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Molecular Insights into the Identification and Phylogenetic of the Collector Urchin, *Tripneustes gratilla* (Linnaeus 1758) from the Red Island Beach, East Java, Indonesia

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ABSTRACT

The collector urchin, *Tripneustes gratilla* is an ecologically crucial sea urchin community with typical habitats consisting of shallow seagrass beds or reefs. Samples were obtained from the Red Island Beach, Banyuwangi Regency, East Java, Indonesia. Nucleotide sequencing analysis was conducted on mitochondrial *cytochrome oxidase subunit I* (*coi*) DNA fragments amplified by PCR. Nucleotide sequences ranging from 605 to 606 base pairs were collected from 16 specimens at the sample site. Through DNA barcoding analysis, it was determined that all samples are of the *Tripneustes gratilla* species. Phylogenetic studies were conducted by comparing the gene sequences of the collecting urchin haplotypes identified in this study with gene sequences of identical base pair length from GenBank and *T. ventricosus*, which served as the reference group. The phylogenetic trees exhibited good consistency and clearly delineated distinct clades for each species. Phylogenetic status of haplotype and the existence of sea urchins were documented. The clarification of the evolutionary position of ecologically important species offers fundamental information for potential future conservation efforts of such species.

INTRODUCTION

Echinoderms are a group of marine invertebrates that exhibit bilateral symmetry in their larvae. However, they have the characteristic pentamerous radial symmetry during adulthood, which a secondary bilateral symmetry may further enhance **(Sonet *et al.*, 2022)**. Echinoidea (sea urchins), Holothuroidea (sea cucumbers), Ophiuroidea (brittle and basket stars), Asteroidea (starfish or sea stars), and Crinoidea (sea lilies and feather stars), are the five extant groups within the phylum Echinodermata. Extant and extinct species represent around 7000 and 13000, respectively **(Pawson, 2007)**. However, it is essential to note that it is likely an underestimate, as taxa are still being described on a yearly basis. This study focused on examining the Echinoidea class, specifically the species

Tripneustes gratilla, often known as the collector sea urchin **(Wainwright *et al.*, 2013; 2019)**. Three species of the sea urchin genus *Tripneustes* have been reported **(Kroh, 2015)**. These species have different features in shape, environment, and body functions **(Lawrence & Agatsuma, 2007; Pena *et al.*, 2010)**. Three species, namely *T. gratilla* (Linnaeus, 1758), *T. depressus* (Agassiz, 1863), and *T. ventricosus* (Lamarck, 1816), are classified within the genus *Tripneustes*. *T. gratilla* exhibits a global distribution across the ocean, encompassing regions such as Indonesia **(Toha *et al.*, 2013; Wainwright *et al.*, 2013)**. Its typical habitats consist of shallow seagrass beds or reefs, which possess significant primary productive potential, but are susceptible to natural and anthropogenic disturbances **(Nomleni *et al.*, 2020)**. *T. gratilla* possesses commercial and ecological significance, justifying its utilization as bio-indicators for assessing intertidal environmental conditions **(Stimson *et al.*, 2007)**. It is commercially collected for its gonads, sometimes known as "roe" because of its significance in sustaining small-scale fishing and facilitating commercial trade.

Previous research by **Toha *et al.* (2015)** stated that *T. gratilla* has morphological variations, including color variations on its spines and body, associated with environmental adaptability. Based on molecular analysis, it is exciting to investigate whether these color differences make them different species or if they remain the same. Molecular analysis of *T. Gratilla* is commonly conducted using DNA barcoding techniques combined with phylogenetic analysis. DNA barcoding and phylogenetic analysis are two genetic tools that are very useful in identifying a species. Using examination of sequence variation in a conserved domain region of DNA, these approaches effectively identify recognized species and uncover new species **(Kaur & Pandit, 2022; Hardianto & Satriyo, 2023)**. Prior studies have been carried out on the integration of DNA barcode methodology with phylogenetic analysis in several aquatic species, such as coral reef **(Wijayanti *et al.*, 2017; Wijayanti *et al.*, 2018)**, crustaceans **(Irwani *et al.*, 2020; Vella & Vella, 2022)**, fish **(Syaifudin *et al.*, 2021; Nursalim *et al.*, 2022; Khansa *et al.*, 2023)** and shellfish **(Castaneda *et al.*, 2023; Hardianto & Satriyo, 2023)**. The integration of DNA barcoding with phylogenetic analysis offers significant advantages for species authentication by revealing early phases that cannot be detected through morphological descriptions and inter-species relationships. Empirical evidence has demonstrated its efficacy in the identification of juvenile, larval, and adult species.

The purpose of this research was to amplify nucleotide sequences of the mitochondrial DNA (mtDNA*) cytochrome oxidase subunit I* (*coi*) in order to investigate the genetic distances, species authentication, protein base composition, and phylogenetic relationships of the collector urchin from Red Island Beach, Banyuwangi Regency, East Java, Indonesia. Therefore, the identification of the species through the *coi* gene is an endeavor to generate genetic data that can serve as the foundation for the development of more effective future conservation strategies for the target species.

MATERIALS AND METHODS

Samples of the species were obtained in 2022 from the Beach region, which encompasses a significant section of the species' natural habitat in the Red Island Beach, Banyuwangi Regency, East Java, Indonesia (Fig. 1). A total of 16 specimens of *T. gratilla* were gathered, and selected based on their morphology and color variation characteristics (Fig. 2). On-site, sea urchin samples were preserved in a solution of 70% ethanol and thereafter transmitted to the laboratory for genetic examination. Muscle tissue weighing 20mg was removed from the samples by dissecting the feed and skin with scissors and forceps. According to the manual protocol provided by the manufacturer, DNA was isolated using the Favorgen DNA Extraction Kit (Biontech Corp.).

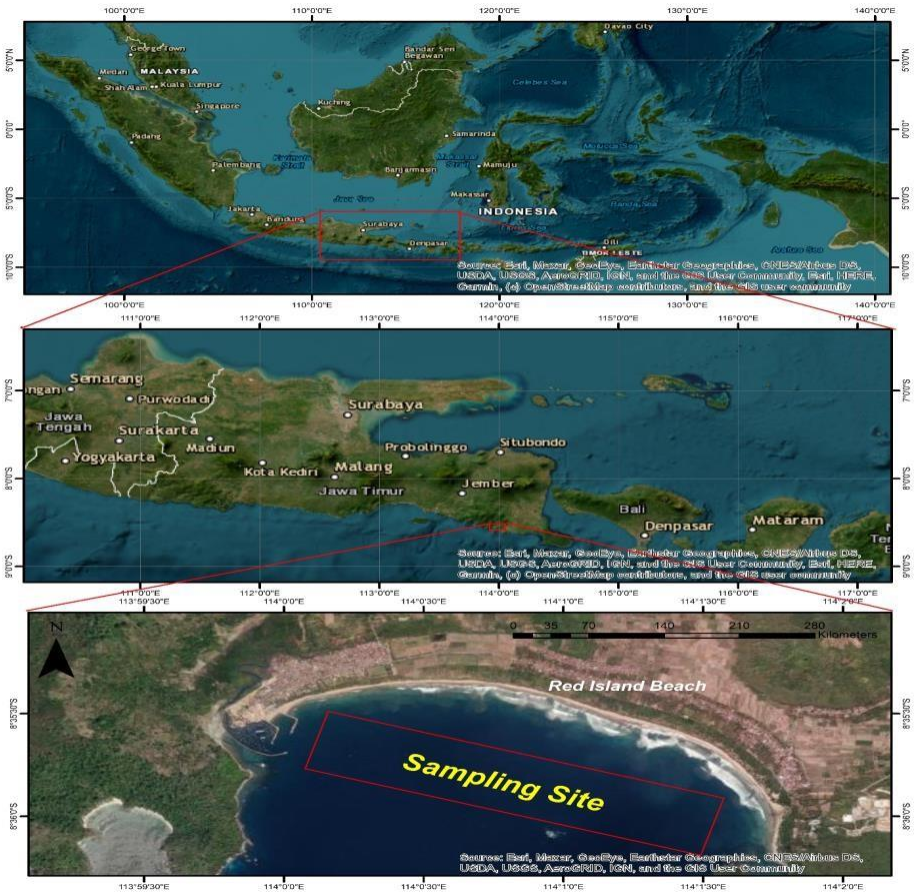

Fig. 1. Map of the sampling sites. The red square indicated of the location site, Red Island Beach, Banyuwangi Regency, East Java Province, Indonesia

Amplification of the partial mitochondrial *coi* gene was performed using polymerase chain reaction (PCR) with universal primers LCO 1490 (5'-GGTCAACAAATCATAAAGATATTGG -3') and HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAATCA -3') as described by **Folmer *et al.* (1994)**. Each

reaction was established in a total volume of 25µL, and the subsequent reagents were introduced into each PCR microtube: 1.0 L of template DNA was combined with 25 picomoles of each primer, and 12.5µL of Bioline master mix Taq DNA polymerase. Each sample was diluted to a volume of 25µL using distilled water. A GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) was used to perform PCR with the following conditions: a hot start at 94°C for 180 seconds, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 48-52°C for 30 seconds, extension at 72°C for 90 seconds, and final extension at 72°C for 420 seconds. Validation of PCR was conducted by electrophoresis on a 1.0% agarose gel. Following electrophoresis, the gels were stained with ethidium bromide and the resulting products were examined using a UV transilluminator from Advanced Scientific Products Pty Ltd. in Queensland, Australia. Polymerase chain reaction (PCR) products were isolated using a PCR product pre-sequencing kit from USB Co., USA. Sequencing of amplified DNA was performed using an ABI 3730xl DNA analyzer with the BigDye Terminator v3.1 cycle sequencing kit from Applied Biosystems, USA.

Alignment of sequencing data was performed using muscle alignment software in MEGA X **(Kumar *et al.*, 2018)** using default alignment parameters. To avoid discrepancies, the sequences were manually rectified. The Basic Local Alignment Search Tool (BLAST), available at NCBI (National Centre for Biotechnology Information) was used to examine the sequences in order to determine their identity. Furthermore, sequencing data alignment was employed to calculate Kimura's two-parameter (K2P) distance by doing 1,000 bootstrap replications. The objective of this work was to investigate the genetic variations and nucleotide composition between pairs and to create a phylogenetic tree using the neighbour joining (NJ) technique in MEGA. An investigation of the haplotype network between the species was conducted using the minimum spanning network (MSN) analytic approach with PopART **(Leight & Bryant, 2015)**.

RESULTS

1. Morphological observation and sequencing were obtained

Based on observing the morphology of *T. gratilla* individuals, four different types of individuals were found based on spine shape, body color, and spine color (Fig. 2). Then, four samples from each type were analyzed based on the DNA sequencing results. A total of 605-606 base pair mtDNA *coi* sequence were obtained in the sequencing results, and six haplotypes were found. The sequence and haplotype were obtained after the alignment of sixteen specimens of sea urchins from the Red Island Beach, Banyuwangi Regency (Fig. 1). Table (1) contains the nucleotide sequences of all haplotypes that were stored in the GenBank with the accession numbers PV124814–PV124829.

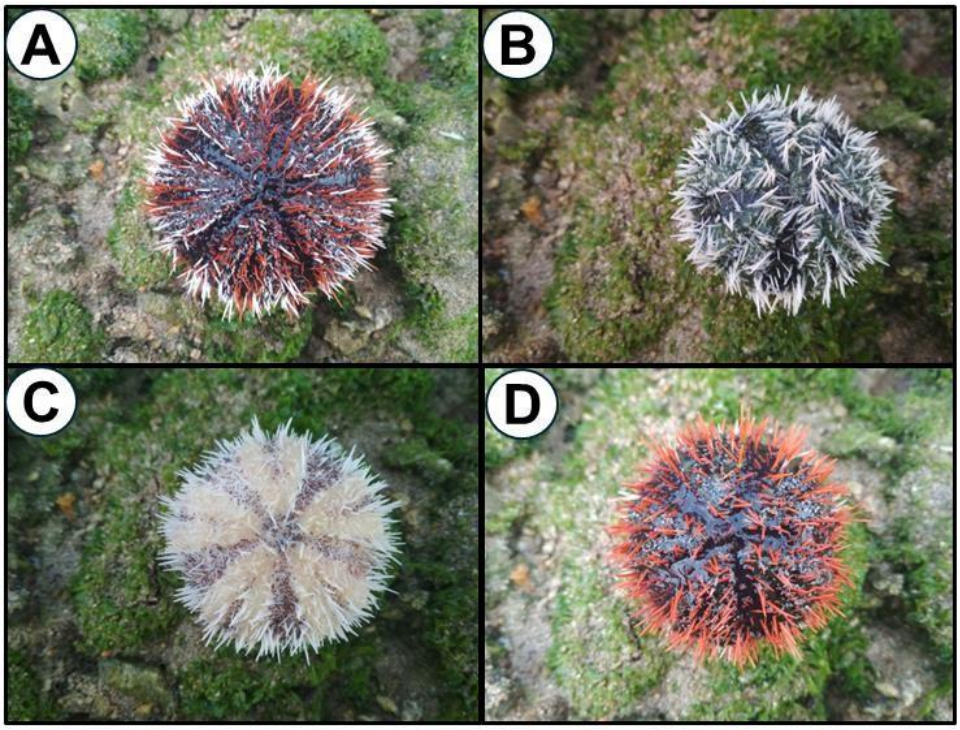

Fig. 2. Morphology and color variation of *Tripneustes gratilla* collected from Red Island Beach, Banyuwangi Regency, East Java Province, Indonesia

Table 1. Details of sea urchins sampling sites, number of samples collected (n), number of haplotypes identified, and accession number for *coi* gene sequence

Location site	Collecting date	n	Sample code	Haplotype Number	GenBank accession number	Species
Red Island Beach, Banyuwangi City, East of Java, Indonesia	October 20, 2022	16	A-1	Hap-1	PV124814	All samples identified as *Tripneustes gratilla*
			A-2	Hap-1	PV124815	
			A-3	Hap-1	PV124816	
			A-4	Hap-2	PV124817	
			B-1	Hap-3	PV124818	
			B-2	Hap-3	PV124819	
			B-3	Hap-3	PV124820	
			B-4	Hap-3	PV124821	
			C-1	Hap-4	PV124822	
			C-2	Hap-4	PV124823	
			C-3	Hap-4	PV124824	
			C-4	Hap-4	PV124825	
			D-1	Hap-5	PV124826	
			D-2	Hap-6	PV124827	
			D-3	Hap-6	PV124828	
			D-4	Hap-6	PV124829	

2. DNA barcoding and genetic distance

The BLAST analysis of the sequencing data has identified a single species of sea urchins. The samples are representative of the species *Tripneustes gratilla*, with a match range of 98-99%, by comparing them with a haplotype from other DNA data banks in NCBI (Table 2). Pairwise analysis generated mean genetic distances ranging from 0.00 to 0.01 (Table 3).

Table 2. BLAST identity percentage of nucleotide of sea urchins based on *coi* gene

No.	Species	Origin	Accession number	Identity (%)
Haplotype name: Hap-1				
1.	*Tripneustes gratilla*	Balayan Bay, the Philippines	KU314864.1	99.51
2.	*Tripneustes gratilla*	Maricaban Island, the Philippines	KU314861.1	99.51
3.	*Tripneustes gratilla*	Zanzibar, Tanzania	KU314854.1	98.51
Haplotype name: Hap-2				
1.	*Tripneustes gratilla*	Balayan Bay, the Philippines	KU314864.1	99.84
2.	*Tripneustes gratilla*	Maricaban Island, the Philippines	KU314861.1	99.54
3.	*Tripneustes gratilla*	Queensland, Australia	MK084944.1	98.33
Haplotype name: Hap-3				
1.	*Tripneustes gratilla*	Balayan Bay, the Philippines	KU314864.1	99.84
2.	*Tripneustes gratilla*	Maricaban Island, the Philippines	KU314861.1	99.48
3.	*Tripneustes gratilla*	Red Sea, Egypt	KU314845.1	98.42
Haplotype name: Hap-4				
1.	*Tripneustes gratilla*	Balayan Bay, the Philippines	KU314864.1	99.74
2.	*Tripneustes gratilla*	Maricaban Island, the Philippines	KU314861.1	99.38
3.	*Tripneustes gratilla*	South Africa	OP898253.1	98.64
Haplotype name: Hap-5				
1.	*Tripneustes gratilla*	Balayan Bay, the Philippines	KU314864.1	99.84
2.	*Tripneustes gratilla*	Maricaban Island, the Philippines	KU314861.1	99.14
3.	*Tripneustes gratilla*	Aqaba, Jordan	KU314838.1	98.54
Haplotype name: Hap-6				
1.	*Tripneustes gratilla*	Balayan Bay, the Philippines	KU314864.1	100
2.	*Tripneustes gratilla*	Maricaban Island, the Philippines	KU314861.1	99.84
3.	*Tripneustes gratilla*	Queensland, Australia	MK084944.1	98.24

Table 3. Pairwise genetic distances of *Tripneustes gratilla* each location sites including out of groups (*T. ventricosus*) and DNA data bank data of mitochondrial DNA *coi* based on K2P defined in this study

No.	Species and sample code	Genetic distances							
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1.	Hap-1								
2.	Hap-2	0.01							
3.	Hap-3	0.02	0.01						
4.	Hap-4	0.01	0.01	0.00					
5.	Hap-5	0.01	0.01	0.01	0.01				
6.	Hap-6	0.01	0.01	0.01	0.01	0.01			
7.	*T. gratilla*-KU314864.1-Philippines	0.01	0.01	0.01	0.01	0.01	0.01		
8.	*T. gratilla*-KU314838.1-Jordan	0.01	0.01	0.01	0.01	0.01	0.01	0.00	
Out of group									
9.	*T. ventricosus* - AY205515.1-Panama	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70

3. Haplotype network and phylogenetic analyses

The haplotype network analysis results clearly showed different gaps between species investigated with the outgroup samples, but not many gaps among haplotypes (Fig. 3). Phylogenetic analysis, which compares the haplotypes of the specimens to data from the gene bank, clarifies that our specimens are from the same clade and belong to the same species, *Tripneustes gratilla* (Fig. 4). This result validates the coherence of the neighbor-joining tree, which exhibits identical topology and findings.

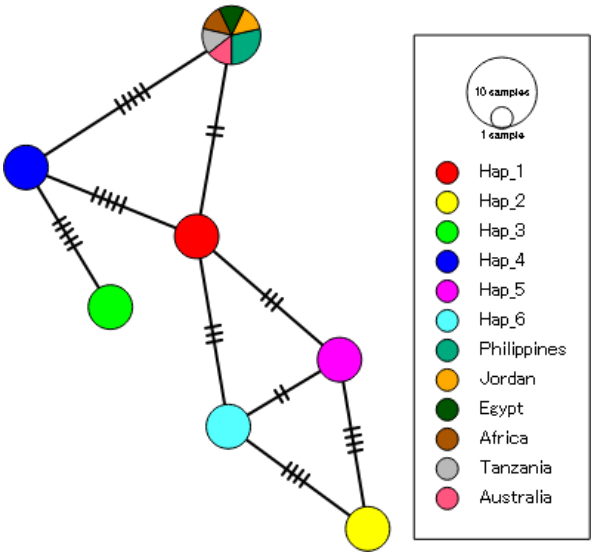

Fig. 4. Haplotype network (implemented in PopART) of *Tripneustes gratilla* based on *coi* gene sequences using minimum spanning network analysis

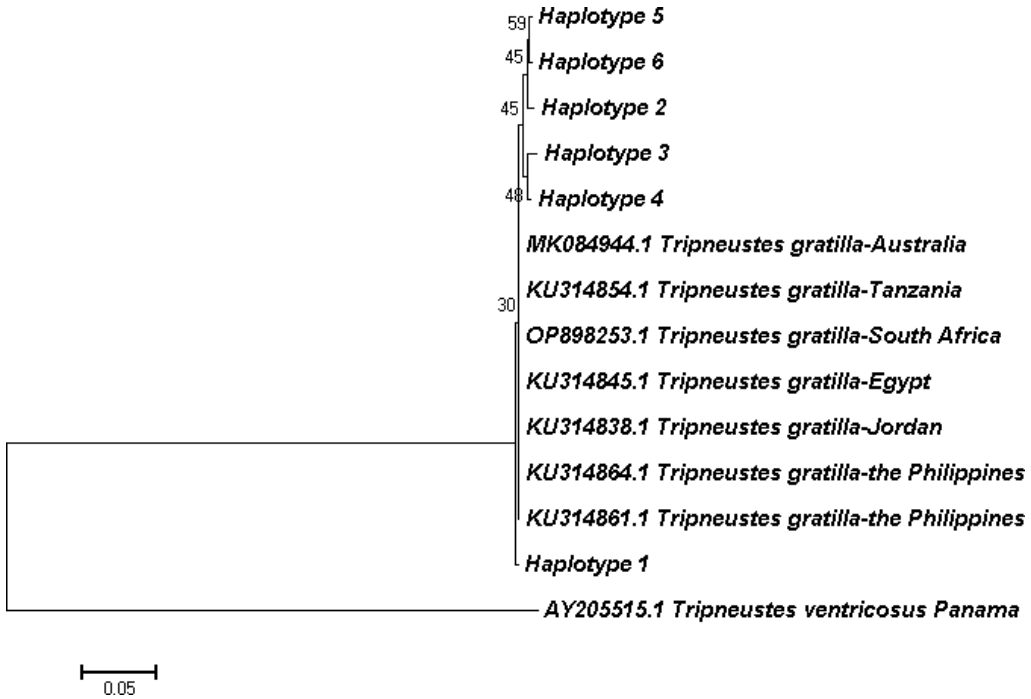

Fig. 3. Phylogenetic relationships of *Tripneustes gratilla* with *Tripneustes ventricosus* as out group. The phylogenetic tree inferred by neighbour joining analysis (NJ) of *coi* gene regions. Haplotype 1 to 6 indicates the sample of this research.

DISCUSSION

The result of BLAST analyses revealed in Table (2) generates that nucleotide sequences of 16 samples with six haplotypes observed in Red Island Beach, Banyuwangi,

Indonesia, were identical, showing a range of 98 to 99% matched to *T. gratilla* from the Philippines (KU314864.1 and KU314864.1), Tanzania (KU314854.1), Australia (MK084944.1), Egypt (KU314845.1), South Africa (OP898253.1) and Jordan (KU314838.1). The requirement for authentication at the species level was a sequence similarity exceeding 97%, while a similarity below that threshold was employed for recognition at the genus level **(Wong & Hanner, 2008)**. This finding indicates that the classification of species using DNA barcoding is highly precise. Due to its low intraspecific variation and high interspecific variation values, particularly in neighboring taxa, the mtDNA *coi* gene is a very effective species authentication method **(Ward *et al.*, 2005; Feng *et al.*, 2011; Lumogdang *et al.*, 2022)**. **Feng *et al.* (2011)**, **Dahruddin *et al.* (2016)** and **Lumogdang *et al.* (2022)** have demonstrated that DNA barcoding serves to authenticate the acknowledged species for global commerce, thereby ensuring the consumption of fishery goods or the practice of ornamental fish trade. This approach is advantageous for species identification and the sustainable management of fishing resources.

Furthermore, the genetic distance was employed in this study to ascertain the genetic correlation between *T. gratilla* specimens collected from Indonesia and other samples from the DNA data bank. The value of genetic distance ranges from 0.00 to 0.01. **Hardianto and Satriyo (2023)** defined a genetic distance difference of 4% (0.04) as a threshold for species that are molecularly similar. Greater population uniformity is observed when the genetic distance between individuals is less. By contrast, a higher genetic distance between individuals in a group will result in a greater level of population diversity. The greatest genetic divergence (0.70) was observed between *T. gratilla* inhabiting Indonesia and the species outgroup *T. ventricosus* from Panama (AY205515.1).

Comparable to the genetic distance analysis, the haplotype network analysis revealed a negligible discrepancy in the species analysis (Fig. 2). Evidently, the barcode variation within *T. gratilla* was somewhat low in comparison to the sequencing variance among species in the genus *Tripneustes*. Genetic distance is a measure of the proportion of genetic differences between species or populations **(Dogan *et al.*, 2016)**. Consequently, a lower genetic distance value results in a more similar partial sequence of the *coi* gene **(Sonet *et al.*, 2022)**. The collecting urchins' phylogenetic tree is schematically shown in Fig. (3). This study assessed the evolutionary dynamics and kinship degree of a species, categorizing them into three distinct clusters. The species *T. gratilla* was isolated from *T. ventricosus* (AY205515.1) with a bootstrap value greater than or equal to 100%. One cluster was formed within the species *T. gratilla* from Indonesia.

Although *T. gratilla* has been documented in several countries in Asia and Africa, research on its evolutionary history is scarce in the literature, particularly in Indonesia. A prior investigation conducted by **Casilagan *et al.* (2013)** documented the genetic

variability, population composition, and demographic background of *T. gratilla* in the Philippines. Furthermore, **Sonet *et al.* (2022)** documented the process of DNA barcoding for several species of echinoderms, including *T. gratilla*, specific to the South Coast of Africa. For instance, **Toha *et al.* (2014)** conducted a comprehensive analysis of the genetic characteristics of collector urchins, specifically *Tripneustes gratilla*, in Indonesia. Moreover, **Toha *et al.* (2015)** documented the range of colors and the geographical range of *T. gratilla* in Cendrawasih Bay, located on Papua Island. In their recent study, **Dailami *et al.* (2023)** documented the DNA barcoding of *T. gratilla* in Papua Island. The determination of the phylogenetic status of an organism is crucial for informing identification and resource management research. *Tripneustes gratilla* is a species that offers valuable insights on ecological and environmental conditions and contributes to the marine food chain **(Toha *et al.*, 2017)**.

The molecular study revealed a strong genetic relationship between all samples and *T. gratilla*. In a prior investigation employing sequencing data of the identical molecular markers (*coi* gene), comparable findings were reported **(Sonet *et al.*, 2022)**. A bootstrap score above 70% suggests that the data possess a high level of stability **(Lemey *et al.*, 2009)**. The reconstructed phylogenetic tree yielded a scale bar of 0.05. Based on the findings of **Syaifudin *et al.* (2021)**, a phylogenetic tree with a 0.01 scale bar indicates a genetic distance where nucleotides change every 100 base pairs. The acquired phylogenetic structures reveal a genetic distance characterized by nucleotide sequence variations occurring five times every 100 base pairs. Diverse aquatic species have been subjected to DNA barcoding analysis, which has shown greater variance within congeneric species than among conspecific individuals **(Ward *et al.*, 2005; Castaneda *et al.*, 2023; Hardianto & Satriyo, 2023)**. Thus, it is capable of accurately differentiating a group of morphologically unique species in marine organisms **(Castaneda *et al.*, 2023; Hardianto & Satriyo, 2023)**.

CONCLUSION

In the light of species identification, molecular techniques such as DNA barcoding and phylogenetic analysis are more accurate and dependable than morphological methods. Using DNA barcoding and phylogenetic analysis, the current effort aimed to identify the specimens collected from the Red Island Beach, Banyuwangi Regency, as belonging to a particular species. This finding would clarify the specimens' evolutionary position and provide essential information for further species research. DNA barcoding analysis compared haplotypes from other DNA data banks at NCBI; the analyses verified that the samples identified as *T. gratilla* match range 98-100%. Based on these findings, the research in Indonesia might incorporate the programs set up in the regions, where phylogenetically connected haplotypes are observed.

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