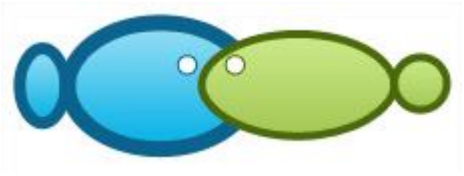

The phenotype comparison and genotype analysis of five Indonesian *Laevistrombus* sp. variants as a basis of species selection for aquaculture

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Abstract. Five morphological variants of Indonesian *Laevistrombus* sp. were successfully identified from Madong-Tanjungpinang marine waters. At first glance there are similarities in the collection of the conch, but when carefully observed there is a morphometric difference among the five. The condition had caused taxonomic uncertainty. For the purpose of selecting cultivation species, it was necessary to identify precisely by combining morphometric and genotype analyses. This study aimed to identify the species of Indonesian *Laevistrombus* sp. from Madong-Tanjungpinang city based on morphometrics and DNA sequence. The study was conducted in September-November 2017. Sampling collection was done along Madong waters. Traditional morphometrics was measured and was analysed by discriminant function analysis (DFA). Genotype analysis was conducted with specific primers of histone H3 gene. The DFA results showed significant morphometric differences in the five *Laevistrombus* groups, especially the thickness of outer shell lips. However, based on all morphometric and other physical characteristics, the five variants were one species of *Laevistrombus turturella*. The result was supported by a phylogenetic tree from DNA sequences of histone H3 gene that showed five variants having same histone H3 sequence. But, the sequences were different with *Strombus canarium, S. vittatus*, and *S. epidromis*. Histone H3 sequences of *Laevistrombus turturella.* had been submitted in the gene bank (submission ID: 2097081).

Key Words: morphometric, genetic, *Laevistrombus turturella*, siput gonggong, Tanjungpinang City.

Introduction. Indonesian *Laevistrombus* sp., called as "siput gonggong" is a name for sea conchs belonging to genus *Laevistrombus* (Gastropoda: Strombidae) from Malay community in Riau Islands Province. The species was famous seafood with delicious and chewy meat taste that served as the best menu for local and foreign tourists. Gonggong conch is an icon of Tanjungpinang city, as evidenced by the construction of the gonggong conch monument and building. Gonggong conch contained high protein, which is of 38.91% in muscle and 46.65% in visceral part (Muzahar 2013), but their fat and cholesterol levels were low, 0.78-2.26 mg/100 g and 9.89-24.95 mg/100 g, respectively (Muzahar & Viruly 2013). Gonggong conch was an important food and alternative protein source for the community along the coast of Bintan Island (Amini 1986). The species has high economic value in Tanjungpinang City markets with the price of 35,000 IDR/kg for live conchs.

The population of gonggong conch faced pressure especially intensive exploitation that can lead to overfishing. This condition was based on increasing number of enthusiasts due to the increase of Tanjungpinang population from 202,215 people in 2015 to 204,735 people in 2016 (BPS 2016). However, there was no data on the number of siput gonggong recorded (Viruly 2011), so it was feared the extinction of population like the large snail (*Strombus gigas*) in some Caribbean regions, America, occurred (Cala

et al 2013). Therefore, it was necessary to take action to preserve the population with cultivating it.

Morphologically there are similarities in the collection of siput gonggong and this condition has caused taxonomy uncertainty among researchers. For example, Amini (1986), Erlambang & Siregar (1995), and Nasution (2011) wrote *Strombus canarium*, while Arianti et al (2013) wrote *Laevistrombus turturella*. But, all of the above researchers did not explain the scientific basis of the use of the scientific names for gonggong conch. In Indonesia, there was no research on genotype analysis for cultivation purpose, so that this research needs to be done. The species hopefully can be determined precisely by combining of morphometric and genetic analysis in its identification.

Material and Method

Sampling location. This research was conducted in September-November 2017. The sampling location was along the Madong-Tanjungpinang City sea waters at 0°97'37"-0°98'30.83" S and 104°43'38.63"-104°48'19" E. Two hundred and twenty eight (228) conch specimens were collected manually by hand at low tide. In locations with a depth more than one meter, the specimen was collected by diving. The morphometric characteristics were measured at Marine Biotechnology Laboratory, Raja Ali Haji Maritime University and genetic aspect was analysed at Fish Reproduction and Genetics Laboratory, Department of Aquaculture, Bogor Agricultural University.

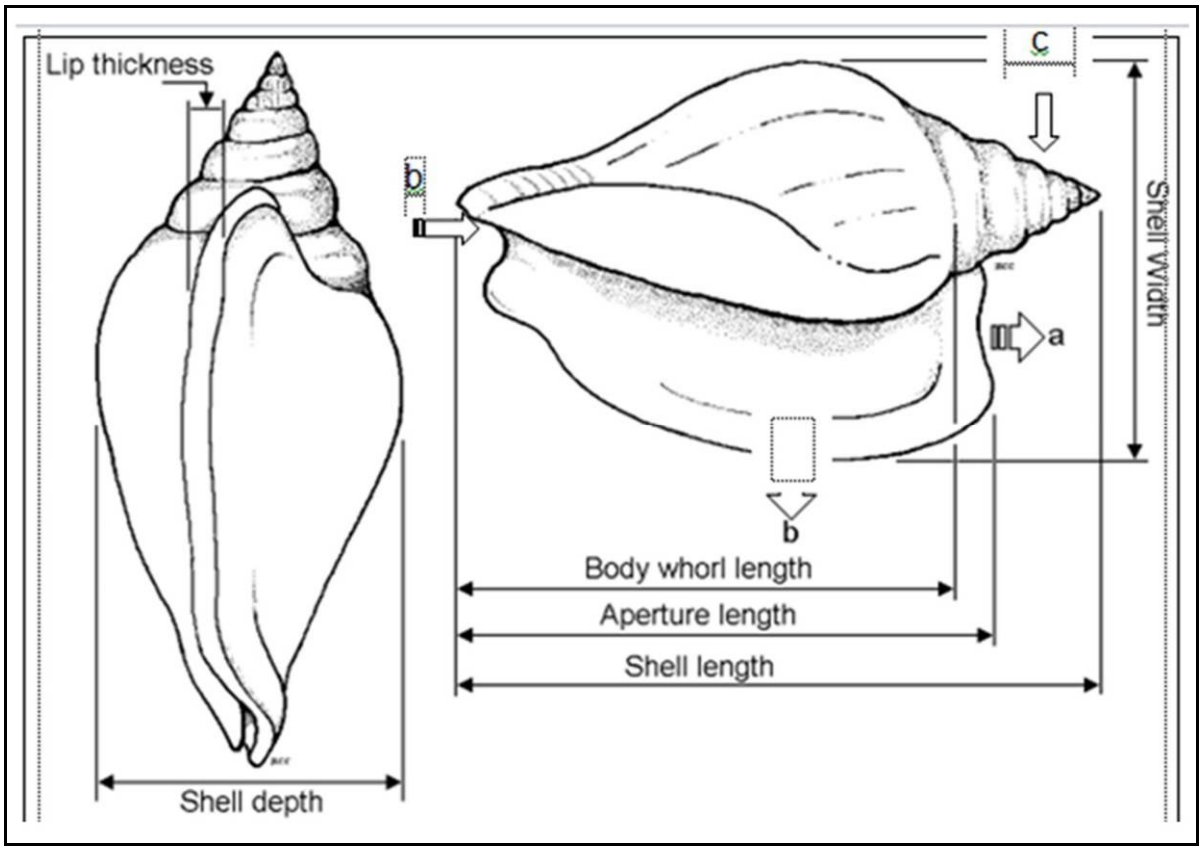

Figure 1. Traditional morphometric analysis of gonggong conch, stromboid notch (a) and siphonal notch (b) and number of the tower (c) (modified from Cob et al 2008).

Genomic DNA extraction and PCR amplification. Genomic DNA was extracted from 15-20 mg muscle tissues of each conch variant. Extraction was carried out using Gentra Puregene Tissue Kit (Qiagen, USA) following manual procedure. DNA was diluted with 30 µL Nucleases-free water (1st Base, Singapore) and stored at -20°C before analysis. DNA

concentration and purity were measured with spectrophotometry at 280/260 nm using GeneQuant® (Pharmacia Biotech, USA).

Gene amplification was employed using polymerase chain reaction (PCR) method with 20 µL total volume consisted of 10 µL MyTaq™ Red Mix DNA polymerase (Bioline, USA), 1 µL (10µM) of each primer, 7 µL nucleases-free water and 1 µL of DNA template. The primer used in this study was presented in Table 1. PCR program for H3 gene and β-actin as internal control was set as follows: 95°C for 3 min; 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; 72°C for 5 min and 20°C for 1 min. PCR product was separated using electrophoresis on 1.5% agarose gel and then visualized using ethidium bromide under UV light.

Table 1

The primer used in this study

Primer name	*The sequence ('5-'3)*	*Product size (base pairs)*	*Application*	*References*
H3A H3B	ATGGCTCGTACCAAGCAGACVGC ATATCCTTRGGCATRATRGTGAC	350	Histone H3 amplification	Latiolais et al (2006)
βAct F βAct R	TATGAAGGTTATGCT CTGCCC CATACCCAGGAAAGATGGCTG	100	β-actin amplification for internal control	Kusrini et al (2016)

Sequence analysis. For gene sequencing, PCR product was sent to 1st Base Laboratory (Singapore). To see the sequence homology, obtained sequences were compared with other conch H3 gene available at GenBank using BLAST® program (https://blast.ncbi.nlm.nih.gov). Phylogenetic analysis was employed on MEGA v.7 software and a phylogenetic tree was constructed with a Neighbor-Joining method with 1000× bootstrap replication.

Results

Phenotype (morphometrics and meristics). This study found five variants of conch morphology characters that lived in the sea waters of Madong-Tanjungpinang city waters. Variations were found on the thickness and the nature of outer lip surface, the direction of aperture opening, shape of the stromboid notch and siphonal notch of the shells. Based on shell thickness, the specimens were grouped into two, *i.e.* thick and thin shell. The difference in thickness and surface outer lip were grouped into four, *i.e.* dull blunt lipped, dull lips, thin blunt lips, and thin lips sharp. Differences in direction of aperture opening were divided into two, *i.e.* expand and bud aperture. Finally, the difference between the stromboid notch and siphonal notch was divided into two, *i.e.* present and absent. So based on all these differences, siput gonggong were categorized into five variants (Table 2), namely:

1) variant 1 (V1): thick-shell, expand aperture, thick blunt outer lips, stromboid and siphonal notch present (51 individuals);

2) Variant 2 (V2): thick-shell, expand aperture, medium blunt outer lips, stromboid and siphonal notch present (34 individuals);

3) variant 3 (V3): thin-shell, expand aperture, thin blunt outer lips, stromboid notch absent, but siphonal notch present (28 individuals);

4) variant 4 (V4): thin-shell, expand aperture, thin outer sharp lips, stromboid notch absent, but siphonal notch present (48 individuals);

5) variant 5 (V5): thin-shell, bud aperture, sharp outer lips, stromboid and siphonal notch absent (67 individuals).

Especially on variant 5 (V5), there was a special character. If the ventral body position faced the observer, then shell would immediately reverse and the dorsal faced to the observer. This might be due to anterior stromboid notch position starting from the bottom of the last tower, while the other four groups starting from the top of the last tower. The average morphometric and meristic values of the five conch variants were presented in Table 2.

Table 2

Comparison of morphometric and meristic values of five variants of *Laevistrombus* sp. from Madong-Tanjungpinang City with *S. canarium* (Cob et al 2008)

Category	*Morphometric character*	*Average value (mm)*	*Average value (mm) S. canarium (Cob et al 2008)*
Variant 1 (V1)	Shell length	66.68±6.02	55.24±0.32
	Shell raw length	52.25±5.01	43.82±0.24
	Shell width	41.41±3.70	35.13±0.19
	Shell depth	30.06±3.10	25.47±0.16
	Shell opening length	51.31±5.39	45.85±0.27
	Shell lip thickness	3.88±1.00	3.26±0.11
	Total weight	32.82±9.18	-
	Number of towers	7.27±0.77	10-11
Variant 2 (V2)	Shell length	65.09±5.33	-
	Shell raw length	51.07±4.69	-
	Shell width	40.38±3.29	-
	Shell depth	29.54±2.96	-
	Shell opening length	50.41±4.68	-
	Shell lip thickness	2.08±0.46	-
	Total weight	28.81±7.93	-
	Number of towers	6.97±0.90	-
Variant 3 (V3)	Shell length	63.39±5.67	-
	Shell raw length	49.71±4.85	-
	Shell width	38.66±3.82	-
	Shell depth	28.75±2.81	-
	Shell opening length	48.95±4.59	-
	Shell lip thickness	0.97±0.30	-
	Total weight	23.12±6.62	-
	Number of towers	7.25±0.88	-
Variant 4 (V4)	Shell length	62.00±4.59	-
	Shell raw length	49.89±4.79	-
	Shell width	36.29±3.36	-
	Shell depth	27.52±2.66	-
	Shell opening length	46.42±4.03	-
	Shell lip thickness	0.72±0.20	-
	Total weight	20.61±6.37	-
	Number of towers	8.02±1.01	-
Variant 5 (V5)	Shell length	58.72±7.76	-
	Shell raw length	45.89±6.54	-
	Shell width	30.52±5.72	-
	Shell depth	26.38±4.71	-
	Shell opening length	42.25±7.09	-
	Shell lip thickness	0.37±0.26	-
	Total weight	15.51±5.96	-
	Number of towers	6.85±0.98	-

All morphometric and meristic characters showed a significant difference ($p < 0.05$) and appropriate determination of morphological characters (Table 3).

The result of discriminant function analysis (DFA) showed a significant difference in the four morphometric characters, i.e. shell raw length, shell width, shell depth, and shell lip thickness. The morphological phenotypes were the main characters to distinguish morphology among conch groups. The discriminant function coefficient values were presented in Table 4.

Table 3

Result of morphometric variable average test from five conch categories

No	*Morphometric character*	*Wilks' Lambda*	*F*	*df1*	*df2*	*Sig.*
1	Shell length	0.804	13.614	4	223	0.000
2	Shell raw length	0.829	11.49	4	223	0.000
3	Shell width	0.492	57.558	4	223	0.000
4	Shell depth	0.851	9.768	4	223	0.000
5	Shell opening length	0.697	24.211	4	223	0.000
6	Shell lip thickness	0.137	351.975	4	223	0.000
7	Number of towers	0.914	5.275	4	223	0.000
8	Total weight	0.539	47.756	4	223	0.000

Table 4

Discriminant coeficient morphometric of gonggong conch (*Laevistrombus* sp.)

No	*Morphometric character*	*Function*			
		1	*2*	*3*	*4*
1	Shell raw length	-0.046	0.051	0.407	0.111
2	Shell width	0.208	-0.504	-0.092	-0.121
3	Shell depth	-0.142	0.435	-0.518	0.253
4	Shell lip thickness	1.594	0.914	0.142	-0.091
	Constant	-3.828	2.271	-2.349	-8.026

So, the function equation was:

1. DFA1 = -3.828-0.046X1+0.208X2-0.142X3+1.594X4
2. DFA2 = 2.271+0.051X1-0.504X2+0.435X3+0.914X4
3. DFA3 = -2.349+0.407X1-0.092X2-0.518X3+0.142X4
4. DFA4 = -8.026+0.111X1-0.121X2+0.253X3-0.091X4

From four DFA functions, only one function had eigenvalues (EV) greater than 1. When used functions one and two, have been explained 99% of total variance. The function one had described 88.2% (EV 7.21) of total variant and the function two described 10.8% (EV 0.88) of the total variance (Table 5). Both functions have the highest four characters, consecutively thick outer shell lips, shell depth, shell raw length and shell width.

Table 5

Eigenvalues, percentage diversity and DFA content of conch morphometric

Function	*DFA_1*	*DFA_2*
Eigenvalues	7.21	0.88
Percentage range	88.2	10.8
Lip thickness	0.927*	0.371
Shell depth	0.147	-0.127
Shell raw length	0.151	-0.207
Shell length	0.174	-0.201
Shell opening length	0.245	-0.296
Total weight	0.293	-0.216
Shell width	0.337	-0.491
Number of towers	-0.031	-0.012

*The largest real correlation between each variable and discriminant function.

The DFA1 and DFA2 scatter pots (Figure 2) showed that the five separation categories of conch were divided into five different groups. Categories V1, V2, and V5 showed far distant. While categories V3 and V4 indicated the distance adjacent to several parameters of each other.

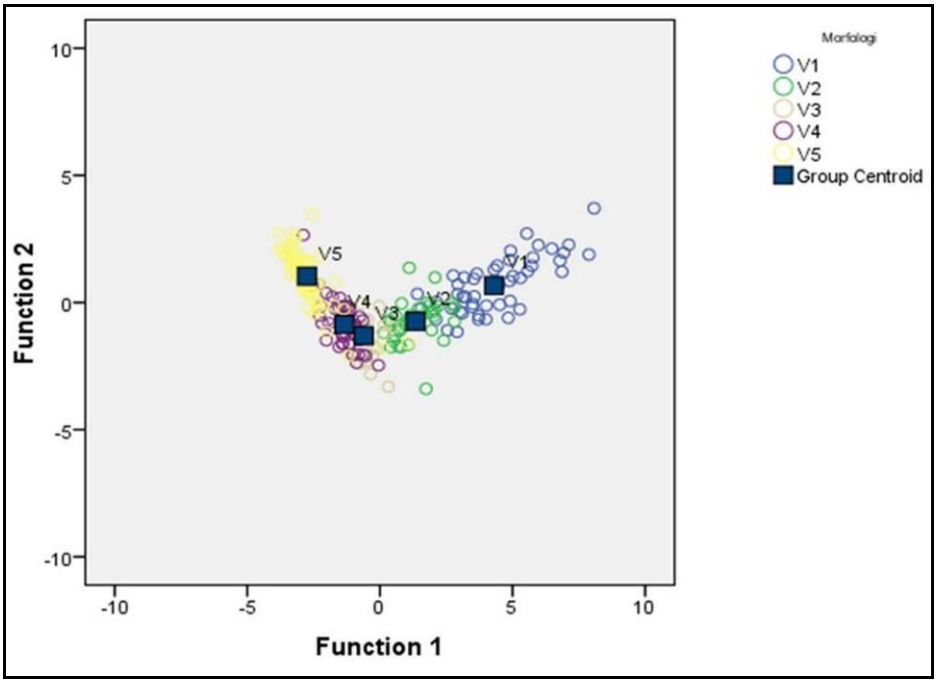

Figure 2. The plot distribution of DFA1-DFA2 function of five conch variants (V1-V5) from Madong-Tanjungpinang with different size of shell thickness, shell width, shell depth, shell raw length, shell lip thickness and aperture direction.

Genotype analysis (histone H3 gen sequence). DNA gene extraction and amplification of all variants were successfully performed using histone H3 primer genes. From the sequencing results, all variants had about 343-378 bp of histone H3 sequence (Figure 3). The phylogenetic tree was constructed by alignment of all variants with other species in one family (Figure 4).

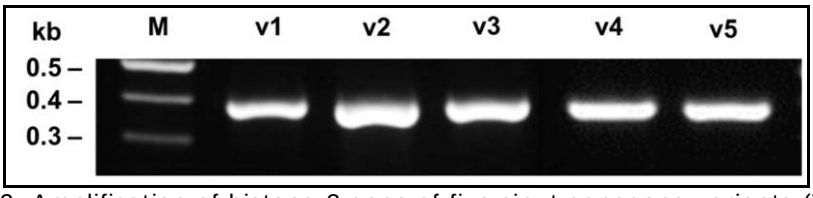

Figure 3. Amplification of histone-3 gene of five siput gonggong variants (V1-V5) (*Laevistrombus* sp.) from Madong-Tanjungpinang, Indonesia; M = DNA marker.

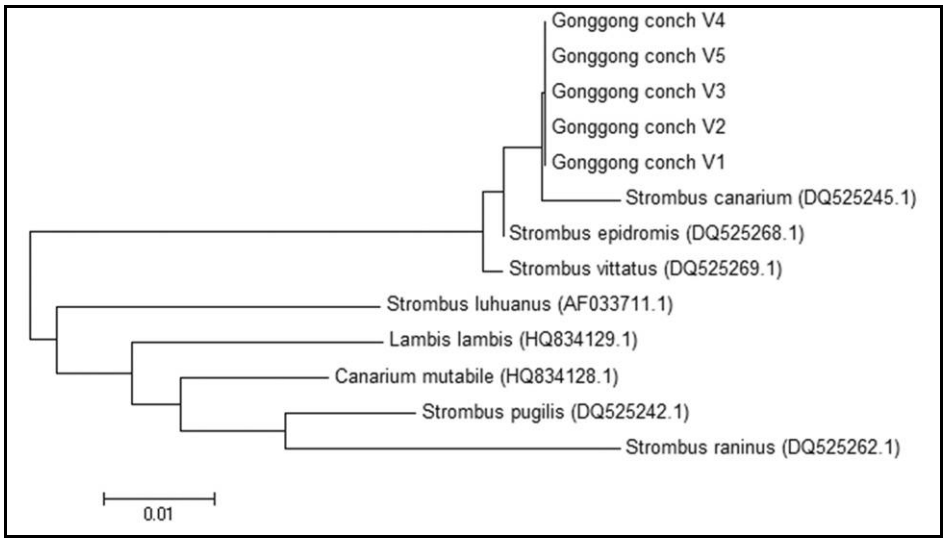

Figure 4. Phylogenetic tree of histone H3 sequence of five conch variants from Madong-Tanjungpinang city, Indonesia.

Discussion. The DFA results, the mean values of morphometric characters, periostracum coating motives, stromboid notch, and a siphonal notch in five groups could be summarized as *Laevistrombus turturella*. This was consistent with the statements of Poppe & Groh (1999), Dharma (2005), and MolluscaBase (2018). Comparison of morphological characters between conch (*Laevistrombus turturella*.) variants from this study and Philippines with *Strombus canarium* and *L. canarium* was shown in Figure 5.

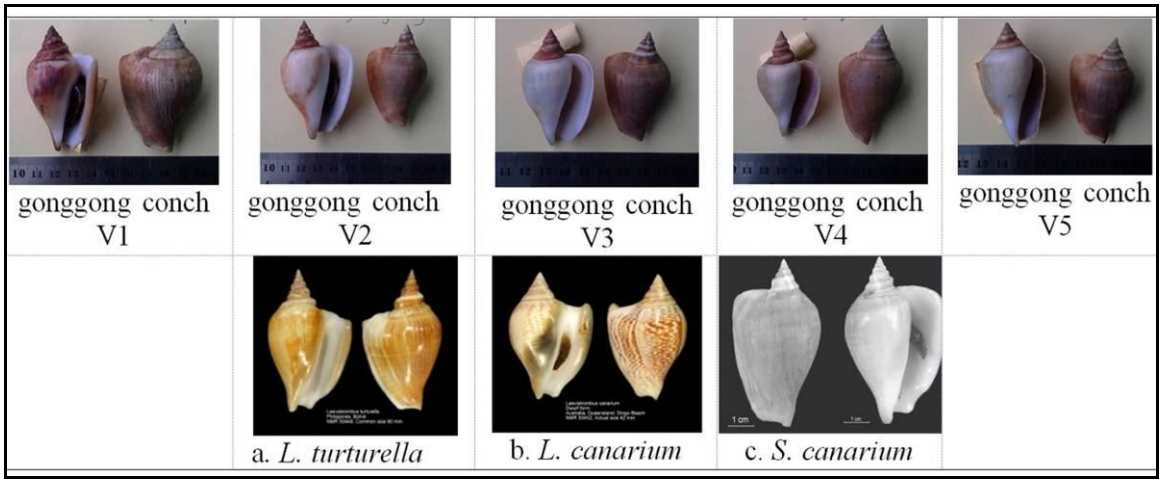

Figure 5. Morphological character comparison of five variants of conch *Laevistrombus turturella* (V1-V5) from Madong-Tanjungpinang city, Indonesia with *Laevistrombus turturella*, *Laevistrombus canarium,* and *Strombus canarium*.

The species of conch from Madong-Tanjungpinang city was *Laevistrombus turturella* that was supported by phylogenetic tree results from the fifth DNA sequence. The condition showed the type of histone H3 protein differs from *Strombus canarium*, *Strombus epidromis*, and *Strombus vittatus* (Figure 4), although genetic distance very closed only 0.01%. The low genetic distance value of species comparison indicated that there were several nucleotide bases variations capable of distinguishing species. But, five variants of conch have same histone H3 protein. The meaning was that the variants were one species of *Laevistrombus turturella*. According to Pamilo & Nei (1988), phylogenetic tree from DNA sequences did not have necessarily agreed with the tree species or by taxonomy compiled from morphological diversity analysis, due to genetic polymorphisms in ancestral species. This research is also in line with Latiolais et al (2006) that used the core DNA sequence (histone subunit 3, H3) and the molecular phylogenetic for the relation of quantitative size of shell shape to examine the relationship between taxonomic and morphological diversity. They concluded that there was no strong phylogenetics barrier between species and taxa *Strombus* and *Lambis*, but the morphological diversity of the positive subclade was related to species richness. This is also supported by the results of examination of SNP (single nucleotide polymorphism) DNA sequence isolates from the five variants of barking there was no difference at all. But, histone DNA sequence of *Laevistrombus turturella* was not yet available in the genebank database. The presence of intergroup variation indicated high variability in all species. The existence of some differences in morphology of conch especially on the character of the outer lip thickness, the length of the raw shell, the width of the shell and the depth of the shell is thought to be caused by differences in age, environmental adaptation, population conditions, nutrient content on different substrate types where the conch live between those in sandy mud and muddy sand, in line with Cadrin (2000) who suggested that morphological differences between the same species were caused by factors such as feeding habits, life histories and environmental physics factors such as water temperatures. Similarly, Silva et al (2013) also stated that in marine gastropods species differentiation was influenced by population history and by complex interactions between oceanic dynamics such as variations of environmental factors along the coast, such as temperature and hydrodynamics as well as other ecological properties. Trussel (2000) mentioned that the pattern of late phenotypic variation for all traits was consistent with

the variation between genetic and environmental influences. The growth rate of juvenile snail *Littorina unifasciata* was largely determined by feeding opportunities rather than the type and amount of food on shell shape patterns (Chapman 1997). Is any of the five variants a sub-species? It might be, but requires further study with the determination of some other markers.

Ecologically, the morphological variation of conch will make the adaptation of the population to the environmental pressure to be higher so that more survive. Hollander et al (2006) stated that there was a degree of plasticity in snails with different ecotypes and phenotypic plasticity will be optimal for enhancing local adaptation.

Conclusions. The five groups of conch from Madong Tanjungpinang marine waters although morphologically different from the main distinguishing feature of the outer shell lining of the shell but all have the same Histone H3 gene sequence of 377-378 bp and belong to species *Laevistrombus turturella*.

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