BIODIVERSITAS
Volume 20, Number 6, June 2019
Pages: 1758-1763

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d200637

DNA barcoding reveals underestimated species diversity of mantis shrimp larvae (stomatopods) in Banten Bay, Indonesia

ABINAWANTO♥, MARIANA D. BAYU INTAN, WISNU WARDHANA, ANOM BOWOLAKSONO
Program of Biology, Postgraduate Program, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia. Depok16404, West Java, Indonesia. Tel.: +62-21-7270163, +62-21-78849009, Fax.: +62-21-78849010, ♥email: abinawanto.ms@sci.ui.ac.id

Manuscript received: 6 February 2019. Revision accepted: 30 March 2019.

Abstract. *Abinawanto, Intan MDB, Wardhana W, Bowolaksono A. 2019. DNA barcoding reveals species diversity of mantis shrimp larvae (stomatopods) from Banten Bay, Indonesia. Biodiversitas 20: 1758-1763.* Larvae of stomatopods can be morphologically identified by linking between larval and adult form, but it's difficult applied in the laboratory because of requirement of condition to rearing larvae collected either from plankton, or spawning adults in the laboratory. Molecular methods are the alternative approach to gather accurate taxonomic identification. One of molecular methods is DNA barcoding based upon sequence diversity in cytochrome c oxidase subunit 1 (COI). Research on stomatopod larvae diversity and distribution at six stations in Banten Bay has been studied from October 2013 to March 2014. This study was conducted to identify the diversity of stomatopod larvae through DNA barcoding and also to examine the distribution of stomatopod larvae in Banten Bay. DNA barcoding was used to identify mantis shrimp larvae in Banten Bay, Indonesia. Partial mitochondrial COI were obtained for 37 larvae, revealing five distinct molecular operational taxonomic units (MOTUs). All MOTUs distinctively fell into Squillidea clade. Two Molecular Operational Taxonomic Units, MOTUs were successfully identified to species level (i.e. *Anchisquilla fasciata*), while three others remained identified to genus level (i.e. *Stomatopoda* spp). The intraspecific variation of COI ranged from 0% to 3.1% (Kimura 2-parameter distance), whereas interspecific divergence ranged from 5.9% to 20.4%. These results imply that COI genes are suitable for species identification of stomatopod larvae in this area, though the number of unidentified species was higher than the identified ones. This is likely because of the limited database in GenBank. In addition, the two identified species represents new records for this area, suggesting that the mantis shrimp in the area have been underestimated based on traditional biodiversity measures. We recommend to develop a taxonomically-comprehensive DNA barcoding database to improve the accuracy and feasibility of using DNA barcoding for species identification of Indonesia mantis shrimps.

Keywords: Banten Bay, Cytochrome c Oxidase Subunit-1, molecular operational taxonomic units, MOTUs, stomatopod larvae

INTRODUCTION

Stomatopods or mantis shrimps (class Malacostraca) are benthic marine crustaceans with high diversity and wide geographical distribution. Over 480 species of stomatopods have been discovered at various types of habitat, and are widespread along tropical and subtropical regions, from a muddy-sand bottom corals to 1500m depth of water (Ahyong et al. 2008). In Indonesia, 23.95% (115 species) of global stomatopod diversity have been discovered widely across Indonesian waters (Hutomo and Moosa 2005; Olavides 2011). Many of them have high economic values, and examples are *Harpiosquilla raphidea*, and *Harpiosquilla harpax*. In addition, having a bipartite life cycle just like many other marine organisms contributes to their dispersals through their planktonic larval stage. However, study on stomatopod diversity and their distribution, especially of their larvae is very uncommon due to the complexity of their life cycle, and lack of morphological descriptions of their developmental stages.

Although the taxonomy of stomatopods has been extensively enhanced, only 10% of known species could be identified in their larval stages (Diaz 1998). Previously, the identification of stomatopods larvae has been done by linking both larval and adult forms, but its application was difficult and time-consuming in laboratories due to the sensitivity and complexity of larvae rearing conditions (Provenzano and Manning 1978; Diaz 1998). Therefore, to cope with the difficulties and complexities of traditional taxonomy methods, especially for groups of species that have multiple developmental stages, many researchers have made use of DNA barcoding methods (Herbert 2003; Vences et al. 2005; Ward et al. 2005; Barber and Boyce 2006; Smith et al. 2008).

DNA barcoding proposed by Hebert (2003) is one of the molecular identification methods hotly discussed in taxonomy study groups. The DNA barcoding method is carried out by analyzing the short genetic sequences in Cytochrome c Oxidase Subunit-1 (COI) gene and comparing them with the sequences stored at the GenBank database. This method has become popular in identifying and studying the genetic diversity of stomatopod larvae by comparing the sequence of stomatopod larvae of the unknown species with the previously described sequence of adult stomatopod (Barber and Erdmann 2000; Barber et al. 2002a,b; Barber et al. 2006; Barber and Boyce 2006; Tang 2010).

As an archipelagic country with five-eighth of its surface area is occupied by waters, the diversity stomatopods in Indonesia waters is suspected to have been underestimated due to inadequate information. The

description of adult stomatopods in Indonesia waters has been studied and already well described by numerous researchers (i.e., Moosa 2000; Ahyong 2002; Ahyong and Moosa 2004). However, the information of larvae stomatopods diversity in Indonesia waters is quite inadequate (Barber et al. 2002a; Barber et al. 2002b; Pujawan et al. 2012). One of the areas where stomatopods are frequently found is in Banten Bay, Indonesia. It is a semi-enclosed inlet marine fishing area located in the northern region of Java which is known to be one of mantis shrimp producing areas. Although Banten Bay is a place commonly known for mantis shrimp fishing, the study of stomatopods has been limited only to commercial species, such as *Harpiosquilla raphidea*, *Harpiosquilla harpax*, and *Miyakea nepa* (Mulyono 2013). This study was therefore conducted to identify and describe the diversity of stomatopod larvae in Banten Bay, Indonesia using DNA barcoding method. Furthermore, this study enriches information on the stomatopod diversity which is presently underestimated.

MATERIALS AND METHODS

Samples collection

Stomatopods larvae were collected by deploying 30x30 cm^2 mouth larval traps with 500 µm mesh size underneath the water surface for approximately 10 minutes. These collections were conducted at six stations in Banten Bay, Java Sea, Indonesia (**Error! Reference source not found.**), in Serang City and Serang District, Banten Province, Indonesia, from October to November 2013.

The larvae samples were preserved in 95% ethanol onboard, and this was replaced by fresh ethanol every 24 hours during the first three days. Prior to the DNA extraction, all stomatopod larvae were sorted from the zooplankton samples in the laboratory under stereo microscopes, and then separated into different morphotypes according to their basic morphological features, such as differences of their body shapes, and carapace characteristics (presence/ absence and shape of the lateral spination, and the rostral spines). Morphological characters are recorded in Supplementary Material 1. Stomatopod larvae were photographed and categorized into 10 distinct morphotypes (morphotype A to J).

Molecular analysis

DNA was extracted from 37 individuals belong to 10 morphotypes of unknown larva species using the Wizard Genomic DNA Purification Kit (Promega). The Mitochondrial cytochrome oxidase subunit-1 gene (COI) was PCR amplified in 25µL of reaction volume using universal primers LCO 1490 and HCO 2198 (Folmer et al. 1994), yielding an amplicon of 650bp. PCR reactions contained 12.5µL of PCR Ready mix (KAPA 2G Robust), 0.625µL of the forward primer and 0.625µL of the reverse primer (final concentrations of 0.5mM), 4µL DNA *template* (40ng/µL), and 7.25µL ddH20. Thermocycling was initiated by 3 minutes of 95°C activation step, followed by 35 cycles of 95°C for 35s, 45°C for 30s and 72°C for 50s, and a final extension of 72°C for 7mins. The size and quality of PCR products were visualized on a 1% agarose gel. PCR fragments were directly Sanger sequenced (Macrogen) without further purification (all PCR fragments showed clean single band on agarose).

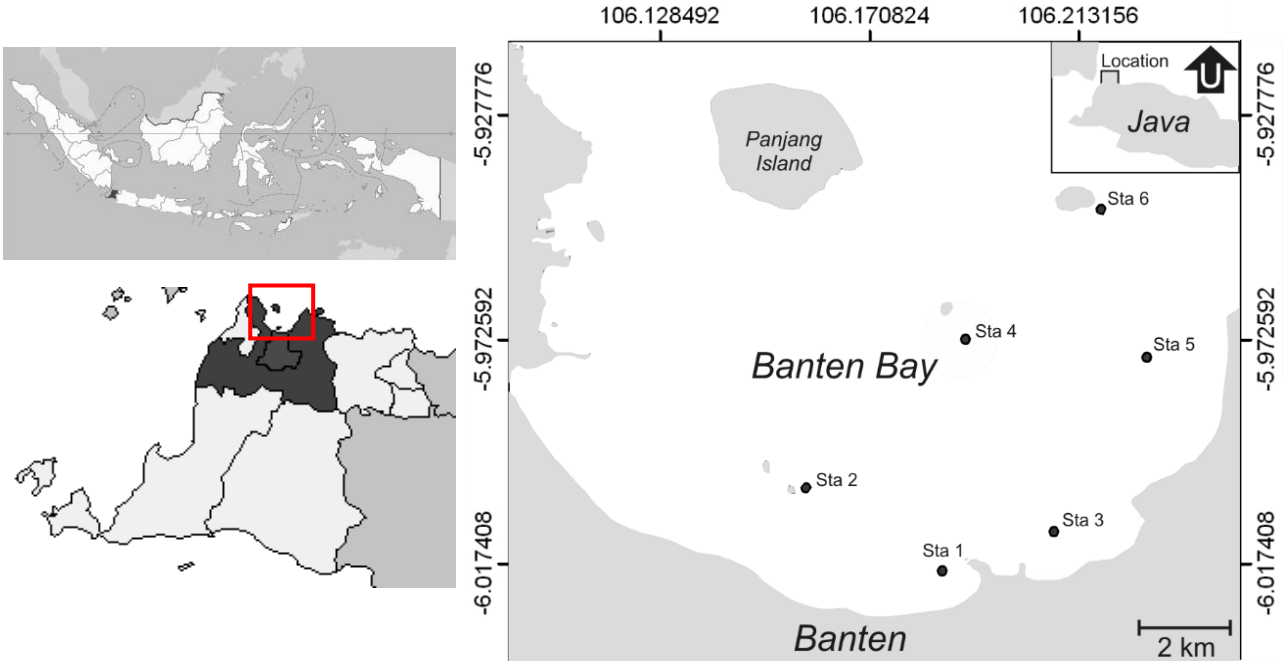

Figure 1. Location of stomatopod larvae sampling stations in Banten Bay, Java Sea, Indonesia. Note: Sta 1: Station 1; Sta 2: Station 2; Sta 3: Station 3; Sta 4: Station 4; Sta 5: Station 5; Sta 6: Station 6.

Electropherograms were evaluated using sequence editing software Chromas Lite 2.1.1 (http://www.technelysium.com.au/chromas.html) and BioEdit v7.0.9 (Hall 1999). The sequences were then compared with those of available database using GenBank BLASTn search (Altschul et al. 1990) and BOLD Identification System tool (BOLD-IDS) (Ratnasingham and Hebert 2007). For species diagnosis, only sequences presenting a minimum of 98% identity with an adult reference sequence were considered to be accurately identified. Multi-sequence alignment of COI sequences was performed using ClustalW (Higgins et al. 1994) on MEGA v6 (Tamura et al. 2013). Neighbor-joining (NJ; Saitou and Nei 1987) trees with bootstrap analysis (1,000 replications) were constructed based on Kimura 2-parameter (Kimura 1980), using MEGA v6 (Tamura et al. 2013). The levels of Kimura 2-parameter sequence divergence were both calculated within clades containing unknown larval samples, as well as between these clades and their nearest sister group using spider package in R programming (Brown et al. 2012).

RESULTS AND DISCUSSION

An approximately 650 base pair (bp) fragment of the mitochondrial cytochrome c oxidase subunit-1 gene (COI) was successfully sequenced from 10 stomatopod morphotypes (n =37) and grouped into five *molecular operational taxonomic units* (MOTU) on the neighbor-joining tree (**Error! Reference source not found.**). All five MOTUs were grouped into one big clade, family Squillidae, and fell outside family Protosquillidae clade. However, only three (60%) were grouped with known reference species. Thus, 45.95% (17 of 37 stomatopod larvae) were successfully classified into species (i.e., *Anchisquilla fasciata* (n = 5; MOTU 1), *Stomatopoda* sp. 1 RWKT-2009 (n = 1; MOTU 3), and *Stomatopoda* sp. PHB-2006 morphotype E (n = 11; MOTU 5).

Kimura 2 parameter differences within and between MOTU were observed within 0% to 3,1%; and between 6 to 20%, respectively (Figure 3, Table 1). Three MOTUs were confidently grouped with reference groups MOTU 1 (morphotype-A and B), MOTU 3 (morphotype-E), and MOTU 5 (morphotype-I and J). This three MOTUs belong to *Anchisquilla fasciata*, *Stomatopoda* sp. 1 RWKT-2009, and *Stomatopoda* sp. PHB-2006 groups respectively. The minimum K2P divergence among clades had its closest sister group of these three MOTUs to be less than 3%, and ranged from 0.3-1.5%. However, the minimum K2P divergence among clades of MOTU 2 and 4 was higher than 3%, and ranged from 4.9-8.8% to its closest sister groups which were *Clorida decorata* and *Stomatopoda* sp. 2 RWKT-2009, respectively.

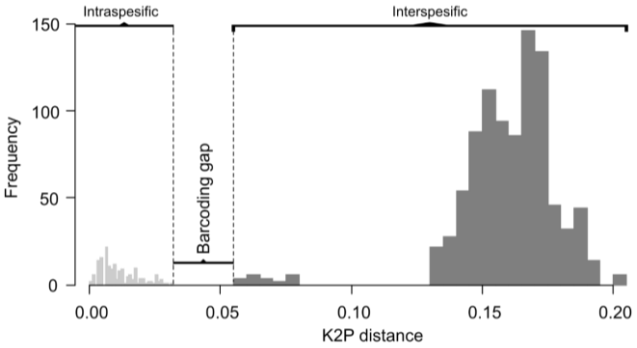

Figure 3. Frequency distributions of genetic variability (K2P distance)

Table 1. Kimura 2-parameter distances within and among clades. MOTUs correspond to distinct larval groups identified in Figure 2. The maximum K2P pairwise sequence divergence is reported within each of these larval MOTUs and the minimum K2P pairwise sequence divergence is reported between each larval MOTU and its nearest sister group.

Morphotypes	MOTUs	Maximum divergence within clades (%)	Minimum divergence among clades (%)	Closest sister group	Minimum divergence among clades (%)	Close sister group
Morphotype-A	MOTU 1*	1.8	0.3	*Anchisquilla fasciata*	14.7	*Clorida decorata*
Morphotype-B					13.4	MOTU2
Morphotype-C	MOTU 2	8.7	8.8	*Clorida decorata*	13.6	*Anchisquilla fasciata*
Morphotype-D					13.4	MOTU1
Morphotype-E	MOTU 3*	n.a.	0.6	*Stomatopoda* sp. 1 RWKT	6.0	MOTU5
					7.3	*Stomatopoda* sp. morphotype E
Morphotype-F	MOTU 4	2.9	4.9	*Stomatopoda* sp. 2 RWKT	14.4	*Harpiosquilla harpax*
Morphotype-G						
Morphotype-H						
Morphotype-I	MOTU 5*	3.0	1.5	*Stomatopoda* sp. morphotype E	5.8	*Stomatopoda* sp. 1 RWKT
Morphotype-J					6.0	MOTU3

Note: * Asterisks indicate larval MOTUs that were successfully identified to species

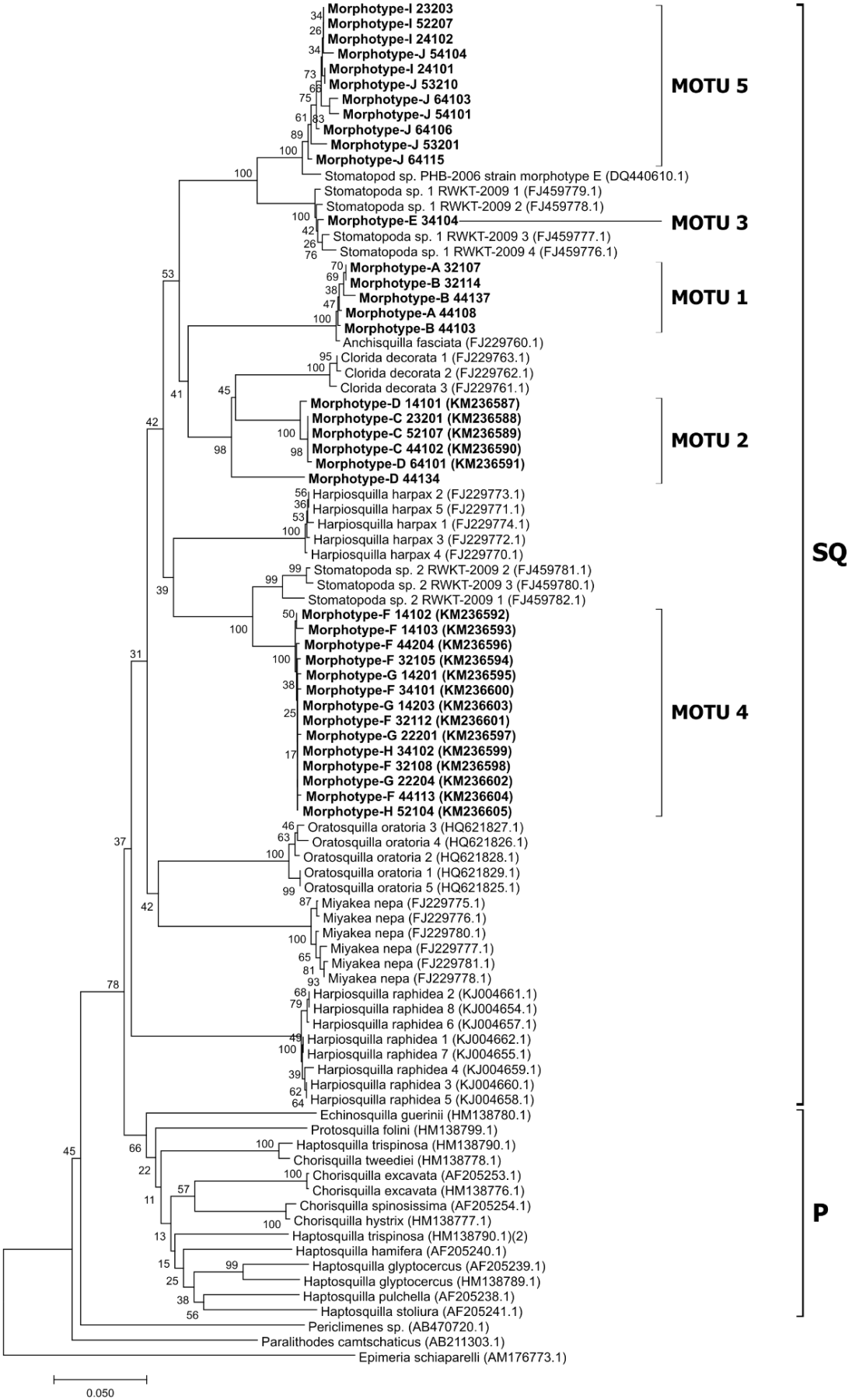

Figure 2. Neighbour-joining (NJ) tree of COI sequences of 37 stomatopod larvae. SQ: Famili Squillidae; P: Famili Protosquillidae.

Discussion

Banten Bay, which is a semi-enclosed inlet in the northern region of Java, Indonesia has various marine ecosystems which support high fish and meroplankton diversity. Stomatopods which are one of meroplankton community in Banten Bay may contribute in maintaining the ocean food chain. Therefore, the existence of stomatopod larvae in Banten Bay is thus important in maintaining the population of pelagic fishes which are the commodity of Banten Bay as marine fishing area. Lack of information on accurate taxonomic identification, which can be a challenging task for the meroplankton, results in limited ecological study on the diversity and distribution of stomatopod larvae in Banten Bay. Larvae of stomatopods can be morphologically identified by linking between information in larval stage and in adult form. Yet, this is difficult applied in the laboratory because of the requirement of condition to rearing larvae collected either from plankton or spawning adults in the laboratory. Molecular methods are the alternative approach to gather accurate taxonomic identification. One of molecular methods is DNA barcoding based on sequence diversity in cytochrome c oxidase subunit 1 (COI).

This study has successfully identified five species of stomatopod in Banten Bay using DNA barcoding. Variation of intraspecies for COI gene based on Kimura-2 parameter were found to be ranged from 0,7% to 2,4%. The COI variation threshold has effectively identified stomatopod larvae in Banten Bay. NMDS ordination and Bray-Curtis cluster showed that the distribution of stomatopod larvae was likely affected by hydrodynamic conditions in Banten Bay. Temperature, salinity and visibility were the main factor affecting larvae abundance in Banten Bay with the score of adjusted R2 94,5% ($P<0,05$). High larvae abundance occurred in Station 4. The result suggested that environmental factors in Station 4 were suitable for stomatopod larvae to grow and there was likely vertical migration of stomatopod larvae affected by environmental factors. Distribution, abundance, and diversity of stomatopods larvae were also affected by sea currents on Banten Bay water which flow to the west of Banten Bay.

This study using COI DNA barcoding was successfully able to identify stomatopod larvae from 3 of the 5 (60%) MOTUs to species level (17 larvae or 45,95%). The accuracy and effectiveness of COI DNA barcoding in the identification process at six stations in Banten Bay were validated by "barcoding gap" between K2P intra- and interspecific variation (Mayer and Paulay 2005). The K2P divergence between intra- and interspecies showed a distinct gap, and was separated by 2.9% differences which ranged from 0-3.1% for intraspecific variation, and 5.9-20.4% for interspecific divergence.

However, only 45.95% of stomatopod larvae can be identified to species level which were grouped in monophyletic clades with known reference species, and the range of minimum K2P variation among clades was less than 3% (Barber and Boyce 2006; Herbert et al. 2003). Meanwhile, MOTU 2 and 4 (n = 20; 54,05%) disputed those criteria, and for that reason, they were considered to be species unambiguously different from reference samples of *Clorida decorata* and *Stomatopoda* sp. 2 RWKT-2009 which is its closest sister group. Barber and Boyce (2006) presumed that when minimum K2P variation values among clades exceed 3%, individuals can be considered to have ambiguous taxonomic identities. These identities may be a result of cryptic species, complexities in species with uncertain number of names or taxonomic status, and widely distributed species with intraspecific geographical variation (Cerutti-Pereyra et al. 2012). The insufficient numbers of taxonomically verified entries on GenBank and BOLD databases can also be the cause of ambiguous identification on DNA barcoding.

This study has shown that the application of DNA barcoding is a valid approach not only for the identification of species, but also to quantify the diversity of stomatopod larvae in Banten Bay, Indonesia. Mulyono (2013) reported the existence of adult *Harpiosquilla harpax*, *Harpiosquilla raphidae*, and *Miyakea nepa* in this area, but the larval stage of these species was not found during this study. This means that, although there was a lack of potential reference species, three species of stomatopod larvae (*Anchisquilla fasciata*, *Stomatopoda* sp. 1 RWKT-2009 and *Stomatopoda* sp. PHB-2006 morphotype E) and 2 unidentified species of stomatopod larvae (MOTU 2 and 4) discovered in this study were not known to settle or recruit in Banten Bay waters. Therefore, the diversity of stomatopod larvae in Banten Bay has been underestimated, even in such an intensively fished area. Further studies are needed to describe the diversity of mantis shrimp larvae by comparing the larvae sequences with that of the adults.

ACKNOWLEDGEMENTS

The authors thank University of Indonesia and Directorate General of Higher Education, the Ministry of Research, Technology and Higher Education, the Government of Indonesia for the financial support through *Proyek Pendidikan Tinggi Penelitian Unggulan Perguruan Tinggi*.

REFERENCES

Ahyong ST. 2002. Stomatopoda (Crustacea) of the KARUBAR Expedition in Indonesia. Zoosystema 24 (2): 373- 384

Ahyong ST, Moosa MK. 2004. Stomatopod Crustacea From Anambas and Natuna Islands. Raffles Bull Zool (supplement) 11: 61-66.

Ahyong ST, Chan TY, Liao YC. 2008. A catalog of the mantis shrimps (Stomatopoda) of Taiwan. National Taiwan Ocean University, Keelung.

Altschul S F, Gish W, Miller W, Myers E W, Lipman D J. 1990. Basic local alignment search tool. J Mol Biol 215:403-410

Barber PH, Boyce SL. 2006. Estimating diversity of Indo-Pacific coral reef stomatopods through DNA barcoding of stomatopod larvae. Proc R Soc London Ser B Biol Sci 273: 2053-2061.

Barber PH, Erdmann MV. 2000. Molecular systematics of the Gonodactylidae (Stomatopoda) using mitochondrial cytochrome oxidase C (Subunit 1) DNA sequence data. J Crustacean Biol 20 (2): 20-36.

Barber PH, Erdmann M V, Palumbi SR. 2006. Comparative phylogeography of three codistributed stomatopods: Origins and timing of regional lineage diversification in the coral triangle. Evolution 60 (9): 1825-1839.

Barber PH, Moosa M K, Palumbi S R. 2002a. Rapid recovery of genetic diversity of stomatopod populations on Krakatau: temporal and spatial scales of marine larval dispersal. Proc R Soc London Ser B Biol Sci 269 (1500): 1591-1597.

Barber P H, Palumbi SR, Erdmann MV, Moosa M K. 2002b. Sharp genetic breaks among populations of *Haptosquilla pulchella* (Stomatopoda) indicate limits to larval transport: patterns, causes, and consequences. Mol Ecol 11: 659-674.

Brown SD, Collins RA, Boyer S, Lefort MC, Malumbres-Olarte J, Vink CJ, Cruickshank RH. 2012. Spider: an R package for the analysis of species identity and evolution, with particular reference to DNA barcoding. Mol Ecol Resour 12 (3): 562-565.

Cerutti-Pereyra F, Meekan MG, Wei NW V, O'Shea O, Bradshaw CJA, Austin CM. 2012. Identification of rays through DNA barcoding: An application for ecologists. PLoS One 7 (6): p.e36479. DOI: 10.1371/journal.pone.0036479.

Diaz G A. 1998. Description of the last seven pelagic larval stages of *Squilla* sp. (Crustacea, Stomatopoda). Bull Mar Sci 62 (3): 753-762.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3 (5): 294-299.

Hebert PDN, Ratnasingham S, deWaard JR. 2003. Barcoding animal life: Cytochrome Coxidase Subunit 1 divergences among closely related species. Proc R Soc London Ser B Biol Sci (Suppl.) 270: S96-S99.

Higgins D, Thompson J, Gibson T, Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673-4680.

Hutomo M, Moosa MK. 2005. Indonesian marine and coastal biodiversity: Present Status. Indian J Mar Sci 34 (1): 88-97.

Kimura M. 1980. A simple method for estimating evolutionary rate of base subtitutiions through comparative studies of nucleotide sequences. J Mol Evol 16: 111-120.

Meyer C P, Paulay G. 2005. DNA barcoding: error rates based on comprehensive sampling. PloS Biol 3 (12): e422. DOI: 10.1371/journal.pbio.0030422.

Moosa MK. 2000. Marine biodiversity of the South China Sea : A checklist of stomatopod crustacea. Raffles Bull Zool 48 (8 Suppl.): 405-457.

Mulyono M, Patria MP, Abinawanto A, Affandi R. 2013. Length-weight relationship and condition factor in giant Harpiosquillid Mantis Shrimp, *Harpiosquilla raphidea* (Crustacea: Stomatopoda) in Banten Bay waters, Indonesia. Intl J Aquat Biol 1 (4): 185-187.

Olavides R. 2011. Crustaceans in Indonesia. In: Palomares MLD, Pauly D (eds.). 2013. Sea Life Base. http://www.sealifebase.fisheries.ubc.ca. 12 March 2013.

Provenzano AJ, Manning RB. 1978. Studies on development of Stomatopod Crustacea II. The later larval stages of *Gonodactylus oerstedii* Hansen Reared In The Laboratory. Bull Mar Sci 28 (2): 297-315.

Pujiawan AANO, Nindhia TS, Mahardika IGNK. 2012. Identifikasi spesies udang mantis (Stomatopoda) di Perairan Pemuteran dengan menggunakan gen Cytochrome C Oxidase Subunit-1 dari DNA. Indonesia Medicus Veterinus 1 (2): 268-280. [Indonesian]

Ratnasingham S, Hebert PD. 2007. BOLD: The Barcode of Life Data System (http://www.barcodinglife.org). Mol Ecol Notes 7 (3): 355-364.

Saitou N, Nei M. 1987. Neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425.

Smith MA, Rodriguez JJ, Whitfield JB, Deans AR, Janzen DH, Hallwachs W, Hebert PDN. 2008. Extreme diversity of tropical parasitoid wasps exposed by iterative integration of natural history, DNA barcoding, morphology, and collections. Proc Natl Acad Sci USA 105 (34): 12359-12364

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA 6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30: 2725-2729.

Tang RWK, Yau C, Chuenng W. 2010. Identification of stomatopod larvae (Crustacea: Stomatopoda) from Hong Kong waters using DNA barcodes. Mol Ecol Res 10: 439-448.

Vences M, Thomas M, van der Meijden A, Chiari Y, Vieites DR. 2005. Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. Front Zool 2 (5): 1-12.

Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PDN. 2005. DNA barcoding Australia's fish species. Phil Trans R Soc Biol Sci 360: 1847-1857.