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Molecular characterization of toxic benthic dinoflagellate, *Prorocentrum lima* in west Indonesian waters using LSU 28S rDNA gene

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Abstract. *Widiarti R, Zamani NP, Bengen DG, Madduppa H. 2022. Molecular characterization of toxic benthic dinoflagellate,* Prorocentrum lima *in west Indonesian waters using LSU 28S rDNA gene. Biodiversitas 23: 3257-3263. Prorocentrum lima* is one of the toxic benthic dinoflagellates known to cause Diarrhetic Shellfish Poisoning (DSP), which is also associated with ciguatoxin-producing species, *Gambierdiscus toxicus* that causes Ciguatera Fish Poisoning (CFP). *P. lima* has a wide range of morphological variability and genetic diversity, but such research has never been reported from Indonesian waters yet. This study aimed to determine the molecular characteristics of *P. lima* in west Indonesian waters, namely Bintan Island, Belitung Island, Seribu Islands, and Karimunjawa Islands. Molecular characterization was conducted by amplification on large subunit (LSU) 28S rDNA gene. Extraction was conducted using freeze-thaw which was continued with single cell PCR method. Genetic distance values and phylogenetic analysis were analyzed using MEGA software. Based on molecular analysis, *P. lima* from this research was divided into two subclades, namely subclade A from Seribu Islands and Belitung Island, and subclade B from Karimunjawa Islands and Bintan Island. *P. lima* from Bintan Island showed a closer relationship with the reference sequence from the Genbank. Observation of molecular characters of *P. lima* showed that the genetic diversity of *P. lima* depended on the variation of the island's morphogenesis type. These findings could support a further study on the distribution of *P. lima* in Indonesian waters, related to the genetic variation and toxin production, since Indonesia consists of many small islands.

Keywords: Diarrhetic Shellfish Poisoning, genetic diversity, morphogenesis type, *Prorocentrum lima*, single cell PCR

INTRODUCTION

Toxic dinoflagellates are generally species-specific because only certain species were able to produce toxins (Durán-Riveroll et al. 2019; Verma et al. 2019). One of toxic dinoflagellates in Indonesian waters is the genus *Prorocentrum*. Determination of the genus is essential because the toxin profile differs significantly between closely related species, such as on species complex (Nascimento et al. 2017). The genus is mostly marine, distributed worldwide in planktonic and benthic habitats, from tropical to temperate oceans (Hoppenrath et al. 2013; Nishimura et al. 2019). About 80 species of *Prorocentrum* have been described, whereas 33 species of which are benthic, and nine species have been shown to produce toxins (Hoppenrath et al. 2013; Hoppenrath et al. 2014; Zhang et al. 2015).

Prorocentrum lima has very high morphological diversity and genetic variation, making it a species complex (Hoppenrath et al. 2013; Chomérat et al. 2018). The species is distinguished from other *Prorocentrum* species based on its oblong oval to ovoid cell, smooth thecal surface with scattered pores, V-shaped periflagellar areas, 8 platelets with a specific arrangement pattern (Hoppenrath et al. 2013; Zhang et al. 2015), and number of pores (Hoppenrath et al. 2013).

Morphological observations using a microscope are sometimes challenging, specifically for athecate (naked) and small Dinoflagellate species (Mordret et al. 2018). Identification based on morphological characters is highly dependent on the life cycle, environmental conditions, and sample preservation procedures (Ki and Han 2008). Therefore, molecular analysis is needed to obtain a more precise and objective result (Mordret et al. 2018). Molecular identification could also be used to avoid errors, such as misinterpreting a toxic species to be non-toxic.

Aside from being associated with the Ciguatera Fish Poisoning (CFP) causing species, *P. lima* could produce Okadaic Acid (OA) and Dinophysis-Toxin (DTX) which causes Diarrhetic Shellfish Poisoning (DSP) (Hoppenrath et al. 2014; Zhang et al. 2015; Durán-Riveroll et al. 2019). Therefore, its phylogenetic information is needed to determine the toxin production and toxicity level since it is essential to evaluate the risk of DSP cases in some areas (Nishimura et al. 2019). Previous studies showed that all *P. lima* strains produce OA, but only a few contain varying amounts of DTX (Hoppenrath et al. 2013; Luo et al. 2017).

Some studies were conducted on the molecular identification of *P. lima* in several locations, such as North Natuna Sea (South China Sea) (Zhang et al. 2015) and Caribbean Sea (Chomérat et al. 2018). However, molecular studies of the species have not been conducted in

Indonesian waters. Most studies were based on planktonic Dinoflagellate species, including *Margalefidinium catenatum* from Lampung Bay waters, having two different ribotypes between cyst forms in sediments and vegetative cells in the water column (Thoha et al. 2019).

The eukaryotic LSU 28S rDNA provides a valuable tool to analyze closely related Dinoflagellate species or even strains with high phylogenetic resolution (Lenaers et al. 1989; Ki and Han 2008; Tawong et al. 2015). Therefore, this study aims to determine the molecular diversity of *P. lima* in the western part of Indonesian waters, which were in Bintan Island, Belitung Island, Seribu Islands, and Karimunjawa Islands waters using LSU 28S rDNA. This research could support a further study on the distribution of *P. lima* in Indonesian waters, related to the genetic variation and toxin production, since Indonesia consists of many small islands with different types of morphogenesis and biophysics characters

MATERIALS AND METHODS

Sampling location

Sampling was conducted in coral reef areas in the western part of Indonesia waters, namely Bintan Island, Belitung Island, Seribu Islands, and Karimunjawa Islands (Figure 1; Table 1) from April to September 2018.

Research materials

The collection of macroalgae as a substrate was limited to *Sargassum* and *Padina*, because both macroalgae genera are mostly attached by benthic dinoflagellates (Widiarti 2002). Thallus was taken from the reef flat area and placed into a plastic bottle with ambient seawater. Furthermore, the bottle was stirred using a vortex machine (1250 rpm for 1 minute) to release the benthic dinoflagellates. The water sample was then filtered through a series of sieve with a pore sizes of 125 µm and 20 µm. Finally, the filtrates were preserved with 96% ethanol for molecular characterization.

Procedures

DNA isolation and extraction

DNA extraction was performed by freeze-thaw and single cell PCR method (Hernández-Rosas et al. 2017; Chomérat et al. 2018) with modifications through the following steps: A target cell is collected under a microscope by a micropipette and transferred into a PCR tube that contained 2 µL of nuclease-free water. The tube containing the target cell is stored in a freezer at -20°C for 18-20 hours. The tube is then removed from the freezer and placed into a water bath at 95°C for 5 minutes, and put in a sonicator for 1 minute. Afterward, it was immediately stored back in the freezer at -20°C until the subsequent DNA analysis treatment.

Amplification and electrophoresis

The PCR mixtures were performed in a volume of 25 µL containing 1 µL extracted cells, 9 µL ddH_2O, 12.5 µL My Taq Red Mix, 1.25 µL primers D1R forward (5'-ACCCGCTGAATTTAAGCATA-3') and 1.25 µL D2C reverse (5'-CCTTGGTCCGTGTTTCAAGA-3') (Laza-Martinez et al. 2011; Tawong et al. 2015). The PCR amplification involves pre-denaturation at 94°C for 5 minutes followed by 37 cycles of denaturation at 94°C for 30 seconds, annealing at 54.4°C for 50 seconds, extension at 72°C for 45 seconds, and final extension at 72°C for 6 minutes (Zhang et al. 2015). The quality of each amplified DNA product was confirmed by electrophoresis, using 1.5% agarose gel and tris-EDTA buffer for 20 minutes at a voltage of 100 V. The PCR products are visualized in the DNA bands on the agarose gel using GelDoc (BioRad, CA. USA). The sequencing process is performed when the PCR results positively contain the desired DNA. Furthermore, the PCR product was then delivered to the PT. Genetika Science Indonesia sequencing service.

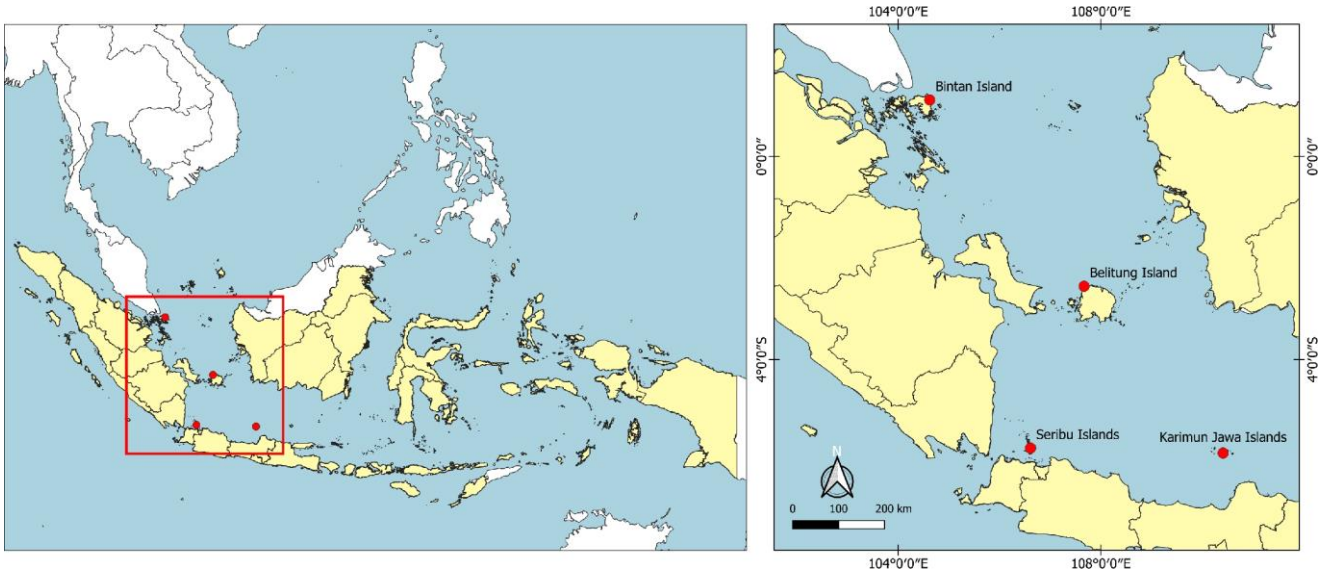

Figure 1. Sampling locations in Seribu Islands (Station I), Bintan Island (Station II), Karimunjawa Islands (Station III), and Belitung Island (Station IV)

Table 1. Research site coordinates in four sampling stations

Location	Sample code	Coordinate
Station IV	3987607_1C	02°33'17.05" S,
(Belitung Island)	3987625_1D	107°40'10.04" E
	3987643_1E	
	3987627_2D	
	3987645_2E	
	3987609_3C	02°33'16.46" S,
	3987647_3E	107°40'04.16" E
Station III	3987611_4C	05°50'22.56" S,
(Karimunjawa Islands)	3987631_4D	110°24'39.77" E
	3987613_6C	05°50'19.40" S,
	3987633_6D	110°24'46.67" E
	3987649_6E	
Station II	3987615_7C	01°06'50.98" S,
(Bintan Island)	3987635_7D	104°37'35.05" E
	3987617_8C	
	3987637_8D	
	3987651_8E	
	3987619_9C	
Station I	3987621_10C	05°44'32.83" S,
(Seribu Islands)	3987639_10D	106°36'49.25" E
	3987653_10E	
	3987623_12C	05°44'29.62" S,
	3987641_12D	106°36'56.47" E
	3987655_12E	

Data analysis

The sequencing result was edited using MEGA 7.0 (Molecular Evolutionary Genetics Analysis) software for reading the base sequences and DNA alignment was edited using tools alignment 18 Clustal W method (Tamura et al. 2011). The data obtained is then adjusted to the reference sequence/database from Genbank (NCBI) using BLAST (Basic Local Alignment Search Tool). The results of the BLAST analysis show the query and similarity percentage. The former is defined as a value that indicates how long the sample sequence aligned with the target sequence in the database (Fassler and Cooper 2011: Newell et al. 2013). Furthermore, the query value becomes 100% when the target sequence in the database covers the entire sample sequences. The similarity percentage is a value that indicates how many characters in the sample sequence are identical to the target sequence (Menlove et al. 2009; Fassler and Cooper 2011; Newell et al. 2013). In other words, the similarity percentage increases with the identicality between two sequences.

Phylogenetic analysis on *P. lima* is carried out with the Maximum Likelihood (ML) method using MEGA software, with 1000 bootstrap replications (Tawong et al. 2015; Zhang et al. 2015). Meanwhile, the difference in evolutionary distance is calculated using the Kimura 2-Parameter. Phylogenetic analysis determines whether species obtained from different locations are in one large clade and have a close relationship or originated from a common ancestor. The values of the genetic distance (both between individuals and between populations from four locations) are analyzed using MEGA 7.0 software and calculated using a 2-parameter Kimura model, on 8 nucleotide sequences.

RESULTS AND DISCUSSION

Molecular characterization of *P. lima*

According to the results of the 24 sequences using the single cell PCR method, only 8 sequences were properly read through alignment using BLAST and have a fairly high query value and similarity (Table 2). BLAST search result in 'no significant similarity found' for the other 9 sequences showed that the alignments may have expected values above the default threshold, and therefore are not displayed. The alignment between the sample sequence and reference sequence showed the number of queries and similarities ranging from 56-67% and 67.48-70.74%, respectively, which were from Station IV (3987607_1C and 3987643_1E), Station III (3987631_4D and 3987613_6C), Station II (3987615_7C and 3987617_8C), and Station I (3987621_10C and 3987655_12E).

The alignment of sample sequence and reference sequence using the BLAST program is conducted by directly writing the target organism, *P. lima*, because the query of 100% was represented by fungi taxon group. The presence of fungi in the sample causes difficulties in obtaining pure sequence results. The query number obtained cannot exceed 50%, because the samples are taken directly from nature, hence, there may be contamination from several groups of fungi. Environmental samples contain various substances or microorganisms that can become contaminants during the DNA analysis process using PCR (Marin et al. 2001). Moreover, the Dinoflagellate shows a high similarity of the LSU rRNA gene sequences with the fungi and ciliate groups (Lenaers et al. 1989).

The single cell PCR method is used to minimize contamination of the sample species because only one cell is collected for amplification and sequencing purposes. The use of common DNA purification methods cannot eliminate contaminants (Marin et al. 2001; Gao et al. 2017). The methods also require large numbers of cells, while Dinoflagellates are a group of microorganisms that are difficult to culture (Marin et al. 2001). The single cell PCR technique is commonly used to study a species-level phylogeny and has been completed with the Chrysophyceae, Dinophyceae, and Bacillariophyceae (Hamilton et al. 2015). Moreover, the genetic information could also be obtained very well from a limited amount and already fixed samples, without going through cell culture stage (Marin et al. 2001; Lim et al. 2014; Annenkova 2018).

The disadvantage of single cell PCR technique is that the number of the PCR product remains unmaximized, resulting in only 15 out of 24 samples that can be read well by electrophoresis results and BLAST sequence reading. This could be due to the number of cell which is taken only one from each sample, increasing the risk of cell not being extracted or wasted during treatment. The success of single cell PCR technique varies widely, and the main problem with methods which is used low cell numbers is the loss of cells during isolation and extraction processes (Marin et al. 2001; Bolch and Percy 2013; Hernández-Rosas 2017).

The other possibility of showing a small query of value is that *P. lima* obtained is different or has not been recorded on Genbank. This is indicated by the very small number of

sequence records from *P. lima*. The sequence data of *P. lima* accounted in NCBI is only 312, 0.0069% and 0.197% of the total Dinoflagellate and *Prorocentrum* sequences, respectively. Meanwhile, *P. lima* data from the BOLD (Barcode of Life Data) System only amounted to 37, and sequences data from the Indonesian waters were never been recorded yet. A species with a low query value could be, because the species has not been recorded in Genbank. Anzani et al. (2019) discovered a new Ascidian species from the waters of Raja Ampat, shown by a low query value.

P. lima is often considered as a species complex, because it has high morphological diversity and genetic variability (Hoppenrath et al. 2013). Zhang et al. (2015) found five ribotype diversity of the species based on sequence results using LSU rDNA (827 bp) and ITS (580 bp) from Hainan waters in the North Natuna Sea. The phylogenetic analysis based on LSU rDNA in the D1/D2 region has also been used severally to classify *P. lima* complex and compare the results with morphological characters (Laza-Martinez et al. 2011; Nagahama et al. 2011; Zhang et al. 2015; Luo et al. 2017: Chomérat et al. 2018). Due to the complex nature of *P. lima*, probably the *P. lima* which is collected from Bintan Island, Belitung Island, Seribu Islands, and Karimunjawa Islands is a new strain that has not been recorded in Genbank.

Genetic distance and phylogenetic tree

The genetic distance showed that the closeness between *P. lima* from Belitung Island and Seribu Islands is 0.036 (Table 3). Meanwhile, the farthest is between the species from Seribu Islands, Bintan Island, and Karimunjawa Islands, which is 0.379. The calculation of the lowest genetic distance between populations (between groups) is also shown by *P. lima* from Belitung Island and Seribu Islands, which is 0.130 (Table 4).

The phylogenetic tree analysis shows that all the discovered *P. lima* belonged to one large clade genetically different from the outgroup, *Gymnodinium catenatum*. It was selected as an outgroup because *G. catenatum* is a Dinoflagellate species belonging to the Gymnodinoid class, which is very different from the Prorocentroid group. Furthermore, the phylogenetic tree generally shows very short branch lengths among *P. lima* in one clade. This shows no divergence because all specimens originate from the same species. The clade is further divided into two subclades, based on the phylogenetic tree. The first is subclade A originating from Station I (Seribu Islands) and Station IV (Belitung Island) and showing a closer relationship than those from other stations. The second is subclade B originating from Station II (Bintan Island) and Station III (Karimunjawa Islands (Figure 2).

The phylogenetic tree also showed that *P. lima* from Station II (Bintan Island) is clustered on the first branch and adjacent to the Genbank reference. This implies that it has a close relationship with the reference. Hence, it could be explained by the location of four stations, whereas Bintan Island is in the northernmost part of the western Indonesian waters, directly adjacent to the North Natuna Sea. The reference sequence also originated from North Natuna Sea area.

Table 2. Percentage of query and similarity of sample sequence from the four locations

Location	Sample code	% Query	% Similarities	Accession number	Description
Station IV	3987607_1C	61.0	69.97	DQ336188.1	
(Belitung Island)	3987625_1D	51.8	93.94	KM266631.1	
	3987643_1E	56.0	68.48	DQ336188.1	
	3987627_2D				*No significant similarity found*
	3987645_2E	62.0	67.98	KY010251.1	
	3987609_3C				*No significant similarity found*
	3987647_3E				*No significant similarity found*
Station III	3987611_4C				*No significant similarity found*
(Karimunjawa	3987631_4D	67.0	67.90	DQ336188.1	
Islands)	3987613_6C	66.0	67.72	KY010251.1	
	3987633_6D	66.0	67.11	KY010251.1	
	3987649_6E				*No significant similarity found*
Station II	3987615_7C	67.0	69.53	MG701857.1	
(Bintan Island)	3987635_7D				*No significant similarity found*
	3987617_8C	67.0	68.87	KY010251.1	
	3987637_8D				*No significant similarity found*
	3987651_8E	65.0	68.5	DQ336188.1	
	3987619_9C				*No significant similarity found*
Station I	3987621_10C	65.0	69.46	KY010251.1	
(Seribu Islands)	3987639_10D	68.0	68.23	KY010251.1	
	3987653_10E	40.0	69.49	KT898173.1	
	3987623_12C				*No significant similarity found*
	3987641_12D	65.0	67.33	DQ336188.1	
	3987655_12E	63.0	70.74	DQ336188.1	

Table 3. Genetic distance between *P. lima* of the four locations

Sample code	1	2	3	4	5	6	7	8
3987607_1C	0.000							
3987643_1E	0.215	0.000						
3987631_4D	0.339	0.354	0.000					
3987613_6C	0.317	0.355	0.201	0.000				
3987615_7C	0.346	0.364	0.181	0.171	0.000			
3987617_8C	0.320	0.364	0.176	0.156	0.048	0.000		
3987621_10C	0.225	0.036	0.379	0.377	0.376	0.379	0.000	
3987655_12E	0.211	0.048	0.376	0.349	0.351	0.357	0.073	0.000

Table 4. Genetic distance between populations from the four locations

Location	Belitung Island	Karimunjawa Islands	Bintan Island	Seribu Islands
Belitung Island	0.000			
Karimunjawa Islands	0.341	0.000		
Bintan Island	0.349	0.171	0.000	
Seribu Islands	0.130	0.370	0.366	0.000

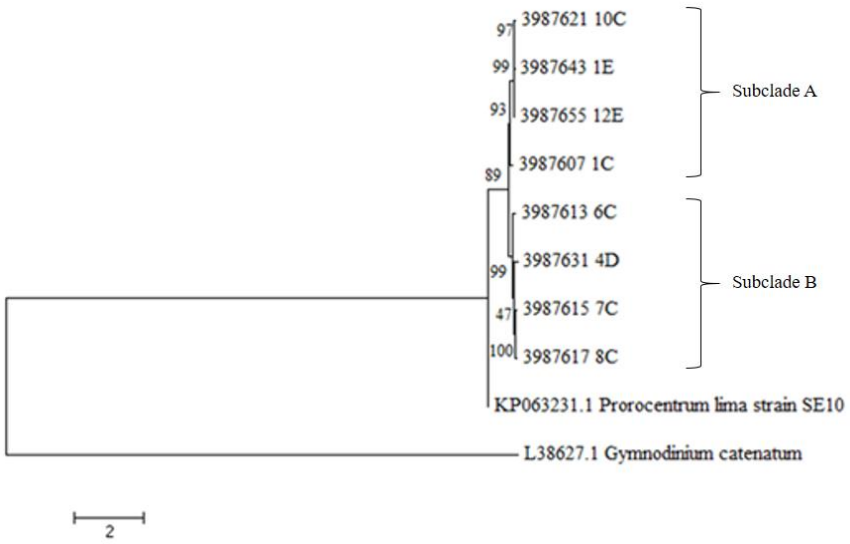

Figure 2. Phylogenetic tree using Maximum Likelihood (ML) analysis on 8 sequences of *P. lima* based on the single cell PCR results. The scale shows the genetic distance between sequences. Numbers in the tree indicate the bootstrap value of each branch

The low value of genetic distance between Station IV (Belitung Island) and Station I (Seribu Islands) indicates the closeness of the population in the two locations. It could be influenced by the distance from the two locations being relatively closer than the other islands, which is 352 km. This led to a close genetic distance between *P. lima* on both islands. Furthermore, the relationship between genetic and geographic distance is shown by Nagahama et al. (2011), which stated that geographically separated populations of *P. lima* could become genetically different, thus allowing the occurrence of allopatric speciation.

Benthic/epiphytic species have a limited distribution, unlike planktonic species which could be directly affected by current movement. Benthic *Prorocentrum* species could be strongly attached to substrates, such as macroalgae or sand grains, which prevent dislocation yet limit distribution (Fraga et al. 2012; Hoppenrath et al. 2013). The distribution of *P. lima* as a benthic species is enhanced by the closer distance between Belitung Island and Seribu Islands. These species generally use media for dispersal, such as macroalgae which are dislocated from the substrates and then washed away to other locations by current or upon floating detritus ("rafting") (Leaw et al. 2001; Durán-Riveroll et al. 2019).

According to the phylogenetic tree analysis, *P. lima* from Karimunjawa Islands and Bintan Islands share a

strong evolutionary relationship, despite that the distance between the two locations is geographically greatest. The genetic distance analysis performed using the single cell PCR method, indicates that the distance between *P. lima* and between populations from the two locations is less than that between the species from Karimunjawa Islands and Belitung Islands or Seribu Islands. These results could be due to the islands morphogenesis type. Karimunjawa Islands and Bintan Island are the hilly islands from monadnock group, and their similar island formation (based on the type of sediment and freshwater input from the mainland) leads to a similar evolutionary process for a species (related to the adaptability of each species to environmental conditions).

In conclusion, using LSU 28S rDNA, *P. lima* in the western part of Indonesian waters which were collected from Bintan Island, Belitung Island, Seribu Islands, and Karimunjawa Islands, is a new strain that has not been recorded in Genbank and it was divided into two subclades, namely subclade A originating from Seribu Islands and Belitung Island and subclade B originating from Karimunjawa Islands and Bintan Island. *P. lima*. Furthermore, *P. lima* from Bintan Island has the closest relationship with the sequence reference from GenBank.

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