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First Report, Prevalence, and Molecular Identification of *Lecithocladium cristatum* in the Black Pomfret Fish, *Parastromateus niger* (Family: Carangidae)

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ABSTRACT

The black pomfret fish, *Parastromateus niger*, has been reported to have a natural range in the oceanic bay of Thailand, one of the biggest commercial fisheries in the Pacific region. It is an economically important marine fish because it is a favored source of high nutritional value for human consumption. A *Lecithocladium* infection is considered a problem for the health of fish, human consumption and commercial fisheries. Fifty specimens of *P. niger* were collected from local markets in Bangkok, Thailand and *Lecithocladium* spp. was recovered from their digestive tracts.The results showed that all parasite specimens were identified as *L. cristatum* via morphological characteristics. For molecular identification, the nucleotide sequence data of *18S rRNA* were used for molecular analysis. *Lecithocladium cristatum* in this study showed 97% sequence identity with *L. cristatum* in the GenBank database. Phylogenetic analysis revealed that this trematode species is deeply embedded within the genus *Lecithocladium*. The prevalence and mean intensity of *L. cristatum* infections were 72.00% and 11.07, respectively. Hence, this work revealed the epidemiological situation of *L. cristatum* in *P. niger* by using the morphologica- and molecular-based methods. Moreover, this is a first report of *L. cristatum* in the black pomfret from Thailand. In addition, these data are important for the prevention of future *Lecithocladium* outbreaks.

INTRODUCTION

The black pomfret fish, *Parastromateus niger* (Family: Carangidae), is commonly found in the Indo-west Pacific region in reefs of South Africa, Mozambique, Kenya, the Arabian Sea, Bay of Bengal, Indonesia, the Philippines, China, Southern Japan, and Australia [1]. The black pomfret fish is considered an important and favored source of high nutritional value, such as protein, for human consumption [2-4] and it is an economically important marine fish for commercial fisheries. There have been several reports about parasitic infections; these parasites can be classified into nine main parasites including, *Bicotyle* sp., *Neoaxine* sp., *Gilquinia* sp., *Lecithocladium bulbolabrum*, *Diplosentis* sp., *Parabomolochus megaceros*, *Thysanote appendiculate,* and *Lemanthropus koenigii* [5-7]. Trematodes belonging to the Family Hemiuridae are commonly found in the digestive tract of several species of marine fish including *P. niger* [1,8]. The Hemiuridae, identified by 19 families, are part of a superfamily containing, 25 subfamilies [9]. At least 83 species of hemiuroid were classified in the genus *Lecithocladium* [10] have been recorded and showed similar morphological characteristics and insufficient accuracy for identification [11-12]. The *Lecithocladium* can be classified by the combination of adult morphological characteristics [6] and molecular analyses [13-16]. Unique characteristics of *Lecithocladium* include a long and slender body, a ventral sucker that is larger than an oral sucker, thick muscle cover around the seminal vesicle, lack of a prostatic vesicle, and a vitellaria with seven tubes [17].

Molecular based methods are used for characterizing genetically distinct but morphologically similar individuals. Target genes are typically *28S rDNA* or *18S rRNA*. These are useful for studying the phylogenetic tree of the *Lecithocladium* [18]. The species *L. cristatum*, *L. angustiovum*, *L. parviovum,* and *L. excisum* have been generally reported in fish [5,19]. The prevalence and intensity of *Lecithocladium* spp. infections depend on various factors, such as environmental factors, including water temperature and habitat [20-21]. For example, the prevalence of *L. cristatum* and *L. parviovum* infections increased to 48.0% during the winter when the water temperature was lower and fell to 16.0% in the summer when the water temperature was higher [5,19]. Furthermore, parasitic infection can be mediated by host resources or the host immune system [22]. For example, large-sized fishes may have lower levels of parasite infection because of immunological responses of the hosts [17,23,24]. Additionally, it depends on the presence of intermediate hosts, such as different species of copepods [25]. The effect of *Lecithocladium* spp. infection in marine fish has been an attractive theme for a long time [26-27] because parasitism is a normal phenomenon in the marine environment. It is probable that all marine fish are infected with parasites belonging to the helminthes [28]. *Lecithocladium* spp. can cause gastro-intestinal tract histopathology in marine fish and commercial fisheries worldwide [29-30]. Currently, parasites are used as indicators for the differentiation of ecology, and

parasitic fauna might show a distribution parallel to the host distribution [11]. Therefore, this study aimed to investigate the epidemiological situation of *Lecithocladium* spp. in black pomfret fish by using morphological and molecular based methods. These data may prove to be an invaluable tool for detection and epidemiological investigation, important for the prevention of future *Lecithocladium* spp. outbreaks.

METHODOLOGY

Specimen collection

A total of 50 specimens of the black pomfret fish, *Parastromateus niger* were collected from February to October 2019 from local markets in Bangkok, Thailand. All fish specimens were transported in ice barrels to the Applied Parasitology Research Laboratory of the Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok. Specimens were examined under a stereomicroscope for parasitic infections within 24 hours. The prevalence and mean intensity of parasitic infections were calculated. Parasite specimens were separated into 2 sections; (I) fixed in 10% formalin solution for morphological studies [31] and (II) kept in 70% ethanol and stored at -20°C for molecular analysis. All experimental methods related to the use of animals were proceeded by the National Research Council of Thailand and approved by the Committee for Animal Care and Use Committee Srinakharinwirot University (License No SWU-A-001-2562).

Morphological studies

The parasite specimens were stained with Semichon's Acetocarmine, then dehydrated through an ascending alcohol series (70%, 80%, 90%, and 95%), cleared in xylene, and mounted on canada balsam. Body length and width were measured and important organs were stained to identify the species by morphological characters. Measurements were taken in millimeters and presented as mean ± standard deviation followed by a range in parentheses [32].

Molecular studies

Genomic DNA was extracted using PureDireX DNA extraction Kit, according to the manufacturer's recommended protocols. All polymerase chain reactions (PCRs) were carried out in a reaction mixture for a final volume of 25 µl comprising; 1.5 U of *Taq* DNA polymerase, 10X buffer, 50 mM $MgCl_2$, 2.5 mM dNTPs, 10 µM each of forward primers and reverse primers, and 5 ng DNA template. The primers were Primer BD 18S forward: 5'-GGT GAC AAG ATA TAA CGA TGT AAC A-3' and Primer BD 18 S reverse: 5'-CAG TCA GAG ACT AGT TAG GAA GT-3'. Primers were designed by selecting the regions of *18s rRNA* gene generated with software tools, then searched against an appropriate nucleotide sequence in the database with BLAST. We found that the potential target accession numbers were MK993436.1, MN566131.1, MN970010.1, MN970005.1, MN217108.1, and MN700961.1. The product was 1,211 base pairs. PCR amplification was completed in a thermal cycler, with a program of 30 cycles of denaturation (94°C, 1 min), annealing (59°C, 1 min), extension (72°C, 1 min), and final extension (72°C, 7min). After that PCR products were electrophoresed on a 1.0% agarose gel in Tris–acetate-EDTA buffer and stained with SYBR Safe DNA Gel Stain, following which bands of the expected size for *18S rRNA* were purified using standard techniques and direct sequencing.

Sequence alignment and phylogenetic analysis

Sequences data were compared with other sequences available in GenBank using BLAST. Alignment was corrected manually using the alignment editor available with the MEGA 10.1 software. DNA sequence similarities were calculated with the sequence identity software. Data were analyzed with maximum parsimony and bootstrap of 10,000 replications.

RESULS ADN DISCUSSION

The black pomfret fish specimens, 36 of 50 were found to be infected with *Lecithocladium cristatum* in the digestive tract. The prevalence of *L. cristatum* infection was 72.00% and mean intensity was 11.07. After being classified by morphological characteristics (n = 50), mean body length was found to be 5.46±0.63 mm (4.47 to 6.62 mm) and mean ecsoma was 1.75±0.42 mm (0.95 to 2.12 mm). Body length was 68% of the total length and the ecsoma was 32.0% of the total length (ratio between body and ecsoma was 2 : 1). The ecsoma tapered posteriorly and was usually extended with a blunt shape. The maximum body width at the level of the ecsoma invagination was 0.88±0.14 mm (0.71 to 1.07 mm). Cuticular denticulations were present on the entire surface of the body proper. A ventral sucker was in the center of the body and measured 0.40±0.04 mm (0.33 to 0.46 mm). The oral sucker was at the anterior of body and measured 0.33±0.03 mm (range 0.30 to 0.38 mm). The ventral sucker was larger than the oral sucker, with a ratio with the body of 1 : 1 : 24. The seminal vesicle was located on the posterior of the body and measured 0.46±0.15 mm (range 0.16 to 0.66 mm) in length and 0.23±0.04 mm (range 0.17 to 0.35 mm) in width. The pharynx was cylindrical and measured 0.35±0.04 mm (range 0.29 to 0.40 mm) in length and 0.19±0.03 mm (range 0.15 to 0.25 mm) in width. Seven tubes of the vitellaria curled around the center of the body and parts of the intestinal caeca were bifurcated. Anterior and posterior testes were oval-shaped and measured 0.30±0.080 mm (range 0.25 to 0.49 mm) in length and 0.27±0.09 mm (range 0.15 to 0.41 mm) in width, positioned obliquely on each side of the body. The two ovaries were oval, post-testicular, and measured 0.23±0.02 mm (range 0.20 to 0.26 mm) in length and 0.28±0.05 mm (range 0.22 to 0.40 mm) in width. The uterus extended more than half of its length into the ecsoma and coiled between the testes and ovaries **(**Figure 1**).** For the molecular analyses, partial sequence data of the 1*8S rRNA* gene was obtained to investigate and accurately identify the *Lecithocladium* specimens. The 1,211 bp specific DNA fragment was amplified and the obtained sequences were compared with other sequences available, revealing that these sequences exhibited similar identity to *L. cristatum,* which had been previously sequenced. In this study, 97% sequence identity was found with *L. cristatum* in the GenBank database. Phylogenetic tree analysis was performed and data was generated by maximum parsimony analysis of the *18S rRNA* sequences together with *Lobatostoma manteri* as a outgroup. These results revealed that the recovered trematode species were deeply embedded within the genus *Lecithocladium* (Figure 2).

In 2002, *Lecithocladium* was found in the black pomfret fish, concurring study conducted in Thailand, which was identified as *L. bulbolabrum.* Notably, *Semenispa irregularis* was also previously found in the black pomfret fish from the gulf of Thailand [33]. Therefore, detection of *L. cristatum* in the black pomfret fish is for the first report in Thailand so far as the literature concerned.

Trematodes belonging to the genus *Lecithocladium* [10] could infect the digestive tract of different kinds of marine fish [12]. The prevalent of present study was slightly different when compared with other *Lecithocladium* spp.such as the report of Forcep Rio Indaryanto et al. [12] that reported the prevalence of *L. angustiovum* identified from the stomach of Indonesian short mackerel (*Rastrelliger brachysoma*) was 87.33% because Thailand and Indonesia are close to the equator and the temperature between summer and winter does not differ drastically, therefore, water temperature did not significantly different to affect the prevalent. However, the prevalence of present study was higher than Abdel-Gaber et al. [34] that reported the prevalence of *L. cristatum* infected Greater lizardfish (*Saurida tumbil*) from Red Sea, Egypt was 12.0%.

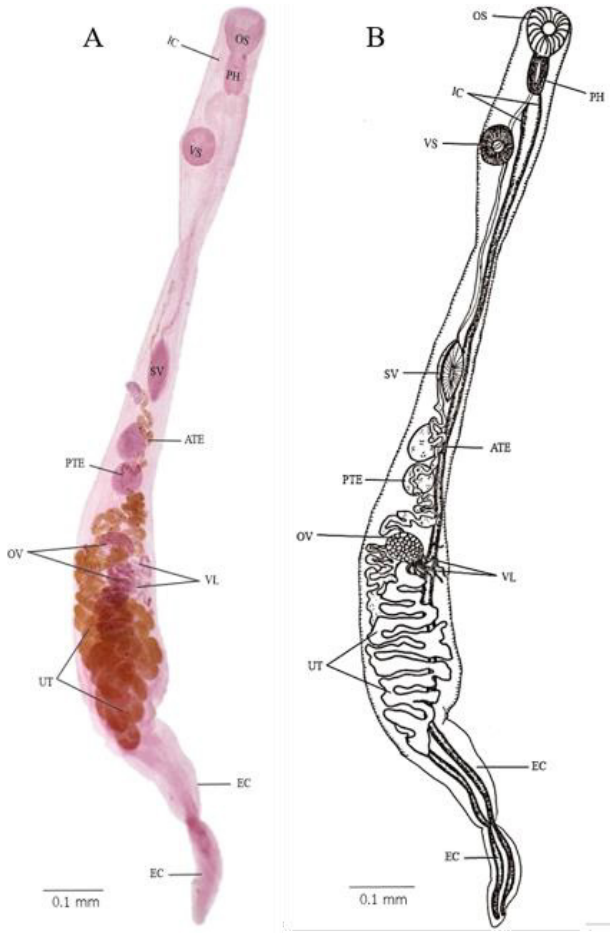

Figure 1. *Lecithocladium cristatum* (A) Whole-mount preparation and (B) line drawings of lateral view (OS: oral sucker, PH pharynx, IC: bifurcates intestinal caeca, VS: ventral sucker, ATE: anterior testis, PTE: posterior testis, SV: seminal vesicle, OV: ovary, UT: coiled uterus filled with numerous of eggs (EG), VL: seven tubulars vitelleria and EC: ecsoma.

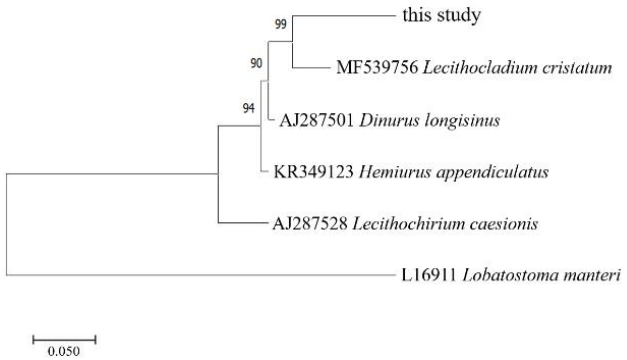

Figure 2. Phylogenetic analysis generated by maximum parsimony analysis of the *18S rRNA* sequence data. GenBank accession numbers are given before the species names together with *Lobatostoma manteri* (outgroup).

In addition, there are other different factors that affect the prevalence and mean intensity of infection. Size of host is one of important factor affected parasite infection because parasite interactions can be mediated by the host immune system [22]. For example, Adams, Luque and Oliva, Ferrari-Hoeinghaus et al. [17,23-24] suggested that the large-sized fishes may have lower levels of parasite infection because of immunological responses of the hosts. In this study, *L. cristatum* infection of individuals size between 4.92-8.27 inches (lower normal size) was higher than *L. cristatum* infection of individuals' size between 9.84–12.20 inches (upper normal size) when normal size of black pomfret was between 8.66–9.45 inches that reported by Bloch [1].

Lecithocladium spp. can be classified by morphological characteristics in adult helminthes [6]. The specific features used to classify this species were length and width of body or ecsoma, an elongated pharynx, a seminal vesicle with a thick muscle located in the posterior part of the body, a ventral sucker that was as large as the oral sucker, a vitellarium with 7 elongated tubes, and the absence of a prostatic vesicle [13,17,35]. *Lecithocladium cristatum* specimens were classified by morphological characteristics and the mean body: ecsoma length ratio was 2:1 closely resembled that of *L. cristatum* from *Seriolella porosa* in San Matías Gulf, Argentina as recorded by Guagliardo et al. [36] and from the greater lizardfish (*Saurida tumbil*) in the Red Sea as recorded by Abdel-Gaber et al. [34]. The maximum body width at the level of the ecsoma invagination was 0.88±0.14 mm closely resembled that of *L. cristatum* from *S. violacea* in Peru and Chile as recorded by Luque and Oliva [17]. We found that *L. cristatum* in the present study, has characteristics closely resembled that of *L. cristatum* from other related species that were described previously with slight differences in body and organ measurements. These results consistent with Guagliardo et al. [36] stated that all *L. cristatum* had the characteristically generic features of that genus but with slight differences in body and organ measurements. Generally, only morphological data provide less resolution than molecular data because hemiuroid in the genus *Lecithocladium* were recorded and had similar morphological characteristics, which was insufficient identification of species belonging to this genus [10-12]. Therefore, the *Lecithocladium* spp. can be classified by the combination between morphology [6] and molecular data [13-16]. For molecular based methods used for characterization, the target genes are typically *28S rDNA* or *18S rRNA,* which are useful for studying gene sequences and the phylogenetic tree of *Lecithocladium* [14,18] reported that *18S rDNA* or *18S rRNA* gene sequences significantly enhanced the chances of accurately identifying new species and supported this with phylogenetic analysis. In the present report, we used the *18S rRNA* gene for molecular analysis because the *18S rRNA* gene is particularly useful for elucidating relationships in this group and it is highly variable even in very closely related species [37]. The result showed that the present investigation revealed at least a 97% sequence similarity to other *L. cristatum* described previously in the GenBank database. By the study of Blair et al. [38], phylogenetic relationships were determined within the family Hemiuroidea, which is a group with a complex taxonomical history, when analyzed with maximum parsimony. These results revealed that the recovered trematode species were deeply embedded within the genus *Lecithocladium* consistent with the report of Abdel-Gaber et al. [34].

CONCLUSION

This is the first report of *L. cristatum* infecting the black pomfret fish in Thailand. This study has provided a description of *L. cristatum* by clarifying some of the body and organ measurements. Moreover, this report contributes to knowledge on identifies the role of the black pomfret fish, as a definitive host of hemiurids in the ecosystem.

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