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Colpoda steinii ASSOCIATED WITH HYBRID GROUPER FROM KG. GONG BATU SETIU, TERENGGANU

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ABSTRACT

This research was carried out to examine the presence of protozoa that inhabit on the gills of hybrid grouper. This phylum can act as ecto and endoparasites in fish and in turn are responsible for many diseases. Observation on cultured samples by using swabbing techniques on the gill surface indicated the presence of ciliated protozoan. The morphological features of the vegetative stage ciliates were kidney shaped and highly motile with 49.12 µm in length, whereas the resting stage of the ciliates was round shape with 38.42 µm in length as shown by light microscopy. Observation on the cell surface by scanning electron microscopy indicated unfamiliar silverline system for ciliates and the resting cyst surface structure was mildly wrinkled. Further specific species identification was verified by conducting Polymerase Chain Reaction (PCR). The ciliate gene in the 18S SSU ribosomal RNA isolated was very similar (99% sequence identity) to *Colpoda steinii* DNA sequence at GenBank under accession number of KJ607912.1 with 100% query cover. Although there were no reports on hybrid grouper mortality in Setiu, due to gills infection by ciliate *C. stenii*, it was important to obtain information on microorganism inhabitants in hybrid grouper fish gills. It is hoped that this data collected might be an indicator for fish health state for our fish farmers and as a precaution for conserving healthy environment in Setiu for better aquaculture-related production.

Key words: *Colpoda stenii,* hybrid grouper, ciliate, cyst, gill

INTRODUCTION

Protozoa has been reported as one of the most prevailing pathogens in fish population but less research has been conducted related to the diseases in fish caused by protozoan infection especially in Malaysia (Sayuthi, 1993). A research previously conducted by Smit *et al*. (2004) had shown that fish disease cases that occurred were inflicted by protozoan species. Furthermore, compared with other protists, especially parasites such as *Eimeria*, *Toxoplasma* and *Plasmodium*, *Colpoda* ciliates has been poorly studied, as colpodeans have little influence on humans or domestic animals (Shen, 1999). In fact, ciliate protozoans are identified as the most economically important parasites for fish aquaculture (Bastos *et al*., 2017). Due to that, the surveillance of ciliates present in the hybrid grouper is the focus of this study by identifying the protozoan species contained in the gills of hybrid grouper. Cilliate is an organism that might acts as an indicator of the quality of aquatic ecosystem (Debastiani *et al*., 2016). Colpodean ciliates are extremely common and found to be living in terrestrial habitats such as soils, leaf litters and mosses. Foissener (1997) reported Colpodean were found in most soil sample collected from all over the world and despite of that, ciliates were also reported to be residing in animal faeces that may have been resulted from the animal drinking water, consumed food or the air dust contaminated with the ciliates in its resting state (Tuanyuan *et al*., 2014). Identification of protozoan existing in the gills and more detail study about its dispersal factor and its persistency in the aquatic environment is crucial in order to combat potential disease in aquaculture industry. The actual pathogens and pathogenicity occurred must be investigated since the disease resulting from the infections will cause tremendous impact on fish production, consumption, trade and aquaculture practice in any country (Gomes *et al*., 2017). The presence of any protozoan species from

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gills of hybrid grouper is to be essentially proven as it is crucial for economy and health of fish in a comprehensive aquaculture with hope that it can provide useful information about the diseases that might be caused by ciliates.

MATERIALS AND METHODS

Ciliates isolation and cultivation

Ciliates isolation and maintenance

Five samples of hybrid groupers were obtained from a fish cage farmer at Kg. Gong Batu, Setiu, Terengganu. Sterile cotton buds were used to swab the protozoan samples from the gills of the fishes and then were spread onto non-nutrient agar plates. One to two drops of heat-killed *Escherichia coli* was later provided as a source of food for each plate of isolated ciliates. Page's Amoeba Saline (PAS) solution was then added to the non-nutrient agar plate to keep the surface of agar plate watery.The cultured ciliates were incubated at 30°C and their vegetative cells and cyst stages were observed on a daily basis through inverted microscope.

Ciliates subculture technique

Vegetative cell and cyst stages of ciliates were observed and identified under the inverted microscope. The location of the vegetative cell and cyst located was marked at the bottom of the plate by using marker pen. The agar region in the plate containing cystic colony of the ciliates were excised and transferred onto new non-nutrient agar plate. A few drops of heat-killed *E. coli* were provided for the maintenance of protozoan culture and its proliferation was monitored on a daily basis.

Ciliates morphological identification

Observation of ciliates under light microscope

Ciliates were harvested with PAS by using sterile hockey stick. The harvested ciliates were centrifuged at 7.5 rpm for 10 minutes. The pellets obtained were transferred onto glass slide and a cover slip was placed on top of the glass slide. The specimens were observed under light microscope (NIKON Eclipse E200, Japan) from low to high magnification and the images were captured and the diameter of the 50 cysts form were also measured.

Observation of ciliates under scanning electron microscope (SEM)

The ciliates from the agar culture plate were harvested and transferred onto cover slip in the 6-well plate followed by overnight incubation. SEM preparation procedure by Fatimah and Nakisah (2013) was followed for ciliates detailed morphology.

Species identification by polymerase chain reaction (PCR) method

Deoxyribonucleic acid (DNA) extraction

DNA extraction of ciliate cultures was carried out using protocol by InnuPREP DNA Mini Kit. Approximately, 50mg of cell cultures was lysed with 400 uL of TLS and 25 uL of proteinase K. The culture was vortexed and incubated at 50°C before centrifuged at 11,000 rpm for 5 min. Then, the supernatant was transferred to new 1.5 mL tubes before adding 400 uL TBS buffer and vortexed. The mixture was transferred to spin filter and centrifuged at 11,000 rpm for 2 min. Next, the spin filter was washed twice with 500 uL and 750 uL of HS buffer, respectively. The DNA was then eluted with 200 uL Elution Buffer before being stored at -20°C for further use.

PCR for amplification of 18S rRNA genes

18S rRNA gene of 1700 bp was amplified by PCR using genomic DNA extracted from the *Colpoda* species as a template. A 18S rRNA gene was amplified from DNA using universal eukaryotic primer Euk-A and Euk-B (Koid *et al*., 2012). The universal eukaryotic forward and reverse primers for 18S rRNA gene sequence is 5'-ACCTGGTTGATCC TQCCAGT-3' (euk-A) for forward primer and 5'-GATCCTTCTGCAGGTCACCTAC-3' (euk-B) for reverse primer, with expected PCR product size of 1700bp. PCR mastermix (Promega), forward and reverse primers and extracted DNA of ciliates template were mixed to prepare PCR reaction. The components of PCR reaction for amplification of 18S rRNA genes were 5x Green Gotaq (10µL), 4µL of 2.5mM $MgCl_2$, 1µL of 250mM dNTPs, DH_2O and 0.25µLTaq polymerase. PCR cycling condition included pre-denaturation (95°C, 2min), denaturation (95°C, 30sec), annealing (55°C, 30s), extension (72°C , 2 min) and final extension (72°C, 7min). The amplified PCR products were electrophoresed on a 1.2% agarose gel stained with ethidium bromide (EtBr) and bands were visualized by UV transillumination. The PCR products were sent for sequencing to the First Base Laboratory Sdn. Bhd. Then, the nucleotide sequences obtained were submitted to GenBank in a BLAST search for identification.

RESULTS AND DISCUSSION

Nowadays, increasing human population has caused greater attention to expand the aquaculture sector in order to meet the food demands. Initiative had been taken to provide large scale production of fish by practising cage culture. According to FAO (2014), high value fishes such as groupers, snappers and barramundi population have increased due to rearing by the fish farmers in small cages in the inshore environments. Groupers are one of the highly in demand fish due to their significant price and exquisite taste (Guerrero, 2014). Grouper aquaculture is one of the source of daily income generated by fishing activities (Sheriff, 2004). However, practising cage farming having many economic advantages potentially contributes to fish disease outbreaks that usually associates with pathogen, host fish and environmental problems. In addition, other factors that lead to the fish diseases is lack of attention given on the fish cages condition and environment. Usually, disease occurrence depends on water quality and feed quality given to fishes. Conditions such as high stocking density, poor water quality and suspended particulate matters in the fish cages could contribute to the outbreak of infectious and non-infectious diseases due to stressed conditions hence causing the fish to be more susceptible to disease. FAO (2014) supported that parasites exist or live together with the fish in the water without causing any significant problems to the fish health, but the problems arise when fish are in stress conditions. In this particular condition, fish immunity is compromised and the parasites get oppurtunity to establish and multiply in numbers. Gills are the main organ focussed on isolating the protozoa because it has large surface area in contact with the external aquatic environment that contains various type of organism (Mallat,1985). There were positive cultures of ciliates isolated from the five samples of hybrid groupers, which were isolated from their gills. Gills play the crucial role in respiration and excretion because their role as filter feeder and sites of gaseous exchanges are indirectly having much exposure with the particular organism. This fact is proved with the presence of the ciliates isolated from the gills of hybrid grouper as shown in Figure 1 and Figure 2.

Observation of morphology and their behavior such as locomotion style and reproduction was completed using inverted microscope, which signified different morphologies of vegetative cells and cysts. Ciliates observed under cysts stage were spherical in shape and coated with a single cell wall as shown in Figure 1 (a) while vegetative cells were observed to be in slightly reformed (kidney shaped), that is high convex on one side and concave on the other side as shown in Figure 1 (b). Ciliates in their resting state (cysts) were showing no movement while the vegetative cells were observed to be actively swimming on the agar plate and its locomotion pattern resembled the spinning ball. Cysts existing in unfavorable conditions (after few days living on the agar plate) resulted from encystment process meanwhile vegetative cells were seen after incubation period of the ciliates subcultured onto a new agar plate. Ciliates vegetative cells fully covered the agar surface after 24 hours of incubation and the number was gradually decreased in days as the number of cysts increased. Figure 1 shows the morphology of ciliates obtained in this study. To confirm the genus of the expected unknown ciliates by morphological observations based on the shape and size, the diameter of the cysts form were measured. Fifty random ciliates were measured in a population and the average diameter of its cyst was 38.416 ± 3.52 µm and average length of vegetative cells 49.17 ± 6.14 µm. Table 1 shows the average length of vegetative cells and average diameter of cysts of ciliates.

In this study, the ciliates isolated from the gills of the hybrid grouper showed that they also can live in the freshwater environment. The ciliates in the form of cyst were observed to be of rigid spherical shape and coated with a single cell wall as shown in Figure 1 (a). This characteristic gave the ciliates a good mechanical protection in order to withstand adverse conditions and with this adaptive help, increased the rate of ciliates survival rate. In contrast, the vegetative cells were examined to be equipped with more complex structure as shown in Figure 1 (b) compared to cysts. Vegetative cells equipped with minute projection of cilia enabled it to move freely and became highly motile and potentially harm its host because of its penetration activity. In this study, only the body shape of the species was obtained as shown in the Figure 2 despite of ciliary rows that is used for locomotion purposes. However, Foissener (2014) suggested the most suitable method used to study the structures of ciliates organism is by using silver impregnation method. The life history was observed more specifically when the ciliates were cultivated in non-nutrient agar added with *E. coli* as its food source. The isolated ciliates were observed to be in two forms; cysts and vegetative cells state, both equipped with different morphology and locomotion patterns resulting from its strategy to keep surviving in its host. In this study the vegetative cell number was observed to reduce gradually after 48 hours of incubation because the vegetative cells undergoes encystment and thus it remains in dormant state as the condition in the agar no longer fulfils the requirement of the ciliates to survive. In addition, encystment was often resulting

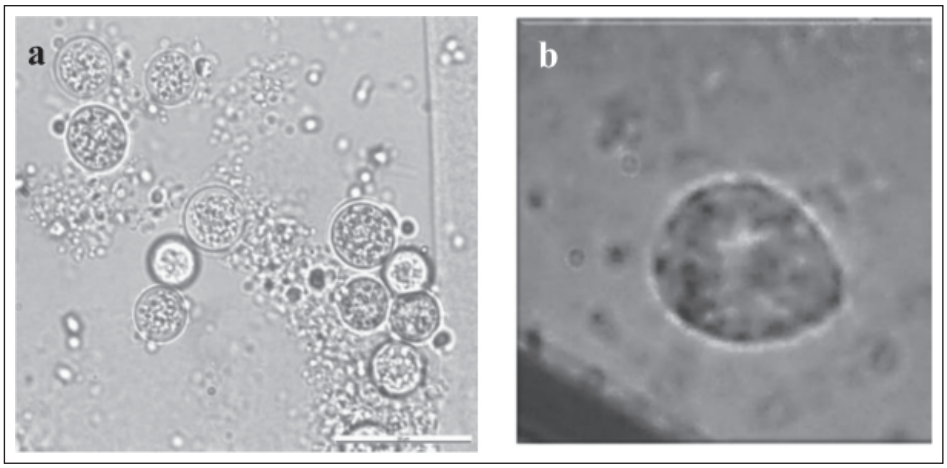

Fig. 1. Morphology of ciliates observed. (a) Ciliates in cysts state and (b) ciliates in the form of vegetative cells (400x).

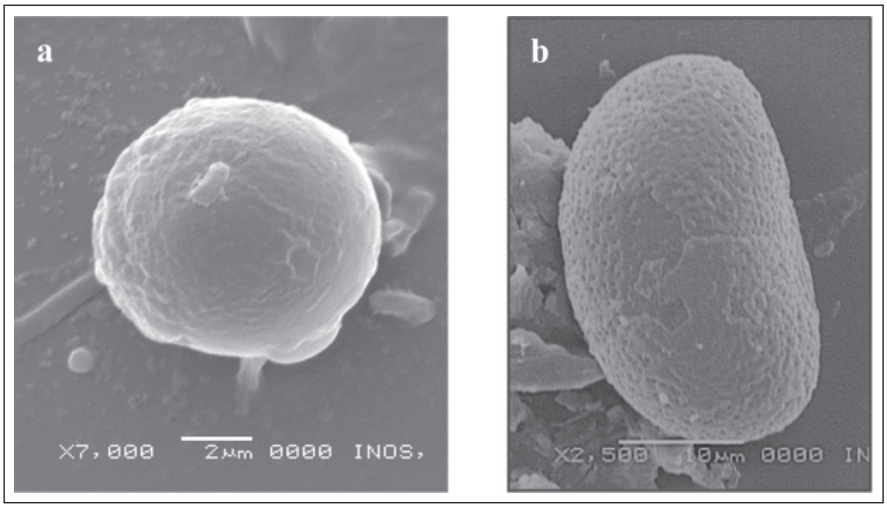

Fig. 2. Morphology of ciliates I cysts and vegetative cells state. (a) Cysts of ciliates and (b) vegetative cells of ciliates.

Table 1. The average length of vegetative cells and average diameter of ciliates

	Vegetative cells	Cyst
	Length (µm)	Diameter (µm)
Cilliates	49.17 ± 6.14	38.416 ± 3.52

from an overpopulation of vegetative cells or by rapid drying up of the medium. The ciliates were observed to undergo excystment after 24 hours of incubation (22°C) in the new agar plate that were nourished with minerals, nutrient and heat killed *E. coli* as source of food. Supported by Verni and Rosati (2011), excystment occurred within ciliates when cysts were ready to undergo cell division, after all of its requirements were met. Cell division in ciliates is initiated by the movement of its cell component that actually rotates within the cysts and thus creates an inner pressure causing the cell wall to rupture while enabling the fully formed vegetative cells to release form the cysts. Vegetative cells were observed living on the agar surface of the first day of incubation. However, after the next day of incubation, the ciliates were seen residing in the agar because of its penetration activity. Active penetration activity of the ciliates enable it to invade other parts of its host once it enters the host body as was reported to exist in the patient urinary tract and were excreted in the urine (Costache *et al*., 2011). Fifty random ciliates selected and measured had characterized cysts diameter and vegetative cell length in this study, which were 38.416 (13.36-29.75) ± 3.52 µm and 49.17 (40.21-65.05) ±6.14 µm, respectively. The cysts size gradually

increased from the first day onwards of incubation period as it underwent successive binary fission while vegetative cells that enter the excystment process of cysts were observed to be increased in body length from day one onwards as it grew and survived on the agar plate until it reached the carrying capacity on the plate such as crowded space or overpopulation and limited food source. Lynn *et al*. (2000) revealed ciliates' size measurement of 14-26 µm in diameter and 42-56 µm in body length thus supported the size of ciliates isolated in this study. Scanning electron microscope was able to confirm the shape and morphological characteristics of ciliates that were viewed under the light microscope as it provided the 3D view of the ciliates basic structures and its body shape. Any distinct and hidden features can be seen under SEM. Observation under SEM revealed the structures of ciliates in the cysts stage that is spherical in shape and having grumbled cell wall surface. The vegetative cell structures studied using SEM, shows that the cells are oval in shape and its surface is fully covered with tiny pores. The morphology of ciliates under both cysts and vegetative cells state are shown in the Figure 2.

In this study, DNA of the ciliates was successfully amplified using 18S rRNA. The DNA sequence of ciliates was used for species identification purposes using BLAST. BLAST enables a researcher to compare a query sequence with a library or database of sequences and identify library sequences that resemble the query sequence. The species identification using the BLAST successfully identified the DNA sequence of ciliates isolated from hybrid grouper as *Colpoda steinii* as it showed the highest percentage of the identity similar to the *Colpoda steinii* strain PABBc-11 18S ribosomal RNA gene, partial sequence provided by the Genbank. *Colpoda steinii* strain PABBc-11 18S ribosomal RNA gene, partial sequence provided by Genbank is a viable ciliate isolated from Late Pleistocene permafrost sediments. PCR conducted successfully amplified the ciliates DNA with the size of PCR products between 1500 bp to 2000 bp that closely matched the size of expected band, 1700 bp. Figure 3 shows positive result of 18S rRNA genes with expected size of 1700 bp run on 1.2% agarose gel.

The sequence results obtained indicates that the amplified PCR product has high identical percentage with *Colpoda steinii*. Table 2 shows the result of the BLAST of the sequencing analysis. The DNA from the ciliates shows 99% identical to the gene sequence GenBank under accession number: KJ607912.1 with 100% query cover.

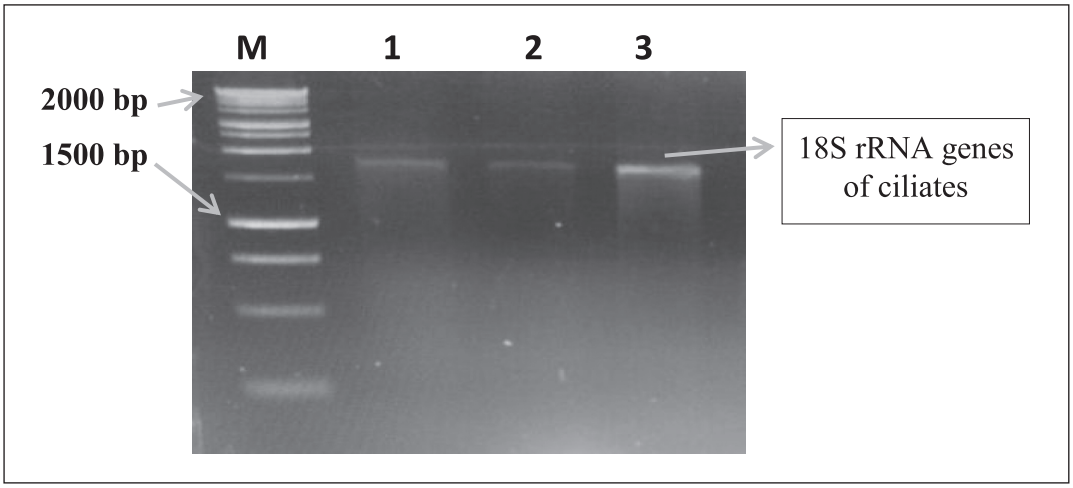

Fig. 3. Visualization of PCR products on 1.2% agarose gel stained with ethidium bromide (EtBr). Lane M: 1kb bp DNA ladder (Promega). Lane 1, 2, 3 shows the band of the amplified 18S rRNA genes of ciliates.

Table 2. Basic Local Alignment Search Tools (BLAST) sequences analysis

No	Species	Accession Number	% of identity	Query cover
1	*Colpoda steenii* strain PAPBSc-418 S ribosomal RNA gene, partial sequence	KJ607912.1	99%	100%

CONCLUSION

In conclusion, all of the five samples of hybrid grouper studied indicated and proved positive for culture of ciliates as a genus that have been putatively identified based on the morphological characteristics observed under microscopy. The morphological characterization as well as the movement pattern have resulted in the identification of *Colpoda steinii*. This was confirmed again with results obtained from molecular approach, PCR and sequencing. Further studies about this project may be carried out for identification and degree of pathogenicity of these ciliate species in order to prevent any significant loss in aquaculture industry.

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