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Research Article
Molecular Identification of Lobster Species Based on Cytochrome Oxidase Subunit I Gene characters

[1]Yusnaini, [2]Muzuni and [1]Indriyani Nur

[1]Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Halu Oleo University, Campus Bumi Tridharma Anduonohu, Kendari, Southeast Sulawesi, Indonesia
[2]Department of Biotechnology, Faculty of Mathematics and Natural Science, Halu Oleo University, Campus Bumi Tridharma Anduonohu, Kendari, Southeast Sulawesi, Indonesia

Abstract

Background and Objective: In the puerulus phase, which is not pigmented, identification of lobster species based on morphological characteristics is still difficult identity, so it is necessary to identify based on molecular characters. This study aimed to analyze the mitochondrial subunit I cytochrome oxidase (COI) gene characters of the puerulus of lobster species. **Materials and Methods:** The data can be useful for developing lobster seed identification methods based on DNA characteristics. Location of lobster sampling in Staring Bay, coastal waters of Moramo District, South Konawe Regency, Indonesia. The molecular characterization method is carried out in several stages, namely specific primer design, DNA preparation, PCR with specific primers, DNA sequencing and DNA sequence analysis. Characteristics of COI gene fragments were analyzed using BLAST analysis, restriction enzyme analysis and phylogenetic tree analysis. **Results:** The results showed that DNA was successfully isolated with a high level of purity. The results of the amplification of the COI gene fragment showed thick and firm bands and formed a single band measuring 751 bp with 249 amino acids. Based on the BLAST analysis shows that the COI gene fragment has 99% similarity with *Panulirus homarus*. Based restriction enzyme analysis shows that the site of recognition and restriction enzyme cutting position in the *Panulirus* COI gene fragment is the same as *Panulirus homarus* in Genebank, the *Ase*I enzyme in position 392 and *Psi*I in positions 47 and 106. **Conclusion:** Based on phylogenetic tree analysis, the COI gene fragment is in one group with *Panulirus homarus* and has a bootstrap value of 100% which shows that the nucleotide sequence is stable. The three analyzes show that the DNA source organism is the same species as *Panulirus homarus*.

Key words: Molecular identification, *Panulirus homarus*, PCR, phylogeny, nucleotide

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Corresponding Author: Muzuni, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Halu Oleo University, Campus Bumi Tridharma Anduonohu, Kendari, Southeast Sulawesi, Indonesia

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Marine lobster is a fishery commodity with important economic value, besides being consumed domestically, it is also has economic value for export. Lobsters consist of two large groups, namely clawed lobster (nipples) such as Nephropidae and spiny lobster (not pinned) such as Palinuridae, Scyllaridae and Synaxidae. The diversity of lobster is high, consisting of 6-10 species that have economic value living in Indonesian waters[1-3]. Lobster is a renewable resource, thus developing cultivation through restocking and sea ranching have been done[4].

The diversity of genetic levels is very important for crustaceans including lobsters. Information on genetic diversity at the individual and population-level needs to be known as a basis for information in resource management and conservation and other uses. Assessment of lobster genetic diversity can be analyzed using molecular DNA and based on morphology or anatomy. Several types of identification with this method have been carried out, such as lobster phylogeny clawed (Homarida)[5] and identification of lobster larvae with molecular approaches[6,7].

Lobster identification based on phenotypic or morphology data such as colour and carapace shape can generally only be done on juvenile or adult lobsters. In some cases, there are some difficulties in distinguishing lobster species because they have a high degree of morphological similarity, especially in simpan tric species. Sympatric speciation occurs when two groups of the same species live in the same geographical location but they develop differently until they cannot mate and are considered different species[8-10].

Staring Bay precisely in the coastal waters of Beroro Village, Moramo Subdistrict, South Konawe Regency is a water in Southeast Sulawesi Province, Indonesia as a producer of seeds (puerulus/baby lobster) from which broodstocks spawn naturally. This location has high lobster seed productivity, especially Family Palinuridae lobster, spiny lobster, including *Panulirus homarus*. The presence of lobsters appears seasonally in Staring Bay the numbers are very volatile and the species that are caught are often mixed.

In the puerulus phase, the identification of morphology is still difficult because it does not yet have a pigment as a differentiator. Ornate lobster and sand lobster at the puerulus phase have a brown and black carapace, they are still difficult to distinguish between species. This can be overcome by conducting basic research on lobster identification based on molecular characteristics that are specific to each species. DNA barcoding technique is one of the most popular molecular identification techniques that can be used. The technique is designed to identify species quickly and accurately as a species marker[11]. The barcoding technique of DNA can be used to identify an organism from species to subspecies that are accurately performed against different species that are hard to distinguish morphology[12]. The Cytochrome Oxidase Subunit I (COI) gene is part of mitochondrial DNA that is frequently used for both barcoding of species and subspecies. According to Makowsky *et al.*[13], relationships using mitochondrial DNA can be analyzed phylogenetically. For characterization of puerulus lobster relationships, the mitochondrial Cytochrome Oxidase Subunit I (COI) gene has been selected. The advantages of the COI gene include that the gene is conserved and does not have significant differences between other metazoa, so, it can be used universally to group organisms into one species or between species and can construct phylogenetic relationships at the broad taxon level[14].

Information on identifying lobster species is still limited to juvenile or adult lobsters, so, the results of this study can be used to compile methods to identify puerulus lobsters or to identify lobster seeds earlier. With the knowledge of molecular markers, lobsters captured at that location can be identified as originating from similar breeds or lines that are captured annually and whether the species are similar to lobsters in different topical waters or other waters. From these data, the method of early identification of lobster types based on morphological and molecular characteristics can be further developed. Thus, the objective of this study was to characterize the mitochondrial Subunit I Cytochrome Oxidase (COI) gene of the puerulus of Lobster species, to provide data for molecular identification. This study will provide information about certain species of lobsters found in the Staring Bay, South East Sulawesi, Indonesia based on genetic markers Cytochrome Oxidase Subunit I (COI).

MATERIALS AND METHODS

Site of research: The location of the lobster sampling was carried out in Staring Bay, South Konawe Regency. Then, the morphological and molecular analysis of samples was carried out at the Fisheries Laboratory of Halu Oleo University and Molecular Biology Laboratory, Faculty of Mathematics and Natural Sciences Halu Oleo University, Indonesia from March to September, 2020.

Collection of specimens: The lobster specimen collection was carried out by carrying out lobster cached in the Staring Bay Waters of South Konawe Regency, Southeast Sulawesi Province. The collection of specimens in this study was carried

out by taking parts of lobsters to observe lobster seeds (puerulus) or cutting the right leg or lobster gills using a scalpel. Then the specimen was put into a 1.5 mL micro centrifugation tube and filled with 100% ethanol as much as 1 mL and labelled. The specimens were then taken to the Molecular Biology Laboratory for DNA extraction.

Primer design: The primer was designed by collecting sequences of lobster COI gene. Furthermore, the sequence is aligned with the software of Bioedit (Biological sequence alignment editor) and the highly conserved area of the sequence is determined. The highly conserved area around the end 5' is used as the forward primer and those around the end 3' are used as reverse primer. The two primers that have been designed are CoxIp-F: 5'-CAGARCTMGGWCAACCAGGAA GACT-3') as forwarding primer and CoxIp-R: 5'- GCTCATACTAT RAATCCTARWAGTCC-3' as a reverse primer.

DNA extraction, COI gene amplification and electrophoresis: DNA extraction using the CTAB method with slight modifications[15]. Before isolation, lysis buffer is prepared in advance according to the number of samples to be extracted. Samples were weighed as much as 0.1-0.2 g and then crushed with the help of quartz sand. Samples were inserted into a microtube 1.5 mL and added 600 mL of lysis buffer. Samples were incubated for 30 min at a temperature of 65°C and inverted every 5 min. The samples were then put in ice for 5 min and then centrifuged at 10,000 rpm for 10 min. The supernatant is taken and put in a new microtube and added to 1 x volume Phenol-Chloroform-Isoamyl Alcohol that serves to separate contaminants such as proteins and organic compounds with DNA. Furthermore, the suspension was centrifuged at 10,000 rpm, a temperature of 40°C for 10 min. The supernatant is taken and transferred in a 1.5 mL microtube then added with 0.1 volume of sodium acetate 3 M pH 5.2 and 2 volumes of absolute ethanol and then incubated for 2 hrs and centrifuged for 20 min at 10,000 rpm 4°C so that DNA pellet was obtained. Furthermore, the DNA pellet was washed with 0.5 mL of 70% ethanol, then dried and finally dissolved in 20 mL H_2O. To remove RNA, the solution was added to 100 ug mL^{-1} RNase and incubated at 37°C for 12 hrs. DNA solution is then stored at a temperature of -20°C.

PCR reactions use DreamTaq Green PCR Master Mix (2X). The composition of the PCR reaction is 100 ng DNA templates, 0.5 µM CoxIp-F, 0.5 µM CoxIp-R, 1x Master Mix and dH_2O to reach 10 µL. PCR reactions were performed in 30 cycles with the following conditions: initial denaturation, for 5 min. at a temperature of 94°C, denaturation, for 1.5 min at a temperature of 94°C, annealing for 1 min at 55°C, extension, for 1.5 min at a temperature of 72°C and final extension, for 5 min at a temperature of 72°C. The PCR reaction requires a time of ±2.30 hrs. The result of amplification was then performed electrophoresis on a 1% agarose with a voltage of 100 volts and 80 mA for 30 min and then visualized on top of a UV Transilluminator.

Sequencing and analysis of DNA: DNA sequencing of amplification product using automated DNA sequencer tool (automated DNA sequencer ABI Prism 310, Perkin-Elmer). DNA sequencing was done by the Sanger method, using a dye terminator in the form of fluorescent dye rhodamine (PRISM dyedoaxy reaction terminator cycle sequencing kit). After getting the results of sequencing, DNA sequences were then aligned using the NCBI Blast program.

Characterization of nucleotide sequences of lobster COI gene carried by some analysis: Local alignment analysis of DNA sequencing results with existing data in GeneBank done with BLAST (basic local alignment search tools) provided by NCBI (National Center for Biotechnology Information) through http://www.ncbi.nlm.nih.gov/blast, restriction enzyme analysis using Bioedit (Biological sequence alignment editor) for Windows 95/98/NT/2000/XP and phylogenic tree analysis based on nucleotide sequences using Mafft Katoh version 7 (https://mafft.cbrc.jp/alignment/server/). The amino acid was analyzed using the Expasy program (https://web.expasy.org/translate/). Based on these characteristics, *Panulirus* samples can be determined its species.

RESULTS AND DISCUSSION

Genomic DNA isolation and amplification of the gene fragment of COI: The quality and quantity of genomic DNA are known by testing using electrophoresis and spectrophotometry methods. DNA isolation results showed very good quality because they did not show a smear pattern at the bottom of the target DNA band in Fig. 1a with a concentration of 1,500 ng μL^{-1}. According to Muzuni *et al.*[15] stated that good and not degraded DNA quality would show clean bands on electrophoresis results and did not show a smear banding pattern.

The measurement of genomic DNA purity can be calculated using a spectrophotometer with a wavelength of λ 260 and 280 nm. DNA is said to be pure if it has an absorbance ratio value of λ260 nm and λ280 nm between 1.6 and 2.0. Ratio values greater than 2.0 indicate RNA contaminations and this must be considered to avoid DNA over quantification[16]. While a ratio value of less than 1.6 indicates the presence of

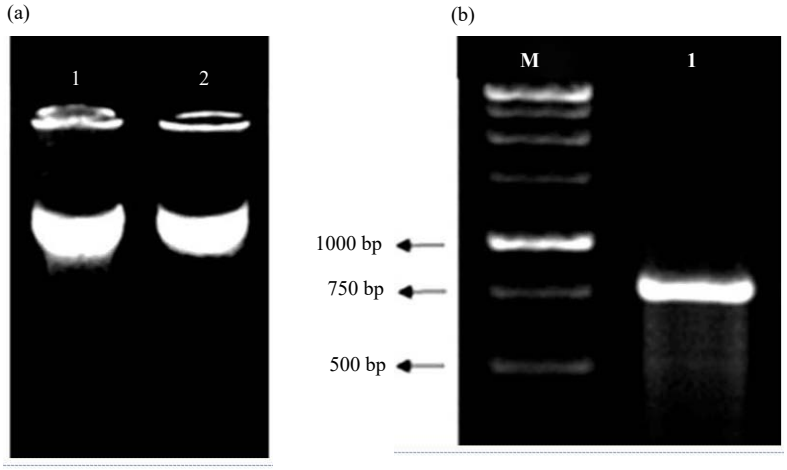

Fig. 1(a-b): Electrophoresis, (a) *Panulirus genomic* DNA (line 1 and 2: Two replications) and (b) PCR products of DNA *Panulirus* sample using CoxIp-F and CoxIp-R primers in 1% agarose gel (line 1)
M: 1 kb ladder marker

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  1 CAGAGCTAGGCCAACCAGGAAGACTGATTGGAGACGACCAAATTTATAAT 50
 51 GTAGTAGTAACAGCCCACGCTTTTGTGATAATTTTCTTTATAGTTATGCC 100
101 CATTATAATTGGAGGATTCGGAAACTGGCTCGTTCCTATTATGTTAGGTG 150
151 CCCCAGATATGGCATTTCCCCGAATGAATAACATAAGATTCTGACTTTTA 200
201 CCTCCCTCTCTAACGCTTCTTCTAGCTAGTGGTATAGTGGAGAGGGGAGT 250
251 AGGAACTGGCTGAACAGTTTATCCCCCCCTAGCAGGAGCAGTGGCCCATG 300
301 CCGGAGCATCAGTAGATTTGGGTATTTTCTCCCTCCATCTTGCCGGTGTG 350
351 TCATCAATTCTAGGAGCCGTAAATTTTATTACAACAGTAATTAATATGCG 400
401 ATCTTCAGGTATAACATTCGACCGAATGCCTCTATTTGTATGATCCGTGT 450
451 TTATTACTGCCATTTTACTTCTACTTTCTCTTCCCGTACTAGCTGGAGCT 500
501 ATTACTATACTTCTTACTGATCGTAATTTGAACACATCATTCTTTGACCC 550
551 AGTAGGAGGGGGAGATCCAATTCTCTATCAACATCTATTTTGATTCTTCG 600
601 GACACCCAGAAGTTTATATTCTCATCTTACCAGCATTCGGTATGATTTCT 650
651 CACATTGTATCCCAGGAATCTAACAAAAAAAAACCTTTCGGAGCTTTAGG 700
701 AATAATTTATGCCATGTTATCGATTGGACTTCTAGGATTCATAGTATGAG 750
751 C
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Fig. 2: Nucleotide sequence of COI gene fragment fragments

protein, phenol, or other contaminants that absorb strongly at 280 nm[16,17]. The results of measurements of isolated DNA showed pure results because they had a ratio of absorbance of 1.88, therefore, the DNA had good quality and was suitable as a template for COI gene amplification using the PCR technique.

The isolated DNA was amplified using the CoxIp-F primer and CoxIp-R. The results of the amplification of the COI gene fragment from mitochondrial genome DNA *Panulirus* samples showed thick and firm bands and formed a single band measuring around 750 bp in Fig. 1b. Good PCR products from the DNA sample were purified so that they were of good quality and were suitable as a print in the sequencing process to obtain COI gene fragments sequenced from the lobster genus Panulirus. According to Settanni *et al.*[18] and Muzuni *et al.*[15], DNA amplification using the PCR technique is successful if the PCR product shows a single band with a size that matches a previously known marker. The success of the DNA amplification process using the PCR technique is influenced by several factors, namely DNA quality, primary selection, PCR condition, PCR cycle and PCR reagent composition[18,19]. Based on the DNA sequencing results showed that the DNA successfully amplified by CoxIp-F and CoxIp-R primers sized 751 bp in the order shown in Fig. 2 while the amino acids were 249 with amino acid sequences as shown in Fig. 3. The amplification of COI gene fragments can

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  1 ELGQPGGLIGDDQIYNVVVTAHAFVMIFFMVMPIMIGGFGNWLVPIMLGA 50
 51 PDMAFPRMNNMGFWLLPPSLTLLLASGMVEGGVGTGWTVYPPLAGAVAHA 100
101 GASVDLGIFSLHLAGVSSILGAVNFITTVINMRSSGMTFDRMPLFVWSVF 150
151 ITAILLLLSLPVLAGAITMLLTDRNLNTSFFDPVGGGDPILYQHLFWFFG 200
201 HPEVYILILPAFGMISHIVSQESNKKKPFGALGMIYAMLSIGLLGFMVW  249
```

Fig. 3: Sequence of amino acids fragments of COI gene fragments

Table 1: BLAST analysis results from the nucleotide sequence of PCR products with DNA sequences in genebank

Description	Max score	Total score	Query coverage (%)	E-value	Max identity (%)	Accession
Panulirus homarus mitochondrion, complete genom	1275	1275	98	0.0	96	JN542716.1
Panulirus homarus voucher NBFGR-CHN-PHH-KL6 COI gene	1140	1140	81	0.0	99	JQ229919.1
Panulirus homarus voucher NBFGR-CHN-PHH-CH5 COI gene	1135	1135	81	0.0	99	JQ229912.1
Panulirus homarus voucher NBFGR-CHN-PHH-CH1 COI gene	1129	1129	81	0.0	99	JQ229883.1
Panulirus homarus voucher NBFGR-CHN-PHH-CH3 COI gene	1123	1123	81	0.0	99	JQ229910.1
Panulirus homarus voucher NBFGR-CHN-PHH-Vzg2 COI gene	1123	1123	81	0.0	99	JQ229888.1
Panulirus homarus voucher NBFGR-CHN-PHH-Vzg7 COI gene	1117	1117	81	0.0	98	JQ229926.1
Vaccine Panulirus homarus COH22 COI gene	1112	1112	78	0.0	99	KF715546.1
Panulirus homarus voucher NBFGR-CHN-PHH-KL8 COI gene	1112	1112	81	0.0	98	JQ229921.1
Panulirus homarus voucher NBFGR-CHN-PHH-KL4 COI gene	1112	1112	81	0.0	98	JQ229917.1

produce gene fragments of different sizes depending on the primer used. Ptacek *et al.*[20] amplified the COI gene using L-CO1490 and H-CO2198 primers and produced a 650 bp COI gene fragment. While Chow *et al.*[6] amplified 1300 bp COI gene using COI65F1 and COI1342R1 primers.

BLAST analysis of genes fragments Cytochrome Oxidase Subunit I: The BLAST analysis (Basic Local Alignment Sequence Tools) was done by comparing the nucleotide sequence data of PCR results with the nucleotide sequences found in GeneBank. The result of the sequence identity analysis was shown in Table 1.

The BLAST analysis results showed a 99% similarity between the COI gene fragment and *P. homarus*, which shows that the DNA source organisms are the same species as *P. homarus*. This identity is reinforced by the Query cover value of 99% with an error value of 0. Pearson[21] stated that a nucleotide sequence was said to be the same species when the Query coverage and max identity values approached 100% with error values approaching 0. The identity similarity was seen from the similarity value of the nucleotide base sequence that was owned. Similarity values can be determined from the parameters of the bit score and identities[22,23]. The higher the value of identities, the more similar it is to the reference sequence in Genebank.

Restriction enzymes: The sequence of mitochondrial DNA is regarded as the strongest strategy for solving taxonomic uncertainties. The significant result of this inquiry is the fact that peurulus larvae's profile was comparable to that of adult *P. homarus*. The findings of this study show that it is possible to use the restriction enzyme to define and differentiate *P. homarus* pueruli from those of other lobsters. Restriction enzymes are enzymes that can cut DNA segments that are recognized in the nucleotide sequence. According to Muzuni *et al.*[24] restriction enzymes can cut DNA segments specifically so that the cuts are directed. The use of restriction enzymes as a means of identifying an organism because each restriction enzyme recognizes and cuts in a typical sequence. Before carrying out restriction analysis, the nucleotide sequence of the *Panulirus* sample is aligned with the comparative *Panulirus* nucleotide sequence obtained from Genebank, namely: *Panulirus homarus* (GeneBank Acc. KF548576), *Panulirus versicolor* (GeneBank Acc. AF339472), *Panulirus penicillatus* (GeneBank Acc. AF339468) and *Panulirus ornatus* (GeneBank Acc. AF339467). The alignment results must show the same DNA fragment length so that several nucleotides from the Panulirus sample and Genebank must be cut to the same length, which is 587 bp.

Based on restriction enzyme analysis shown that the recognition site and the cutting position of the restriction enzyme of the *Panulirus* COI gene fragment are the same as the *Panulirus homarus* in Genebank, namely *Ase*I enzymes in position 392 and *Psi*I in positions 47 and 106. This shows that the species of *Panulirus* sample is *Panulirus homarus*. Restriction enzymes that can be used as special markers of *Panulirus homarus* and differentiate with other species are *Ase*I enzymes which have an AT'TA_AT recognition site while restriction enzymes are used as special markers for all Panulirus species namely *Psi*I enzymes with TTA'TAA recognition sites in Table 2.

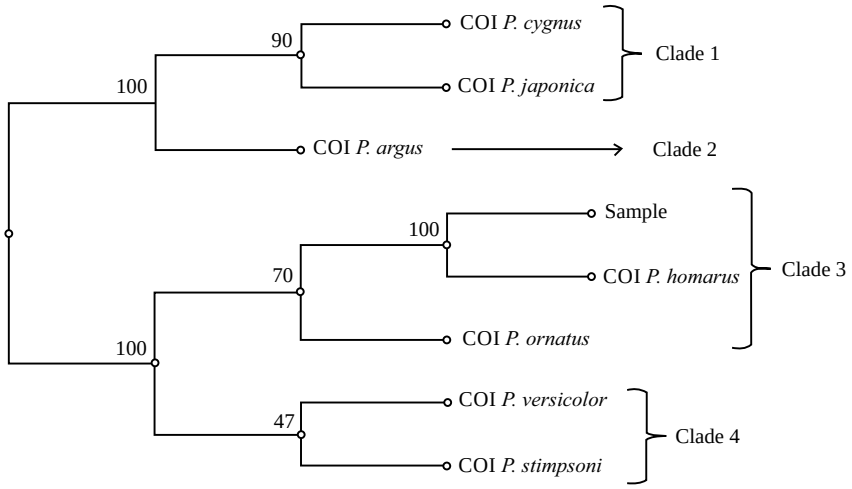

Fig. 4: Phylogenetic tree based on the nucleotide sequence of the COI gene fragment in the *Panulirus* sample compared to the COI *Panulirus* gene fragment available at genebank
(*Panulirus cygnus*, NC_028024, *Panulirus japonica*, NC_004251, *Panulirus argus*, NC_039671, *Panulirus homarus*, JN542716, *Panulirus ornatus*, HM446347, *Panulirus versicolor*, NC_028627 and *Panulirus stimpsoni*, GQ292768)

Table 2: Restriction enzyme sites for the COI gene segment of the sample compared with other *Panulirus* gene segments

		Nucleotide position				
Restriction enzymes	Recognition sites	I	II	III	IV	V
**Ase*I	AT'TA_AT	392	392	-	-	-
*Eco*ICRI	GAG'CTC	-	6	-	151	-
***Pst*I	TTA'TAA	47, 106	47, 106	106	79, 106	106
*Sac*I	G_AGCT'C	-	8	-	153	-
*Bc*II	T'GATC_A	-	-	36, 442	-	36
*Bgl*II	A'GATC_T	-	-	315	-	-
*Eag*I	C'GGCC_G	-	-	284	-	-
*Pvu*I	CG_AT'CG	-	-	423	-	522
*Bse*YI	C'CCAG_C	-	-	-	-	283
*Kpn*I	G_GTAC'C	-	-	-	257	-
*Alu*I	AG'CT	6, 226, 493, 499	6, 226, 493, 499	499	151, 499	6, 295
*Taq*I	T'CG_A	419	419	419, 423	-	545

**Restriction enzymes that can be used as molecular markers for all *Panulirus* species, *Restriction enzyme special markers *Panulirus homarus*, I: *Panulirus samples*, II: *Panulirus homarus* (GeneBank Acc. KF548576). III: *Panulirus versicolor* (GeneBank Acc. AF339472), IV: *Panulirus penicillatus* (GeneBank Acc. AF339468). (V) *Panulirus ornatus* (GeneBank Acc. AF339467)

Restriction enzyme analysis can also be used as a reference in the identification of *Panulirus* using RFLP (Restriction Fragment Length Polymorphism) which is used as a species identification tool[6,25,26]. *Alu*I and *Taq*I enzymes can be used to distinguish *Panulirus longipes* from *P. longipes femoristriga*[6] while *Alu*I, *Taq*I, *Bsp*LI, *Rsa*I and *Hha*I enzymes are used to identify *Panulirus* homarus using the RFLP technique[25]. Based on the results of restriction enzyme analysis in this study, enzymes that could potentially be used to identify *Panulirus homarus* using the RFLP technique were *Ase*I, *Pst*I, *Alu*I and *Taq*I.

Construction of phylogenetic trees based on nucleotide sequences: Phylogenic tree analysis can analyze changes that occur in the evolution of different organisms. Analysis of segments that have proximity can be identified by occupying adjacent branches in the tree[15,27]. Analysis of phylogenetic trees was conducted to determine kinship between *Panulirus* samples and other *Panulirus*. This analysis used the Mafft Katoh program (online). The result of the phylogenetic tree analysis shows that the tree has 4 clades, namely clad I (*P. cygnus* and *P. japonica*), clade 2 (*P. argus*), clade 3 (samples *Panulirus*, *P. homarus* and *P. ornatus*) and clade 4 (*P. versicolor* and *P. stimpsoni*) in Fig. 4. This grouping is the same as the results of the research of Ptacek *et al.*[20] who showed *P. Cygnus* in association with *P. japonica*, *P. homarus* with *P. ornatus* and *P. versicolor* with *P. stimpsoni*.

Based on the phylogenetic construction of the COI gene fragment showed that the *Panulirus* sample was in one group with *P. homarus* and had a boot value of 100% indicating that

the sequence was truly stable. According to Hoang *et al.*[28], the bootstrap value of ≥95% in a branch can show the level of trust of a branch. Therefore, it could be ascertained that *Panulirus* samples included the species of *P. homarus*.

CONCLUSION

Based on the results of the study it can be concluded that the characteristics possessed by the *Panulirus* sample are as follows: Produce a PCR product measuring 751 bp with 249 amino acids, showing 99% similarity with *Panulirus homarus*, having a restriction enzyme cutting site similar to *Panulirus homarus* and located in one group with *Panulirus homarus* with a bootstrap value of 100%. Based on these characteristics indicate that the DNA source organism is the same species as *Panulirus homarus*.

SIGNIFICANCE STATEMENT

This study discovered the *Panulirus* scattered in Staring Bay Waters, South Konawe Regency and Southeast Sulawesi Province. The lobster sample used in this study was the lobster seed (puerulus) whose species could not be determined. Therefore, this study will help researchers to determine the type of lobster that is scattered in the sampling area. It is suspected that other samples scattered in other areas can also be determined using this method because the primers used are designed using DNA sequences that are conserved in several types of lobster, namely *Panulirus homarus*, *Panulirus ornatus* and *Panulirus versicolor*.

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