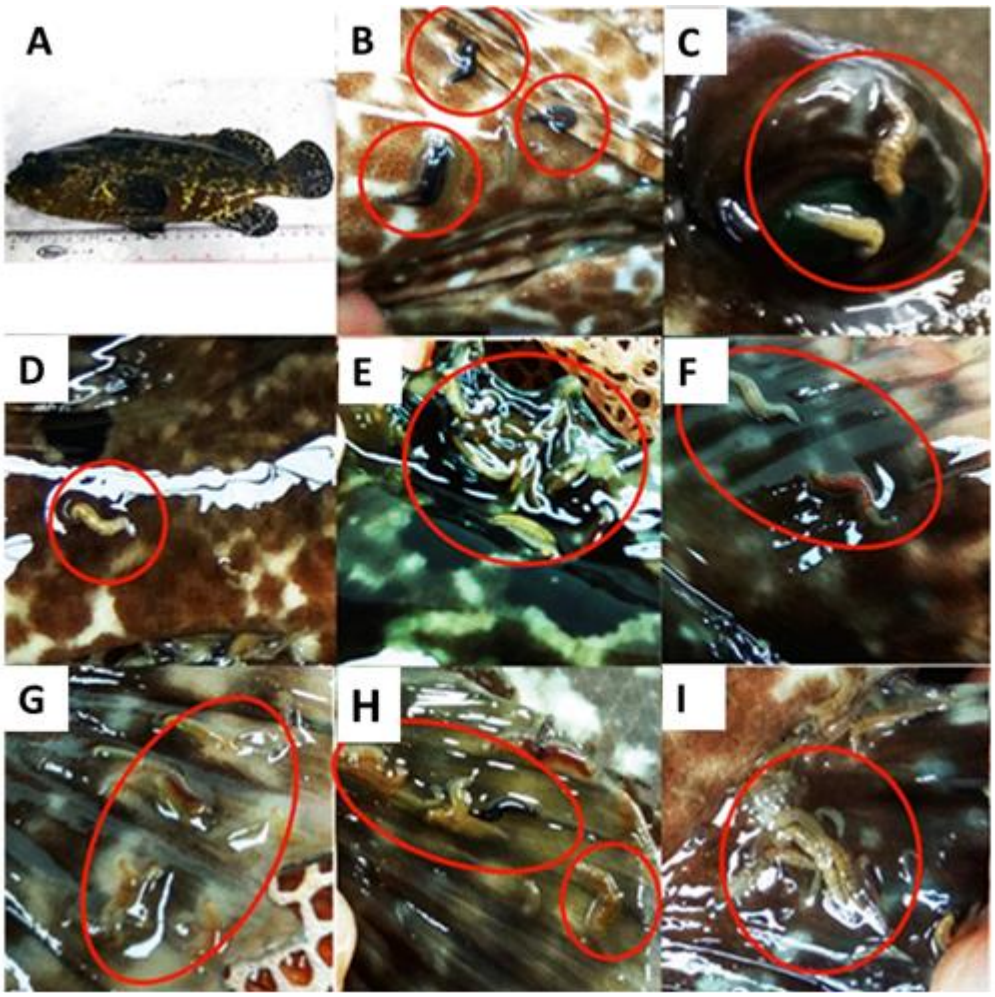


Figure 1. Infection by leech on fish body part (A: Infected grouper, B: Infection on operculum, C: Infection on eyes, D: Infection on the body surface, E & F: Infection on dorsal fin, G: Infection on caudal fin, H: Infection on pectoral fins and I: Infection on anal fin)

Prevalence and intensity

Leeches were found from all observed farms with different prevalence and intensity (Figure 2A). The average prevalence among farms was 62% with the highest prevalence was 86% (Farm C), and the lowest prevalence was 11% (Farm F). The average intensity among farms was 7.1 leeches fish^{-1}with the highest intensity was 11.2 leeches fish^{-1} (Farm F), and the lowest intensity was 0.9 leeches fish^{-1} (Farm D).

The prevalence and intensity levels of leech infection on each grouper sample population were varied (Figure 2B). Prevalence in the populations was also different even on the same farm. The highest prevalence was in population C2 and F1 grouper (100%), and the lowest was in population D2 (0%). The high prevalence variation among the population in farms occurred in Farm G (population G2 of 72.2% and population G1 of 8.3%) Fam F (Population F1 of 100%, population F2 of 19.4%). The intensity of leech infection in the population was also different even on the same farm. The highest inter-population variation in farms occurred in Farm F with population F1 of 21.2 leeches fish^{-1}and population F2 of 0.3 leeches fish^{-1}

The prevalence and intensity of leech infection on cantik grouper were, respectively, 69% and 9.3 leeches fish^{-1}, which were higher than those of cantang grouper with only 42% in prevalence and 2.6 leeches fish^{-1}in intensity (Figure 2C). This result suggests that cantik grouper is more susceptible to leech than cantang grouper. The highest prevalence of 100% was found in large grouper, and then 62% in medium grouper group and the lowest was 46% in small grouper. The highest intensity also showed similar pattern with the highest intensity was found in large grouper and the smallest was found in small grouper (Figure 2.D).

Morphological identification

The leeches can be observed on the fish body using unaided observation. The parasite has cylindrical shape, soft, elastic, and smooth body surface with light brown or black (Figure 3.A, 3.B). This parasite attaches to fish using its sucker and sucks its host blood. Adult of this species has a length of about 8-18 mm and a maximum width of the urosome of 0.5-2.0 mm. Anterior sucker has a diameter of 0.3-0.5 mm, and posterior sucker has a larger diameter that of 1.0-1.8 mm

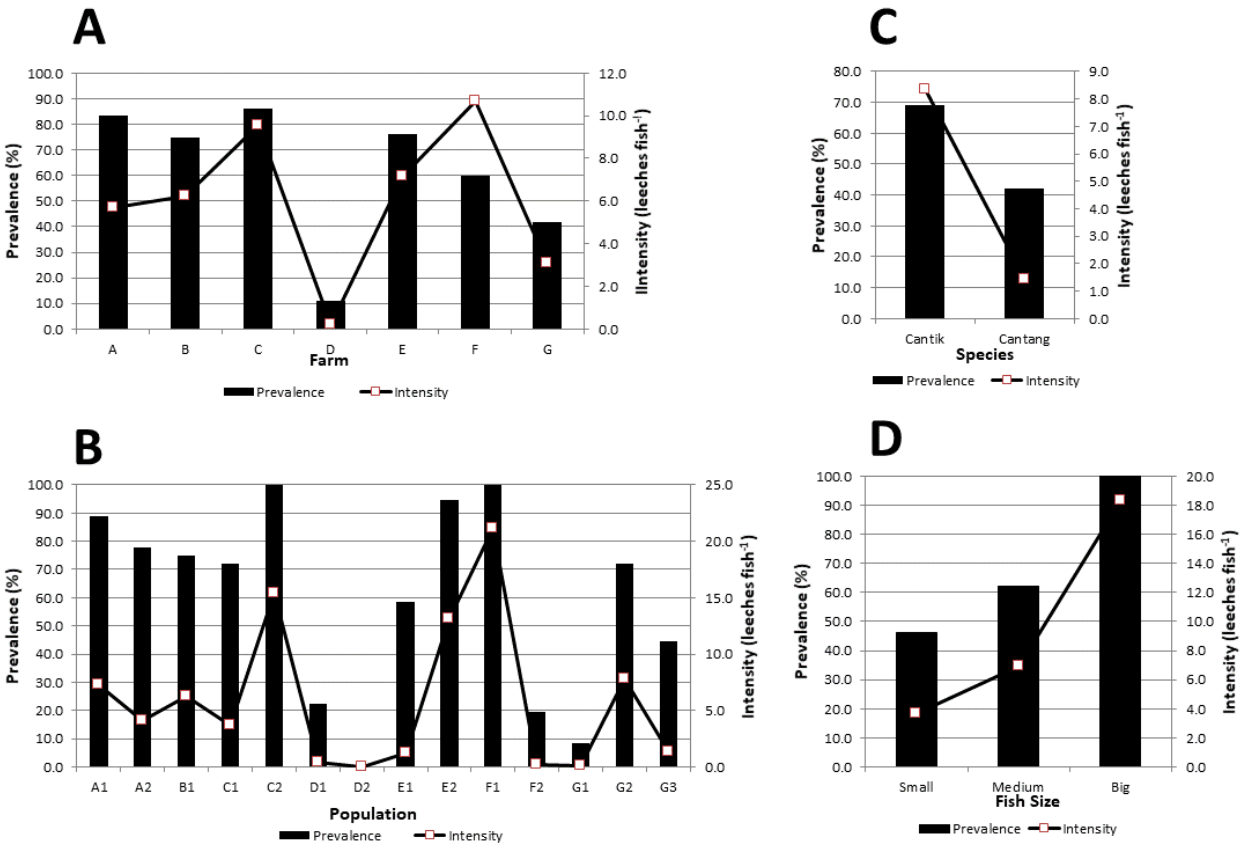
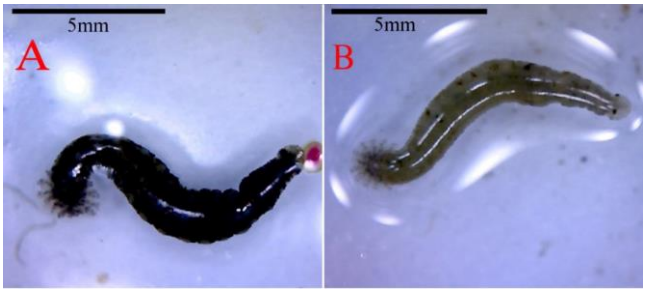


Figure 2. Prevalence and intensity of leech on the farm (A), population (B) species (C) and grouper size (D)



Fig**ure 3.** Leech found in grouper (A; Leech with black color; B: Leech with brown color)

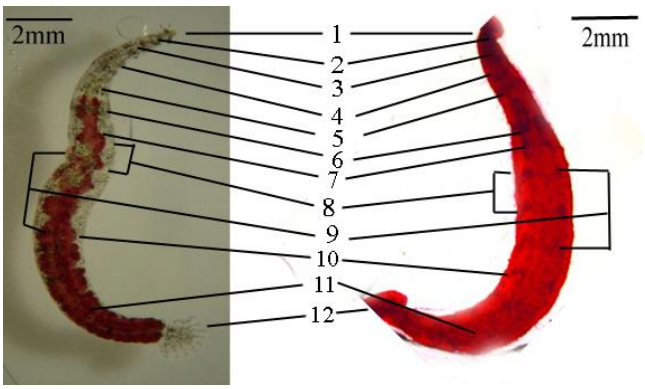


Figure 4. Morphology of leech (1 = Anterior sucker; 2 = Subesophageal ganglion mass; 3 = Proboscis; 4 = Ductus ejaculator; 5 = Ovary; 6 = First testicular ganglion; 7 = First Testis; 8 = Crop Abdomen; 9 = Pulsatile vesicle; 10 = 5th Testis; 11 = Posterior crop caecum; 12 = Posterior sucker)

Table 2. Leech determination according to Chandra (1983)

No	Description
1b	Has eyes and pulsating vesicles
4a	Eye pair
5a	Has no lateral branchiae
6b	Has pulsating vesicles
∴	*Zeylanicobdella arugamensis*

Table 3. Leech determination according to Sawyer et al. (1982)

No	Description
1b	Species that live in seawater or brackish
8b	Has no gill radius
9a	Has 10-12 pairs of pulsating vesicles along the lateral border of the abdomen
10b	Small size of about 1-2 cm
11b	Posterior sucker has a different sleeve with an anterior sucker and large size
12a	The lower body is smooth, about 12 segments in the body
∴	*Zeylanicobdella arugamensis*

This species has a pair of eyespots on the anterior sucker, 12 segments in the body, five pairs of testes and a pair of ovaries. The other part of the body of this species consists of subesophageal ganglion mass, proboscis, ovary, crop posterior caecum, pulsatile vesicles, testis (1-5), crop abdomen, testicular ganglion (1-5), and the ductus ejaculator (Figure 2). Posterior sucker (Figure 4(12)) different sleeve and large size than anterior sucker (Figure 4(1). Based on determination key of Chandra (1983) and Sawyer et al. (1982), this species is *Zeylanicobdella arugamensis* (Table 2 & Table 3)

Molecular identification

The genomic DNA was used as the template to amplify COI gene. The COI gene from leech was successfully amplified as indicated by the presence of a single band of DNA after agarose electrophoresis. This DNA fragment contained 725 nucleotides sequences that have been deposited in Genbank with accession number MH299847. The BLAST analysis showed that the sequence has a 100% identity with *Zeylanicobdella arugamensis* (KY4741721.1), while its homology with *Aestabdella abditovesiculata* (DQ414300.1), *Pterobdella amara* (DQ414334.1), *Myzobdella lugubris* (KY440059.1) was, respectively, 90%, 89%, and 86%**.** This Indonesian *Z. arugamensis* is closely related with *Z arugamensis* from Malaysia (Figure 5).

Discussion

The leeches can be seen visually attached to the fins, tail, body, operculum, mouth, and eyes of fishes. Some fishes showed hemorrhagic on their body surfaces, which is in line with the statements of Johnny and Roza (2006) that leech infection was found in the external part of the fish and caused hemorrhages leading to secondary bacterial infection. According to Ravi and Yahaya (2017), the most frequent effect of leech infection in fish are local bleeding and ulceration in fish tissues. This species is attached to the host using anterior and posterior suckers. They suck the blood of their host using their sucker, and in this study, the leech having fish blood was black (Figure 1). Leech that has sucked fish blood will escape from fish to find a place for spawning (Kua et al. 2010). After detached from the host, leeches were able to swim in the sea and able to survive without host for 5-7 days Cruz-Lacierda et al. (2000).

Leech parasites were found from all observed farms in Pegametan bay. The prevalence and intensity of leech that infected grouper in each farm were varied in values. The prevalence and intensity were also varied between population among farms and within farm. The highest prevalence was found in population C2 and F1 (100%), and the lowest prevalence was in population D2 (0%). The results indicated that infestation of leech was affected by location and or cultivation management. The prevalence and intensity of leech varied between species and size. Cantik grouper was more susceptible than cantang grouper. This shows that cantik grouper has a higher risk of disease infection than the cantang grouper. The bigger fish tended to have higher prevalence and intensity.

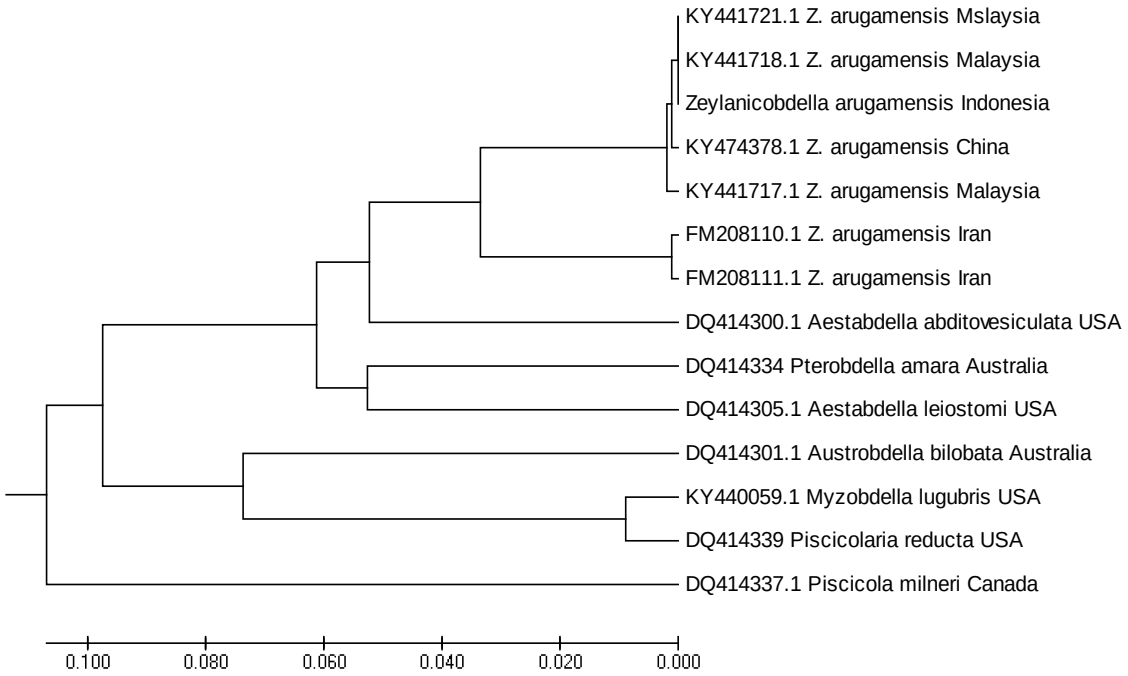


Figure 5. UPGMA tree using the mtDNA COI of Indonesia marine leech and added sequences from GenBank with indicated accession numbers

The average prevalence for these 14 populations was 59%, and average intensity was 6.9 leeches fish^{-1}. This prevalence and intensity were higher than that reported in muddy grouper in the Philippines with a prevalence of 30% and intensity of 2 leeches fish^{-1} (Cruz-Lacierda et al. 2000), and in red snapper with study of ectoparasite prevalence was 11.5% and intensity of 1.48 leeches fish^{-1} (Ravi and Yahaya 2017). This prevalence is lower than the that in white snapper in Malaysia (70%) (Kua et al. 2006).

Morphological and molecular identification based on COI sequence consistently showed that the leech belonged to *Zeylanicobdella arugamensis.* The COI sequences of *Z. arugamensis* on Genbank is limited, where up to April 2018, only ten entries were available. *Z. arugamensis* showed the genetic diversity, and at least 3 clusters are shown in Figure 5, Indonesian-Malaysian, Malaysian-China and Iran clusters. This genetic diversity seems to be correlated with the country location. *Z. arugamensis* had been reported to infect brackish-water fish Mozambique tilapia, *Oreochromis mossambicus* in Okinawa Japan (Nagasawa and Uyeno 2000), amphibious goby (*Scartelaos tenuis*) in southern Iran (Polgar et al. 2009). *Z. arugamensis* had been reported to infect cultured marine fish such as orange-spotted grouper (*Epinephelus coiodes*) in Philippine (Cruz-Lucierda et al. 2000), crimson snapper (*Lutjanus erythropterus*) in Malaysia (Ravi and Yahaya 2017), orange-spotted grouper, (*Epinephelus coiodes*) in Indonesia (Kleinertz and Palm 2015). In this study, we report the first time that *Z. arugamensis* can infect cantang and cantik hybrid groupers.

Several authors have documented study on leech infections on fish in Indonesia. Rosa and Johny (2006) have reported infection of the leech on *Epinephelus bleekeri* and *E. polyphekadion.* However, the species of leech was not reported. The intensity of *Piscicola* sp from tiger grouper (*E. fuscoguttatus*) and spotted coral grouper (*Plectropomus maculatus*) has been reported by Diana et al. (2004). The *Z. arugamensis* in Indonesia was reported by Kleinertz and Palm (2015) from orange-spotted grouper, *E. coiodes* with the species identification was only based on the morphology using microscope observation. Here we report the first time the identification of *Z. arugamensis* in Indonesia based on the COI nucleotide sequences and the sequences have been deposited in Genbank with accession number MH299847. Improvement of culture management should be addressed to control the leech. The lesions form this infection can cause secondary infection by bacteria, and this *Z. arugamensis* has been reported to be able to transmit the hemogregarine and trypanosomes simultaneously between fish (Hayes et al. 2006).

ACKNOWLEDGEMENTS

The authors highly appreciate the farmers for permitting us conduct the research in their farms and for the many technical support they provided.

REFFERENCES

Chandra M. 1983. *Zeylanicobdella arugamensis.* Rec. Zool. Surv. India. 80 : 273.

Chandra M. 1991. A check-list of leeches of India. Rec. Zool. Surv. India. 80: 265-290

Cruz-Lacierda ER,.Toledo JD, Tan-Fermin JD and Burreson EM 2000. Marine leech (*Zeylanicobdella arugamensis*) infestation on cultured orange-spotted grouper, *Epinephelus coiodes*. Aquaculture 185: 191-196

Diana SP, Sunyoto and Danakusumah E. 2004. Derajat infestasi ektoparasit Hirudinea *Piscicola* sp. pada ikan kerapu macan *Epinephelus fuscoguttatus* (Forsskal, 1775) dan kerapu sunu *Plectropomus maculatus* (Bloch, 1790). Jurnal Ilmu-Ilmu Perairan Dan Perikanan Indonesia 11 (1):1-4. [Indonesian]

Hayes PM, Smit NJ, Seddon AM, Wertheim DF and Davies AJ. 2006. A new fish haemogregarine from South Africa and its suspected dual transmission with trypanosomes by a marine leech. Folia Parastitol 53:241-248

Ismi S, Asih YN, Kusumawati D and Prihadi TH. 2012. Grouper Nursery as a business for increasing the income of the coastal community. Prosiding Seminar Insentif Riset SINas (INSINas 2012). PG:312-318. [Indonesian]

Ismi S, Asih YN and Kusumawati D. 2013. Peningkatan produksi dan kualitas benih ikan kerapu melalui program hibridisasi. Jurnal Ilmu Teknologi Kelautan Tropis. 5: 333-342.. [Indonesian]

Johnny F and Roza D. 2006. Infeksi parasit Hirudinea pada induk ikan kerapu lumpur, *Epinephelus bleekeri* dan kerapu batik, *Epinephelus polyphekadion* serta upaya penanggulangannya. BBPBL Gondol. Bali. [Indonesian]

Koesharyani I, Roza D, Mahardika K, Johnny F, Zafran and Yuasa K. 2001. Marine Fish and Crustaceans Diseases in Indonesia. In: Sugama K, Hatai K, Nakai T (eds.). Manual for Fish Diseases Diagnosis. Gondol Research Station for Coastal Fisheries, CRIFI and Japan International Cooperation Agency, Tokyo.

Komar S, Stecher G and Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33:1870-1874

Kua BC, Choong FC, and Leaw YY. 2014. Effect of salinity and temperature on the marine leech, *Zeylanicobdella arugamensis* (De Silva) under laboratory conditions. J Fish Dis. 37 (3): 201-207.

Kua BC, Azmi MA, and Hamid NKA. 2010. The life cycle of the marine leech (*Zeylanicobdella arugamensis*) isolated from sea bass (*Lates calcarifer*) under laboratory conditions. Aquaculture 302: 153-157

Lobo J, Costa PM, Teixeira MAL, Ferreira MSG, Costa MH and Costa FO. 2013. Enhanced primers for amplification of DNA barcodes from a broad range of marine metazoans. BMC Ecology 13: 34.

Murwantoko and Hardaningsih I 2008. Genetic Variation Study of Gourami (*Osphronemus goramy*) Using 5S rDNA sequence Approach. Aquacultura Indonesiana 9 (3):125-134. [Indonesian]

Noda EJ. 2000. Fish Disease Diagnosis and Treatment. Iowa State Press. Iowa.

Polgar G, Burreson EM, Stefani F and Kamrani E. 2009. Leeches on Mudskippers: Host-Parasite Interaction at the Water's Edge. J. Parasitol. 95(4):1021-1025

Ravi R and Yahaya ZS. 2017. *Zeylanicobdella arugamensis* The Marine Leech from Cultured Crimson Snapper (*Lutjanus erythropterus*), Jerejak Island, Penang, Malaysia. Asian Pacific Journal of Tropical Biomedicine 7:473-477.

Roberts RJ, Smail DA, and Munro ES. 2012. Laboratory Methods. In: Roberts RJ (ed.). Fish Pathology. 4th ed. Blackwell Publishing Ltd., New York.

Sawyer RT, Taylor A, and Sahat MJH. 1982. The leeches of Brunei (Annelida: Hirudinea), with a checklist and key to the known and expected freshwater, terrestrial and marine leeches of Borneo. Brunei Mus. 5 (2). 168-201.

Sutarmat T and Yudha HT. 2013. Performance analysis of seed grouper hybrid of cross-breeding between tiger grouper (*Epinephelus fuscoguttatus*) with giant grouper (*Epinephelus lanceolatus*) and camouflage grouper (*Epinephelus microdon*). J. Riset Akuakultur 8 (3): 363-372. [Indonesian]

Walker PJ and Winton JR. 2010. Emerging viral diseases of fish and shrimp. Vet Res 41 (6): 51.