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Zeylanicobdella arugamensis, the marine leech from cultured crimson snapper (*Lutjanus erythropterus*), Jerejak Island, Penang, Malaysia

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ABSTRACT

Objective: To investigate the prevalence, phylogenetics and DNA barcoding of *Zeylanicobdella arugamensis* (*Z. arugamensis*) from crimson snapper (*Lutjanus erythropterus*), Jerejak Island, Penang, Malaysia.

Methods: Experiment was conducted with 200 fish specimens of cultured *Lutjanus erythropterus* from Jerejak Island, Penang, Peninsular Malaysia. The water temperature and length for each fish were measured prior to parasites examination. Next, the morphological identification of parasites was performed. Genomic DNA from parasites was extracted for further molecular analysis. After PCR amplification, phylogenetic tree was constructed. The lowest Bayesian information criterion scores showed that the most compatible model is Tajima and Nei. Finally, data sets of cytochrome oxidase subunit I gene sequence and trace file have been submitted to Barcode of Life Data System.

Results: The prevalence rate of *Z. arugamensis* was recorded to be 11.5%, and the intensity was 1.48. The low intensity was due to the water temperature recorded in this study (32.9–33.2 °C). All the individuals of *Z. arugamensis* recorded in this study showed a close relationship with species that were recorded in NCBI database (*Z. arugamensis* DQ414344, *Aestabdella leiostomi* DQ414305, *Pterobdella amara* DQ414334 and *Cystobranchus meyeri* DQ414315) but less relationship with *Aestabdella abditovesiculata* DQ414300. Finally, the DNA sequences submitted to Barcode of Life Data System in accordance to species have already obtained Barcode Index Number as BOLD: ACM3477.

Conclusions: This study has provided an overview of sequence divergence at cytochrome oxidase subunit I gene, DNA barcodes and parasite prevalence of *Z. arugamensis*.

1. Introduction

The distribution of *Zeylanicobdella arugamensis* (*Ottoniobdella stellata* Moore, 1958) (*Z. arugamensis*) was originally described in Sri Lanka; however, with global ecological changes, it is now known to be distributed throughout the Indian Ocean. From Malaysian waters, *Z. arugamensis* was firstly reported from the seahorse *Hippocampus kuda* and unidentified eel species [1]. *Z. arugamensis* has been reported to be the most dangerous ectoparasite affecting a wide range of fish species. Host mortality usually occurs within a 3-day period following infestation due to secondary infections with pathogenic bacteria such as *Vibrio algnolyticus* [1]. Therefore, marine leeches are an important threat to the aquaculture industry. Hence, a study on the infestation rate, morphology, and molecular diagnosis of this species is essential for the implementation of measures to prevent and control disease associated with leeches infestation in the aquaculture industry. The objective of this study was to analyse the prevalence, morphology and molecular diagnosis of fish parasites collected

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All experimental procedures involving animals were conducted in accordance with ethical and practical guidelines. Research was approved by the Animal Ethics Committee of Universiti Sains Malaysia (1001/PBIOLOGI/855003).

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from *Lutjanus erythropterus* (*L. erythropterus*) cage-cultured at Jerejak Island, Penang, Malaysia. PCR was used to amplify *Z. arugamensis* sequences, and cDNA sequences were compared to those in the NCBI database. Further analysis of sequences was performed using phylogeny tree analysis [2–4].

2. Materials and methods

2.1. Parasite examination on crimson snapper (*L. erythropterus*)

All experimental procedures involving animals were conducted in accordance with ethical and practical guidelines. Research was approved by the Animal Ethics Committee of Universiti Sains Malaysia (1001/PBIOLOGI/855003). The experiment was carried out with 200 specimens of cultured *L. erythropterus* from Jerejak Island, Penang, Peninsular Malaysia (5°18′53.2″ N, 100°19′29.2″ E). The length (cm) of each fish was measured prior to examination for parasites. Water parameters, such as temperature, were measured using a digital hand-held seawater refractometer (S.G), model PAL-07S, Atago (Thailand) Ltd. A solution was prepared containing distilled water and tricaine methane-sulfonate (MS-222) (Sigma–Aldrich) (50 mg/L) to anaesthetise the fish and reduce handling stress. After anaesthetisation, the presence of ectoparasites was determined via external fish body examination and direct observation under a light microscope [5]. The site specificity of the parasite was determined through examination of the head, the body and both sides of the inner operculum.

2.2. Identification of parasite

Parasites were collected from the external skin surfaces of infested areas of the fish using surgical dissecting forceps. Next, the number of parasites collected was recorded, and all parasite specimens were preserved in a 70% ethanol solution and stored in a universal bottle for further examinations [6].

First, morphological identification of parasites was carried out by staining the parasite with a few drops of lactophenol solution (200 mL lactic acid, 200 g/L phenol, 400 mL glycerol and 200 mL deionized water). Two minutes after staining, slides with the specimens were observed under a compound microscope (Leica, USA) [6]. Next, images captured of the parasites were used for identification using morphological keys as suggested previously [7].

2.3. Morphological method using scanning electron microscope

Secondary morphological identification was performed using a Supra 50 VP ultra high resolution LEO analytical field emission scanning electron microscope (Carl Zeiss LEO Supra 50 VP field emission equipped with an Oxford INCA system). Sample preparation was carried out according to the Carl Zeiss LEO Supra 50 VP field emission scanning electron microscope manufacturer procedures and protocol. First, parasite samples were immersed in ethanol and hydrated with serial dilutions of 90%, 80% and 70% ethanol. Then, parasite specimens were placed on carbon film-coated 400 mesh copper grids for 1–3 min. Parasite specimens were then dried using pieces of filter paper. Grids were then placed on filter paper-lined Petri dishes for preservation in a desiccator. Finally, imaging was carried out after 3 days of preservation [8].

2.4. Molecular method using DNA identification

Genomic DNA was extracted from the fish parasites. DNA isolation was performed using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instruction. Isolated DNA was then kept at −20 °C. DNA integrity, optical density and DNA concentration were analysed using ACT-Gene Nano-spectrometer (ASP-2680, Taiwan). Agarose gel (0.5× Tris-borate-ethylene diamine tetraacetic acid) electrophoresis 1% was used to determine the presence of DNA from the sample. Electrophoresis process was started by using a voltage of 50 V for about 10 min in order to make a separation. Then, a voltage of 80 V was used for about 70 min. The gel was occasionally monitored by referring to the dye (bromophenol blue 6×). The gel was then viewed with UV light by using Bio-Rad Molecular Imager Gel Doc TM XR (Bio-Rad, USA) after the staining with ethidium bromide [9]. The isolated DNA was amplified using PCRs targeting the region of the mitochondrial cytochrome oxidase subunit I (*COI*) gene. Universal Folmer primers were used in this study, including LCO 1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) as the forward primer and HCO 2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) as the reverse primer [10,11]. Both primers were prepared by MyTACG Bioscience Sdn Bhd (Malaysia). PCRs were carried out in a total volume of 25 μL of master mix solution (14 μL of double distilled H_2O, 2.5 μL of Promega PCR buffer, 3 μL of Promega $MgCl_2$ solutions, 1 μL of Promega deoxynucleotide solution mix, 1 μL of each forward and reverse primers, 2 μL of DNA template and 0.5 μL of Promega GoTaq DNA polymerase). Standard cycle conditions for PCR were set as follows: initial denaturation for 10 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 50 °C, 60 s at 72 °C and final elongation for 7 min at 72 °C. All PCRs were carried out in MyCycler thermal cycler Bio-Rad PCR systems (USA). PCR products were purified and sequenced by MyTACG Bioscience Sdn Bhd (Malaysia) [5].

2.5. Phylogenetic analysis and DNA barcoding

Clustal W2, MEGA 5 software was used to align all the sequences in this study. All the DNA sequences were checked for stop codons using MEGA 5 before conducting the Bayesian information criterion and polygenetic test. Nucleotide sequences were used in BLAST to confirm the sequence similarities. The phylogenetic tree was constructed based on distance method which uses the evolutionary distance matrix between taxa, using neighbour joining from *COI* gene. Pairwise distance calculation was conducted using MEGA 5 analysis tools and the best substitution model was Tajima and Nei model [2,12]. In addition, analysis of phylogeny was conducted with 1000 bootstrap values. The DNA barcoding was created according to the Barcode of Life Data System web protocol instructions. The sequence data comprised the *COI* sequence and trace file was submitted to Barcode of Life Data System and will be available as public data upon publication [13].

2.6. Prevalence of parasite

Total number of parasite was calculated. The parasite prevalence and intensity were determined using the following formula [6]:

$$\text{Prevalence} = \frac{\text{Number of fish infected}}{\text{Total number of fish}} \times 100\%$$

$$\text{Intensity} = \frac{\text{Number of parasites recovered}}{\text{Number of fishes infected}}$$

2.7. Statistical analysis of temperature

Test for normality with Shapiro Wilk test was conducted and followed by One-way ANOVA test for marine water temperature in this study. Measurements of the water temperature from this study site were done in 4 replicates. These replicates were named as replicate 1, replicate 2, replicate 3 and replicate 4. A total of 48 water samples were collected for replicate 1, replicate 2, replicate 3 and replicate 4, and the samples were collected for 12 weeks [5]. All the statistical analysis in this study was performed with the statistical package for social science software, SPSS version 20.

3. Results

3.1. Morphology of Z. arugamensis

According to Figures 1–3, total body length ($N = 5$) including suckers was 20–25 mm and the maximum body width near middle of urosome was 0.6–2.3 mm. The body was divided into subcylindrical trachelosome and much longer and wider urosome. Oral sucker was 0.3–0.5 mm in diameter and oval in shape. Caudal suckers were larger than oval sucker (1.6–1.8 mm in diameter). Finally, body pigmentation was dark brown dorsally and ventrally with black pigments [7,10].

3.2. DNA and phylogenetic analysis of Z. arugamensis

Based on the results obtained by gel electrophoresis analysis of the DNA templates and PCR products, clearly visible bands were detected around a 700 bp sequence, and subsequent analysis was completed using a Fermentas 1 kb DNA marker. Additionally, the optical density ratio of the DNA was measured at 1.86 and the DNA concentration of purified PCR product was 121 ng/μL. The DNA sequence was then further analysed using Clustal W2 (Bioedit) software. In this study, BLAST search revealed a nucleotide similarity of 93%–96% with *Z. arugamensis* (HQ157566). The *COI* sequence was successfully analysed and recovered from all *Z. arugamensis* specimens, and no stop codon was encountered. Figure 4 displays the constructed phylogenetic tree which shows a monophyletic clade between species. All *Z. arugamensis* specimens collected in this study showed a close relationship with *Z. arugamensis* species maintained in NCBI database, with a 97% bootstrap value for *Z. arugamensis* DQ414344; however, the *Z. arugamensis* specimens also had a 69% bootstrap value for *Aestabdella abditovesiculata* DQ414300 and 97% bootstrap values for *Aestabdella leiostomi* DQ414305, *Pterobdella amara* DQ414334 and *Cystobranchus meyeri* DQ414315.

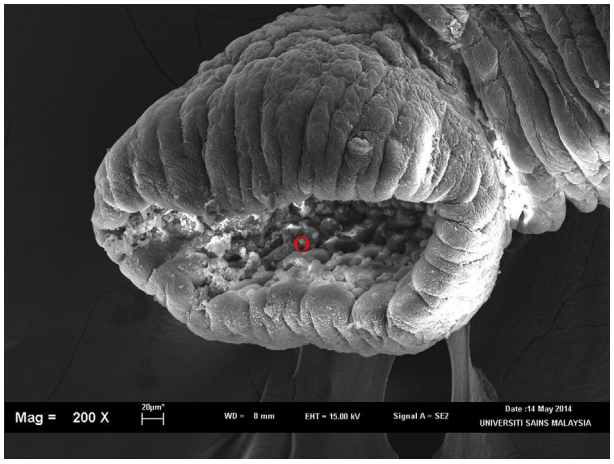

Figure 1. Ventral view of *Z. arugamensis*.
O: Oral sucker; WD: Working distance; EHT: Extra high tension.

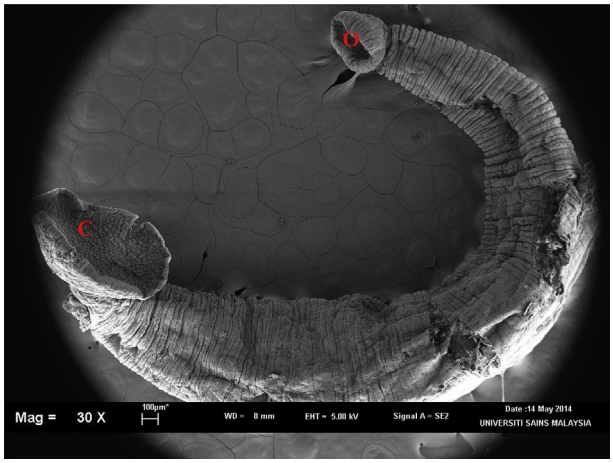

Figure 2. Posterior view of *Z. arugamensis*.
O: Oral sucker; C: Caudal sucker; WD: Working distance; EHT: Extra high tension.

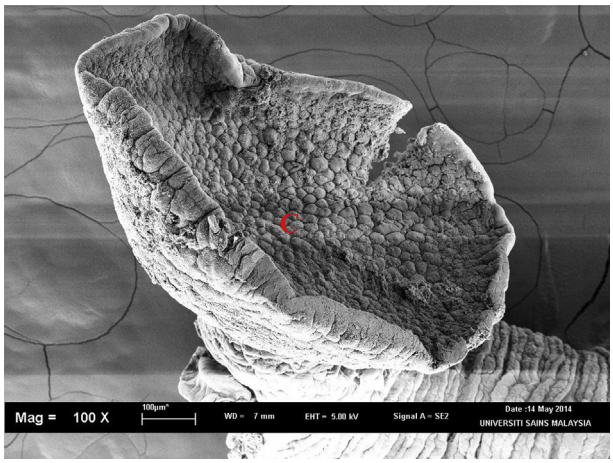

Figure 3. Ventral view of *Z. arugamensis*.
C: Caudal sucker; WD: Working distance; EHT: Extra high tension.

3.3. DNA barcoding accession table of parasites

The DNA barcoding delivers species level identification when taxonomy provides unique *COI* sequence clusters. The DNA sequence submitted to Barcode of Life Data System (www.boldsystem.org) in accordance to species, has already

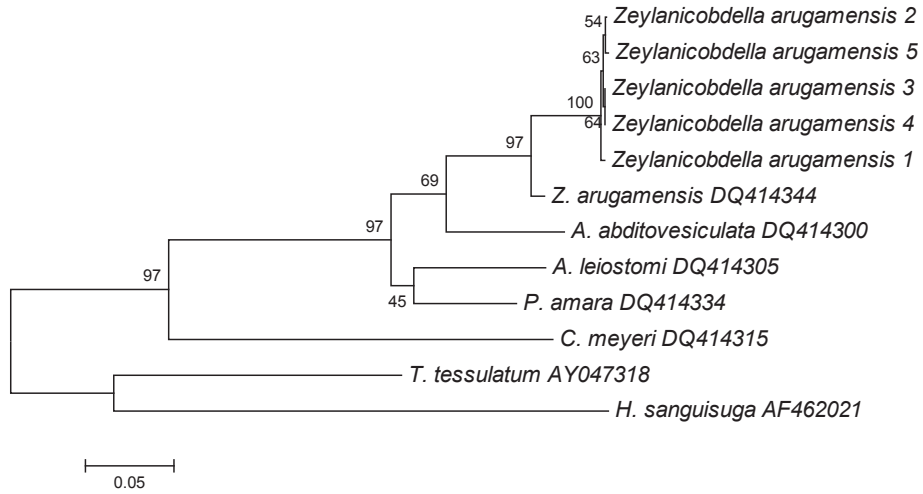

Figure 4. Phylogenetic tree derived from *COI* sequences in this study.

Table 1
Number of *Z. arugamensis* infestation in crimson snapper.

Length (cm)	Fish examined	Fish infected	Parasite recovered	Prevalence (%)	Intensity
26–28	50	10	14	5.0	1.40
29–30	80	8	11	4.0	1.37
31–35	70	5	9	2.5	1.80
Total	200	23	34	11.5	1.48

Table 2
The Shapiro–Wilk test for normality of temperature for each replicate.

Farm cage	Statistics	*P* value
Replicate 1	0.878	0.082
Replicate 2	0.931	0.386
Replicate 3	0.891	0.123
Replicate 4	0.950	0.635

Table 3
One-way ANOVA statistical test.

Farm cage	Sum of squares	*df*	Mean square	*F*	*P* value
Between groups	0.029	3	0.010	0.570	0.637
Within groups	0.750	44	0.017		
Total	0.779	47			

Table 4
Descriptive statistics of temperature from four replicates in this study.

Farm cage	*N*	Mean temperature	SD	SE	95% Confidence interval for mean	
					Lower bound	Upper bound
Replicate 1	12	33.1833	0.12673	0.03658	33.1028	33.2639
Replicate 2	12	33.2333	0.12309	0.03553	33.1551	33.3115
Replicate 3	12	33.2167	0.09374	0.02706	33.1571	33.2762
Replicate 4	12	33.2500	0.16787	0.04846	33.1433	33.3567
Total	48	33.2208	0.12876	0.01858	33.1834	33.2582

obtained BOLD Barcode Index Number as BOLD: ACM3477. Unfortunately, barcode gap analysis using BOLD system could not be performed in this study as there is no other work has been done in BOLD system at present [13].

3.4. Statistics for Z. arugamensis

According to Table 1, a total of 200 fish were examined with a low infestation prevalence rate of only 11.5%, and the intensity of parasites was 1.48.

3.5. Statistical analysis of temperature

Table 2 shows the normality test of temperature for the four replicates. According to Table 3, all the *P* value was above 0.05, hence normality of water temperature had been achieved. Test for homogeneity of variances showed that *P* value was greater than 0.05 ($P = 0.253$), the assumption of homogeneity of variances is met [5,6,8]. According to Table 3, in ANOVA test, *P* value was greater than 0.05; the mean water temperature of different replicates was not significantly different. Table 4 shows the descriptive statistic of temperature in this study and suggests that the mean temperature for all the four replicate groups during the experiments was almost equal within the range of 32.9–33.2 °C.

4. Discussion

In the present study, we conducted a statistical analysis of *Z. arugamensis* parasite. The analysis was conducted according to the methods of previous studies [5,6,8]. The infestation prevalence rate of *Z. arugamensis* was low (11.5%) and intensity of leeches per fish was determined to be 1.48 in this study. The low intensity of leeches is likely due to the water temperatures recorded in this study (32.9–33.2 °C). A previous study [1] found that juvenile leeches were able to hatch at temperatures ranging from 25 to 35 °C but unable to hatch at 40 °C. The survival periods of adult and juvenile leeches ranged from 11 to 16 days at 25 °C, which was comparatively longer than the periods of 5–13 days and 10 h to 5 days observed at 27–30 °C and 35–40 °C, respectively. Hence, a shorter life cycle duration may lead to decreases in infestation rates compared to the infestation levels of other types of parasites. The most frequently identified effect of leeches on fish is local bleeding resulting from feeding

activity of leeches and ulceration at attachment sites [14]. Although histological results are not particularly detailed, microscopic examinations of attachment sites have identified varying degrees of epidermal and dermal layer loss accompanied by profuse bleeding and inflammatory infiltrates. Skin ulcerations create possible routes for pathogens including bacteria and fungi to enter the body, potentially resulting in secondary infections [14]. Secondary infection with the pathogenic bacteria *Vibrio alginolyticus* has been reported in cultured fish infested with *Z. arugamensis* [14]. Host morbidity and mortality due to leech infections vary in different published reports [1,15–18]. In general, leeches are seldom considered as significant parasites of fish and only become a problem when large numbers are present [13]. A study on *Z. arugamensis* infestation in tank-reared orange-spotted grouper *Epinephelus coioides* in the Philippines reported hundreds of leeches per fish but no mortality [14]. Specific factors contributing to overall mortality are not clear but may include factors such as host size and immune system and parasite abundance. The ability of a fish to physiologically endure and survive with leech infestation may also play a role in disease resistance. Ectoparasites can represent stressors for fish and have been associated with decreased food intake, anti-predator behaviour and reduced growth [14]. In general, host–parasite relationships are complex and thought to represent generations of co-evolution and adaptation [10]. Interactions at the host–parasite interface involve the parasite taking advantage at the host environment for its feeding, propagation and avoiding from being recognized by host immunity [7].

In summary, this study utilized analyses of sequence divergence, DNA barcodes, prevalence and phylogenetics and provided additional information related to the documentation and population structuring of fish parasites and a scientific framework for effective parasite identification.

Conflict of interest statement

We declare that we have no conflict of interest.

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