DOI: 10.32663/ja.v21i2.4740

MOLECULAR IDENTIFICATION AND PHYLOGENETIC ANALYSES OF ECTOPARASITES AND ECTOSYMBIONTS THAT INFECT BLUE SWIMMING CRABS (*Portunus pelagicus*)

(Identifikasi Molekuler Dan Analisis Filogenetik Ektoparasit Dan Ektosimbion Pada Rajungan (Portunus Pelagicus))

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ABSTRACT

The decline in blue swimming crab populations can be caused by overfishing and several factors of the aquatic environment, such as anthropogenic pollution, water quality and the presence of ectoparasite in the crab. Molecular studies are essential to validate a particular species of ectoparasites and ectosymbionts that infect Crab. A total of 143 crabs were sampled from fishermen in Demak, Muara Gembong and Labuhan Maringgai. Six ectoparasite and ectosymbionts species, *Chelonibia testudinaria* (Crustacea), *Dianajonesia tridens* (Crustacea), *Octolasmis angulata*, *O. warwicki* (Crustacea), *Ostrea puelchana* (Mollusca) and *Thompsonia* sp. (Crustacea) were isolated. Three ectoparasite and ectosymbionts species that were successfully validated based on the COI mtDNA genes were *C. testudinaria, D. tridens, Oc. angulata*. Validation of isolated ectoparasites infecting *P. pelagicus* was performed using the mtDNA marker COI gene to identify three ectoparasite species molecularly, namely *C. testudinaria, D. tridens*, and *Oc. ang*ulata. The results of the five sample analyses of ectoparasites based on the number of specific nucleotide sites in each sample show that the suspected species *C. testudinaria, D. tridens* and *Oc. angulata* had missense mutations.

Keywords: ectoparasite, blue swimming crab, molecular, *Octolasmis* sp.

ABSTRAK

Penurunan populasi rajungan (*Portunus pelagicus*) dapat disebabkan oleh penangkapan berlebih (*overfishing*) dan beberapa faktor lingkungan perairan, seperti pencemaran antropogenik, kualitas air dan adanya ektoparasit pada rajungan. Studi molekular sangat penting untuk memvalidasi spesies ektoparasit dan ektosimbion tertentu yang menginfeksi Kepiting. Sebanyak 143 kepiting diambil sampelnya dari nelayan di Demak, Muara Gembong dan Labuhan Maringgai. Enam spesies ektoparasit dan ektosimbion, *Chelonibia testudinaria* (krustasea), *Dianajonesia tridens* (krustasea), *Octolasmis angulata, O. warwicki* (krustasea), *Ostrea puelchana* (moluska) dan *Thompsonia* sp. (krustasea) diisolasi. Tiga spesies ektoparasit dan ektosimbion yang berhasil divalidasi berdasarkan gen mtDNA COI adalah *C. testudinaria, D. tridens, Oc. angulata.* Validasi ektoparasit terisolasi yang menginfeksi P. pelagicus dilakukan dengan menggunakan penanda mtDNA gen COI untuk mengidentifikasi tiga spesies ektoparasit secara molekuler, yaitu C. *testudinaria, D. tridens,* dan *Oc. angulata*. Hasil analisis lima sampel ektoparasit berdasarkan jumlah situs nukleotida spesifik pada setiap sampel menunjukkan bahwa spesies yang diduga *C. testudinaria, D. tridens,* dan *Oc. angulata* mengalami mutasi *missense*.

Kata kunci: Blue Swimming Crab, Ektoparasit, Molekular, *Octolasmis* sp.

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INTRODUCTION

The Portunidae family, which includes blue swimming crabs, is commonly consumed by humans due to the soft meat and high nutritional value. Because they have several legs that are particularly suited to swimming, blue swimming crabs from the family Portunidae are frequently referred to as portunid crabs or swimming crabs. P. pelagicus, or blue swimming crabs, are commonly found in Indo-Pacific waters. (Ikhwanuddin et al., 2011; Romano et al., 2012; Santhanam 2018).

One of Indonesia's most expensive fishery products, blue swimming crabs (*P. pelagicus*) have significant economic value and are exported. The over usage of crabs is out of proportion to our understanding of how to manage and preserve these fishery resources. This resulted in a decline in the population of crab stocks, which is a problem that Indonesia is currently facing with regard to various crab fisheries resources. Overfishing and a number of aquatic environment issues, including human pollution, water quality, and the presence of ectoparasites and ectosymbionts in the crab, may be to blame for the population fall of blue swimming crabs. Ectoparasites can have harmful pathogenic effects, particularly when they cause the host to become seriously ill (Shields and Overstreet, 2003). Environmental stressors and deterioration can both have an impact on the presence of ectoparasites. Certain ectosymbionts have a major impact on a fishery's size, productivity, and product consumption. As the water quality drops, ectoparasites will proliferate and mature more quickly in the crab environment (Troedsson et al., 2008; Fidyandini et al., 2024).

Research on ectoparasites that infect blue swimming crabs is required, as is the identification of each type of ectoparasite using morphological and molecular identification techniques. Understanding the parasite infection patterns of blue swimming crabs allows one to understand the true mode of infection among the various crab populations in various regions. Furthermore, knowledge of the previously unidentified distribution and traits of parasites is necessary to develop a more productive and sustainable crab fishery in the future.

Numerous studies have acknowledged that the primary barriers to studying the presence of ectoparasites and ectosymbionts are methodological and taxonomic restrictions. Molecular research is important because it validates specific parasite species. Novelty: Our capacity to identify novel diseases and disease outbreaks, as well as to approximate the patterns of parasitic infection, is hampered by our incomplete understanding of the parasite presence in blue swimming crabs. The study's aim is: The purpose of this work was to identify ectoparasites isolated from P. pelagicus blue swimming crabs using molecular techniques and to conduct a molecular origin analysis of ectoparasites.

MATERIALS AND METHODS

Location research

This research was conducted using blue swimming crabs (P. pelagicus) from Demak Central Java, Muara Gembong West Java, and Labuhan Maringgai East Lampung (Figure 1). The molecular analysis was done at Balai Riset Budidaya Ikan Hias (BRBIH) Depok, West Java.

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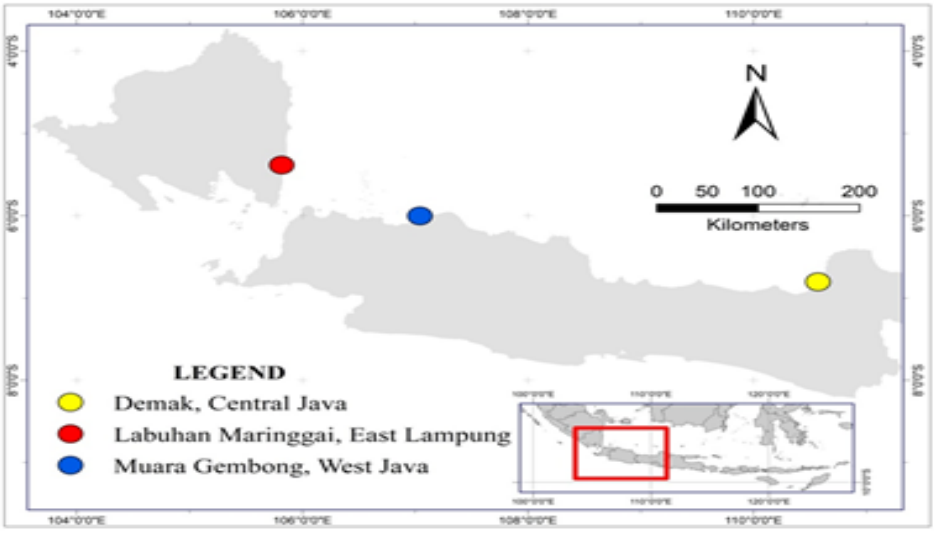

Figure 1. Sampling location for examined crabs (Portunus pelagicus) from coastal waters of Demak, Labuhan Maringgai, and Muara Gembong.

Sample Collection

Samples of the blue swimming crabs (P. pelagicus) were taken by field observation methods from three different locations (Figure 2). Blue swimming crab samples (P. pelagicus) were obtained from traditional fishermen's catch using traps and gill nets. A total of 143 crab samples, consisting of 43 crab samples from Demak, 50 crab samples from Labuan Maringgai and 50 samples from Muara Gembong, were measured and examined in this study. The crabs were individually stored into single plastic bags at temperatures up to 4°C for transport, the isolated parasites were stored in a microtube containing 96% ethanol as a preservative (Shields, 1992).

Molecular Techniques

DNA isolation and extraction

Tissue samples from all P. pelagicus and parasites were preserved in 96% ethanol absolute. The preserved foot samples were washed using distilled water to remove the alcohol content, then dried and put into a 1.5 ml microtubes. DNA isolation and extraction were carried out using the gSYNC DNA Extrasion Kit following the working procedure issued by the manufacturer Gene Aid.

Total DNA quality test

Total DNA quality test was carried out after the DNA was isolated at the extraction stage. Total DNA quality was tested by electrophoresis on a 1.2% agarose gel using a buffer solution of TAE1x and DNA, stained with 5 µl ethidium bromide. The total amount of DNA used was 2.5 µl. Visualization of total DNA was done using an ultraviolet machine.

Amplification and visualization of COI gene DNA fragments

Molecular markers are commonly used to identify genetic structures using mitochondrial DNA (mtDNA) sequence analysis. The mtDNA part that is often used for species validation is Cytochrome oxidase subunit I (COI) (Hubert et al., 2008). Amplification was done using total DNA which had good quality using PCR (Polymerase Chain Reaction) technique. The

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COI gene region was used. For this ectoparasite DNA amplification universal primers, namely LCO1490: 5′-GGTCAACAAATCATAAAGATATTGG-3′ and HCO2198: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′.

The amplification steps included predenaturation of 94oC for 5 minutes, denaturation of 94oC for 45 seconds, annealing 54oC for 1 minute, elongation 72oC for 1 minute, post elongation 72oC for 5 minutes, and storage of 15oC for 10 minutes. Afterwards the PCR product was tested for quality by electrophoresis on a 1.2% agarose gel and visualized using an ultraviolet machine.

DNA sequencing

PCR products that had good quality were used to be continued to the stage of sequencing to determine the exact nucleotide base sequences. Sequencing was done by sending the PCR products to the sequencing service company, namely Macrogen Inc, South Korea.

Bioinformatic analysis

The bioinformatic analysis in this study used MEGA software version X, which consisted of validating nucleotide base sequences. The sequencing results were edited using MEGA software version X. The edited sort results were continued with the Basic Local Alignment Search Tool (BLAST) process through the National Center for Biotechnology Information (NCBI) website. This validation was carried out to see the suitability of the nucleotide sequences of the same species sequences deposited in NCBI.

RESULTS AND DISCUSSION

Seven ectoparasite samples were morphologically suspected to be C. testudinaria, D. tridens, Oc. angulata, Oc. lowei, Oc. warwicki, Os. puelchana and Thompsonia sp. (Figure 2) found to infect the blue swimming crabs (*P. pelagicus*). Molecular identification using the COI mtDNA gene is very effective for identification to the species level (Hebert et al., 2003). Some samples produced quite thick PCR product bands as a result of good total DNA quality (Figure 2).

There were three DNA samples that did not produce PCR product bands namely, *Oc. warwicki. Os. puelchana* and *Thompsonia* sp. so that they cannot be proceeded to the sequencing stage. The success of PCR amplification is determined by the presence or absence of DNA sites. The quality and quantity of DNA highly affect the success of the PCR process (Sunandar and Imron, 2010). According to Suherman (2013), the quality of the DNA template affects the quality of the PCR product which is displayed in the form of a PCR product ribbon. Another factor that affects the appearance of PCR bands is the success of the primers that can attach to the amplicon. The primer will attach to the DNA genome, which has a nucleotide base arrangement that completes the primary base arrangement.

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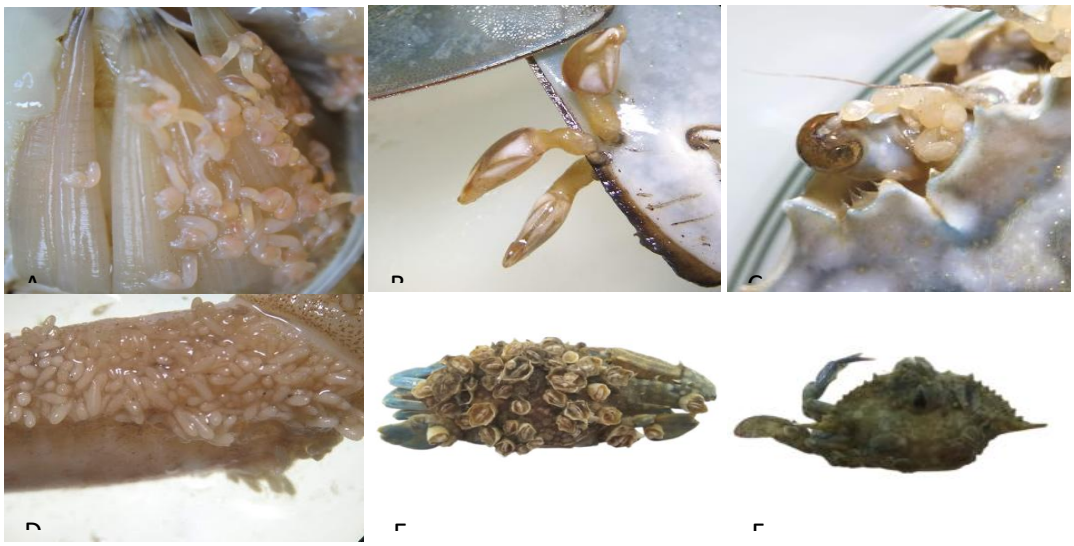

Figure 2. Ectoparasite and ectosymbionts infection of the body of *Portunus pelagicus*: A) *Octolasmis* spp.; B) *Octolasmis warwicki*; C) *Dianajonesia tridens*; D) *Thompsonia* sp.; E) *Chelonibi testudinaria*; F) *Ostrea puelchana*

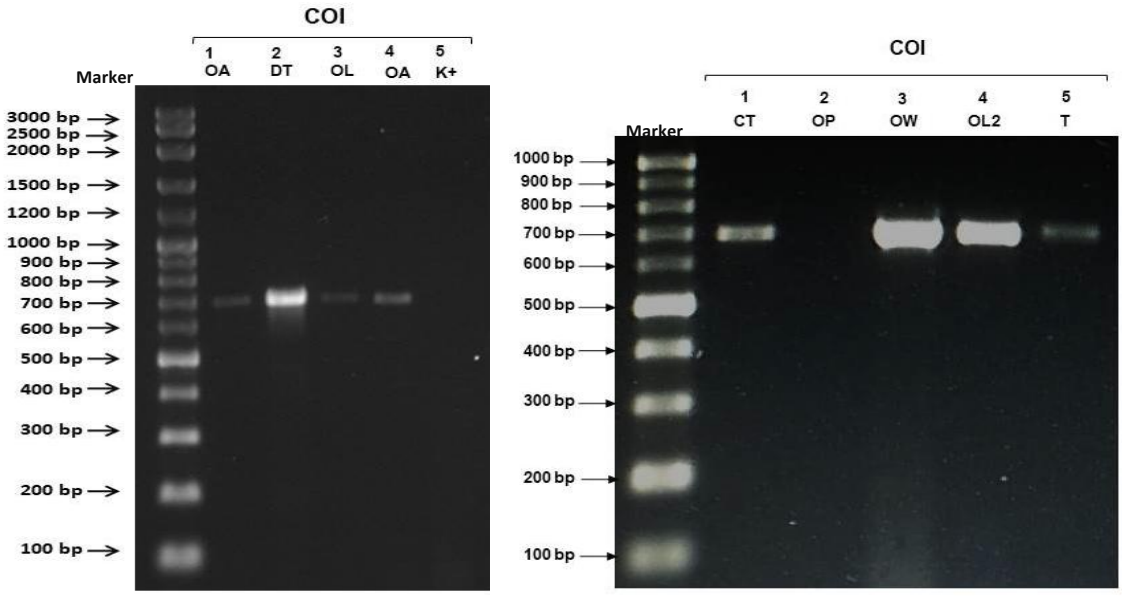

Figure 3. Ectoparasite and ectosymbionts PCR band products in *P. pelagicus* using COI gene markers. Each electrophorensis showed DNA marker (A) *Octolasmis angulata*, (B) *Chelonibia testudinaria*, (2.A) *Dianajonesia tridens*, (2.B) *Ostrea puelchana*, (3.A) *Octolasmis angulata*, (3.B) *Octolasmis warwicki*, (4.A) *Octolasmis angulata*, (4.B) *Octolasmis angulata*, (5.A) fish DNA as a comparison, and (5.B) *Thompsonia* sp.

Jurnal Agroqua
Volume 22 No. 2 Tahun 2024

DOI: 10.32663/ja.v21i2.4740

The nucleotide sequences of the ectoparasite COI gene that infects blue swimming crabs (*P. pelagicus*) were uploaded to the Basic Local Alignment Search Tool – nucleotide (BLASTn) on the National Center for Biotechnology Information (NCBI) website. The BLASTn method was used to validate the sequence results of the target species against the species sequence data listed in NCBI (reference species). The phenomenon of "complex species" appears in samples OL (*Octolasmis lowei*), OL2 (*O. lowei* 2), and OA (*Oc. angulata*). While the three samples differ morphologically, they are molecularly similar to *Oc. angulata* based on the CO1 gene fragment; the CT (*Chelonibia testudinaria*) sample, on the other hand, has a similarity value of 96% and is thought to be a sub-species of C. testudinaria. Only DT (*Dianajonesia tridens*) samples from this investigation were correctly recognized as *D. tridens*. Three samples in this study OA (96%), OL2 (95%), and OL (90%), showed a genetic resemblance to the species Oc. angulata. This is insufficient evidence to conclude that the three samples are Oc. angulata since, as Hebert et al., (2003) point out, many organisms can be grouped together into a single species provided the percentage identity is high enough.The results of BLASTn are presented in Table 1.

Table 1. BLAST results on the NCBI website

Sample Code	Query Cover	Percent Identity	BLAST GenBank ID	Species
OA	77%	93.12%	MN336790.1	*Octolasmis angulata*
DT	30%	98.36%	MH753553.1	*Dianajonesia tridens*
OL	82%	90.66%	MN336754.1	*Octolasmis angulata*
CT	91%	96.23%	KJ754819.1	*Chelonibia testudinaria*
OL2	48%	95.21%	KC138498.1	*Octolasmis angulata*

Specific Nucleotides

The sequence of nucleotide bases that have been obtained from the sequencing results was aligned using the Clustal W method from the MEGA X software (Kumar et al. 2018). The results of the calculation of the number of nucleotide bases from all aligned samples obtained a DNA length of 441 bp with a composition of 324 variable sites and 117 conserved sites. However, each species has a different number of variable and conserved sites. The species *Oc. angulata* had 43 variable sites and 398 conserved sites, while *C. testudinaria* had 281 variable sites and 160 conserved sites, and *D. tridens* had 249 variable sites and 192 conserved sites. This variable sites had at least two types of nucleotides, while the conserved sites was to find out the constant/conserved site without nucleotide changes. Species *C. testudinaria* (KY273914.1, KY273915.1, AY174362.1), *D. tridens* (MH753553.1) and *Oc. angulata* (MN336860.1, MN336856.1, MN336854.1) was used as a comparison to determine the variable and conserved sites.

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Specific nucleotides have shown to be results of mutations to facilitate an organism to adapt to the environment. The physico-chemical characteristic of the nucleotide sequences of some genomic sites can change so that changes in the DNA nucleotide sequences can trigger mutations. Specific nucleotides that are the result of mutations are the basis for evolution. Mutations produce genetic diversity so to know the evolutionary process requires knowledge of the rate and pattern of mutations (Hersberg, 2015).

The diversity of nucleotide bases and the presence of specific nucleotides from five samples of ectoparasite species using the COI gene marker was obtained by sequencing the nucleotide bases using MEGA X software with multiple alignment techniques. The results of multiple alignments show the number of specific nucleotides, which are the specific character of nucleotide bases as differentiators from other species. The sample OL2 had five site-specific nucleotides (22, 270, 300, 301, 328). The CT sample had 146 specific nucleotides. OL had seven site-specific nucleotides (67, 106, 178, 181, 214, 271, 307). DT had 249 specific nucleotides, and OA had two specific nucleotide sites at sites 2 and 277.

The results of this study showed that the sample with the highest specific nucleotide site was DT, while the sample with the lowest specific nucleotide site was OA. Populations with high genetic diversity have a better chance of survival. This is because each gene has a different response to environmental conditions, so the high number of specific nucleotides it has can adapt to environmental changes that occur (Yusron, 2005; Akbar et al., 2014). However, in this study the prevalence values of the OA sample (*Oc. angulata*) was higher than those of the DT sample (*D. tridens*). This happens because *Oc. angulata* infects the gills of the host and spends its entire life cycle there, while *D. trides* which is mostly found to infect the carapace of its host and the parasites life cycle is influenced by the molting cycle of the host.

Genetic Distance

In the process of building a phylogenetic tree, the sequences *C. testudinaria* (KY273914.1, KY273915.1, AY174362.1), *D. tridens* (MH753553.1), and *Oc. angulata* (MN336860.1, MN336856.1, MN336854.1) were used as reference species from GenBank. This phylogenetic tree shows the position of the respective sample with its relative species (Figure 4). The results of the analysis of the phylogenetic tree construction of the *P. pelagicus* ectoparasite species based on the COI gene formed two clades (branching). The first clade consists of several subclades, namely the first subclade samples OL, OL2, and OA, the second subclade consists of species *Oc. angulata* (MN336860.1, MN336856.1, MN336854.1) derived from geneBank, the third subclade consists of samples of *D. tridens* and the fourth subclade consists of samples of *C. testudinaria*. The second clade consists of two subclades, the first subclade consists of samples of *C. testudinaria* (KY273914.1, KY273915.1, AY174362.1) from GenBank and the second subclade consists of samples of *D. tridens* (MH753553.1).

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Table 2. Genetic distance

No	Species	1	2	3	4	5	6	7	8	9	10	11	12
1	OL *Octolasmis angulata*												
2	OL2 *Octolasmis angulata*	0,0515											
3	OA *Octolasmis angulata*	0,0403	0,0201										
4	MN336860.1 *Octolasmis angulata* isolate OA QN32	0,0425	0,0447	0,0380									
5	MN336856.1 *Octolasmis angulata* isolate OA QN28	0,0425	0,0447	0,0380	0,0000								
6	MN336854.1 *Octolasmis angulata* isolate OA QN26	0,0626	0,0805	0,0761	0,0559	0,0559							
7	CT *Chelonibia testudinaria*	0,2192	0,2215	0,2148	0,2125	0,2125	0,2125						
8	KY273914.1 *Chelonibia testudinaria* isolate *Chelonibia patula* 1	0,5850	0,5850	0,5828	0,5873	0,5873	0,5873	0,6190					
9	KY273915.1 *Chelonibia testudinaria* isolate *Chelonibia patula* 2	0,5850	0,5850	0,5828	0,5873	0,5873	0,5873	0,6190	0,0045				
10	AY174362.1 *Chelonibia testudinaria* isolate GR30	0,5941	0,5873	0,5850	0,5896	0,5896	0,5828	0,5964	0,1007	0,0962			
11	DT *Dianajonesia tridens*	0,1857	0,1700	0,1700	0,1655	0,1655	0,1812	0,2327	0,5873	0,5873	0,5850		
12	MH753553.1 *Dianajonesia tridens* isolate OT	0,5805	0,5760	0,5760	0,5760	0,5760	0,5646	0,5896	0,2058	0,2081	0,1969	0,5646	

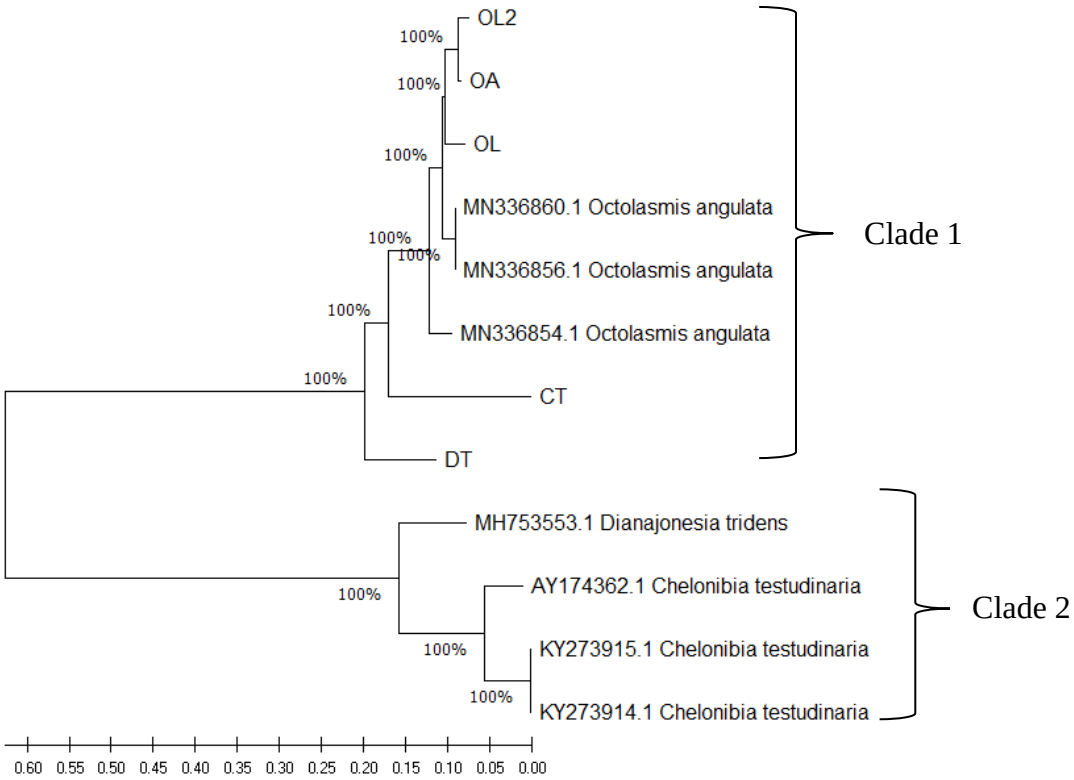

Figure 4. Construction of a phylogenetic tree based on the COI genes

The positions of OA, OL, and OL2 in the phylogenetic tree are located in a subclade with the reference species of GenBank *Oc. angulata* (MN336860.1, MN336856.1, MN336854.1) thus indicating that OA, OL, and OL2 are closely related to species *Oc. angulata* (MN336860.1, MN336856.1, MN336854.1). The position of

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the DT sample (*D. tridens*) is in the third subclade of the first clade, so it seems to have a distant relative from the *Oc. angulata* species even though it belongs to the same family, namely Poecilasmatidae. Initially, *D. tridens* was included in the same genus as *Octolasmis* with the previous scientific name *O. tridens*, then changed to *Poecilasma tridens* and then changed to *Temnaspis tridens* until finally it is now included in the *Dianajonesia* genus, namely *D. tridens*. The phylogenetic tree indicates a distant relationship between the species *Oc. angulata* and *C. testudinaria*. This is because the two species come from different families, *C. testudinaria* belongs to the family Chelonibiidae. In the second clade, there are only reference species from GenBank, namely *C. testudinaria* (KY273914.1, KY273915.1, AY174362.1) and *D. tridens* (MH753553.1). In the second clade, there is a distant relationship between *C. testudinaria* (KY273914.1, KY273915.1, AY174362.1) and *D. tridens* (MH753553.1). The greater the value of genetic distance in the population, the more isolated the varieties from one another. Genetic distance indicates a possible geographic influence on the population (Laltanpuii et al., 2014).

The study's findings demonstrated that seven different species of ectoparasites and ectosymbionts *C. testudinaria, D. tridens, Oc. angulata, Oc. lowei, Oc. warwicki, Os. puelchana,* and *Thompsonia* sp. infested P. pelagicus across all locations. According to Yunarty et al., (2023), the ectoparasite counts in all samples were generally higher than those in *P. pelagicus* that was cultivated in brackish water.

The most frequent ectoparasite that infects crabs is *Octolasmis* sp. The morphology of this parasite is distinct from that of other parasites. The lower portion of the body, which resembles a sprout and is yellowish in color, is linked to the host. The findings of Utari et al., (2017), who also discovered Octolasmis sp. in mangrove crabs, are consistent with this. It is common to find *Octolasmis* sp. infecting Crustacea. According to Suherman (2013), this parasite clings to the hypobranchial portion of the gills, which is the region of the body that has a scutum, tergum, and carina. The signs of this parasite infection include grayish brown moss fibers on the underside of the carapace and darkening of the gills to black (Wardhani et al., 2018).

Morphological analysis revealed that the OL and OL2 samples were *O. lowei*. This is as a result of the two samples' similar morphological features to those of *O. lowei*. The OL, OL2, and OA samples exhibit species similarity, suggesting the presence of a species complex phenomena across the three samples. According to Service and Townson (2004), complex species are those that share the same or extremely similar physical traits and are challenging to differentiate from one another. The morphologies of the members of the complex species are similar to one another, making identification errors frequent. Because complex species share the same morphology but differ genetically, it can be quite challenging to identify between them. But the behavior of these species varies (Elyazar et al., 2013).

The results of the five sample analysis of ectoparasites based on the number of specific nucleotide sites in each sample show that the suspected species *Oc. angulata, C. testudinaria* and *D. tridens* had missense mutations. This is indicated because of the change in nucleotides

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followed by changes in amino acids. According to El-Tonsy and Abaza (2016), a missense mutation is a mutation that occurs due to the replacement of one amino acid with another amino acid. Genetic mutations occur randomly due to the adaptive needs of the organism related to the population from which the organism originates (Merlin, 2010). The presence of high genetic variation can increase the survival rate of ectoparasites so that the infection rate will be higher. This can cause the crab population to decrease due to the high presence of these ectoparasites.

The degree of similarity between species, populations, or individuals is measured by genetic distance. This was done in order to calculate the evolutionary rate between homologous gene copies based on the type and frequency of mutations that had happened since the homologous genes' ancestors' time. The resulting genetic spacing can be used to estimate the time of divergence across populations and to build phylogenetic trees (Beaumont et al., 1998; Nei, 2013). According to Hebert et al., (2003), species that differ from one another or are distantly related have a genetic gap of greater than 3%.

Bootstrap techniques are frequently used in phylogenetic tree construction to assess the stability of the findings and to calculate confidence intervals and standard errors. The bootstrap value is indicated by the number at each clade point. The phylogenetic tree branches' stability was assessed using the bootstrap method. According to Nakano and Ozawa (2004), a branch is considered unstable if the bootstrap value is less than 70% and stable if it is greater than 95%. According to Dharmayanti (2011), bootstrap values can also be used to gauge how confident a phylogenetic tree is. The confidence level in the phylogenetic tree created increases with the bootstrap value (Hall, 2001). Every unit (sample) in the phylogenetic tree construction method is re-sampled and computed several times. Following resampling, each calculation yields a consistency value.

CONCLUSION

Validation of isolated ectoparasites infecting *P. pelagicus* was performed using the mtDNA marker COI gene to identify three ectoparasite species molecularly, namely *C. testudinaria, D. tridens,* and *Oc. angulata*. The results of the five sample analyses of ectoparasites based on the number of specific nucleotide sites in each sample show that the suspected species *C. testudinaria, D. tridens* and *Oc. angulata* had missense mutations. This is indicated because of the change in nucleotides followed by changes in the amino acids. The similarity of species in the OL, OL2, and OA samples indicated that there is a species complex phenomenon in the three samples. Complex species are those that have the same or very similar morphological characteristics that are difficult to distinguish from one another.

Acknowledgments

We are grateful to the Ministry Of Education, Culture, Research And Technology, Directorate General Of Higher Education, Research, And Technology for supporting this research. Based on Decree Number 0667/E5/AL.04/2024 dated 30 May 2024.

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