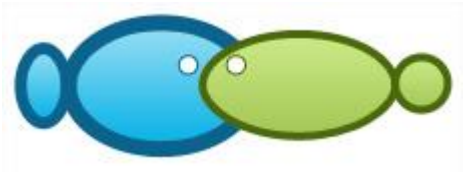

Phylogeny of the spiny lobster *Panulirus versicolor* in Cenderawasih Bay, Papua, Indonesia

[1]Bayu Pranata, [2]Mohamad Fadjar, [3]Feni Iranawati, [4]Abdul H. Toha, [5]Jeni

[1] Graduate School of Fisheries and Marine Sciences, University of Brawijaya, Malang, Indonesia; [2] Department of Aquaculture, Faculty of Fisheries and Marine Science, University of Brawijaya, Malang, Indonesia; [3] Department of Marine Sciences, Faculty of Fisheries and Marine Science, University of Brawijaya, Malang, Indonesia; [4] Fisheries Department, University of Papua, Manokwari, Indonesia; [5] Biology Department, University of Papua, Manokwari, Indonesia. Corresponding author: B. Pranata, pr4n4t4b4yu05@gmail.com

Abstract. The aim of our study was to identify the genetic and phylogenetic characteristics of spiny lobster *Panulirus versicolor* in Cendrawasih Bay, Indonesia and their relationship with *P. versicolor* lobsters elsewhere in several Pacific and Indian Oceans domains based on the cytochrome oxidase I (COI) gene. We collected tissue samples from five *P. versicolor* individuals in Cenderawasih Bay. We detected that there were 5 haplotypes with a diversity value of haplotype (Hd) and nucletides (Pi) respectively Hd = 1.000 and nucleotides Pi = 0.00841. Our data show that some *P. versicolor* individuals from Cenderawasih Bay were closely related to *P. versicolor* lobsters in other regions of the Indian Ocean and the western Pacific Ocean. We observed the *P. versicolor* of Cenderawsih Bay form a monophyletic clade with *P. versicolor* in other part of the Indian Ocean and the western Pacific Ocean based on the reconstruction of phylogenetic trees. As well as the haplotype distribution showed no sample area genetically isolated from the others.
Key Words: *P. versicolor*, COI, diversity, Indian Ocean, genetic isolation.

Introduction. The spiny lobster (*Panulirus versicolor*) is one of the six species of lobster captured in Indonesian waters (Tewfik et al 2009). In Cenderawasih Bay, *P. versicolor* is found abundantly and become an important commodity in the region. The catching of *P. versicolor* in Cenderawasih Bay is conducted intensively. *P. versicolor* is a tropical species that has a complex life cycle with a long and planktonic pelagic phase.

The initial history of the *Panulirus* lobster consists of a period of deep larvae drift in a relatively long time in the open sea which lasts from several months to over a year, with many possibilities to spread through the ocean currents (Tolley et al 2005). Lobster *P. versicolor* hatches phyllosoma larvae (about 1-2 mm long) (Phillips et al 2006). Phyllosoma larvae phase is estimated to last for 6-7 months before morphed into puerulus (Kaillis 2006). The long larval phase causes this species to have a widespread distribution of the area of origin and allow the supply of stocks in the new area (Abdullah et al 2013). A widespread spread of *P. versicolor* lobster phyllosoma larvae allows for gene flow through outbreeding between populations.

Geographically, Cenderawasih Bay is directly affected by oceanographic processes from the Pacific Ocean. The current pattern in the Pacific Ocean allows for the wide spread of lobster phyllosoma larvae between regions. Current tends to create barrier and direction from the spread of lobster phyllosoma larvae (Riginos et al 2011). Therefore, it is important to identify the genetic and phylogenetic characteristics of *P. versicolor* lobster in Cendrawasih Bay and its relationship with *P. versicolor* lobsters from other regions of the Indian and Pacific Oceans. Phylogenetic knowledge is an important prerequisite for understanding the evolution, adaptation, morphology, ecology, and behavior of species (Suresh et al 2012). In addition, it is important for species conservation and the development of marine protected areas.

DNA-based identification techniques have been successfully used to investigate genetic diversity (Thorpe et al 2000), phylogenetics and spatial connectivity between subpopulations and *P. versicolor* lobster populations. One of the molecular markers routinely used in genetic studies is mitochondrial DNA (mtDNA) (Thorpe et al 2000); mtDNA is a contemporary method which is popularly used (Silva & Russo 2000). In this study, the identification of genetic and phylogenetic diversity was performed using gene DNA cytochrome oxidase c subunit I (COI) which is a protein region coding of the mitochondrial genome (Matzen da Silva et al 2011). The COI gene is informative on various levels of taxonomy and has been widely used at the species and population level (Matzen da Silva et al 2011). Several studies on the genetics of the genus *Panulirus* based on COI genetic markers have been performed by Ptacek et al (2001), Ravago & Juinio-Menez (2003), Chow et al (2005), Crivello et al (2005), Li et al (2011), Chow et al (2011), Abdullah et al (2013), Babbucci et al (2010), Sekiguchi & Inoue (2010), Jeena et al (2011), Senevirathna & Munasinghe (2013), Iacchei et al (2014), and Samadi et al (2015). The purpose of this study was to identify the genetic and phylogenetic characteristics of *P. versicolor* lobsters in Cendrawasih Bay and its relation to *P. versicolor* lobsters from other regions of the Indian and Pacific Ocean based on several previous studies.

Material and Method

Sample collection. The research was conducted in Cenderawasih Bay, West Papua Province, Indonesia (Figure). Periopod tissue (leg muscle tissue) from five *P. vesicolor* individuals were sampled and preserved in 95% alcohol. 11 *P. versicolor* lobster sequence of some areas in the Indian Ocean and the Pacific downloaded from GenBank with accession numbers that can be seen in Table 1.

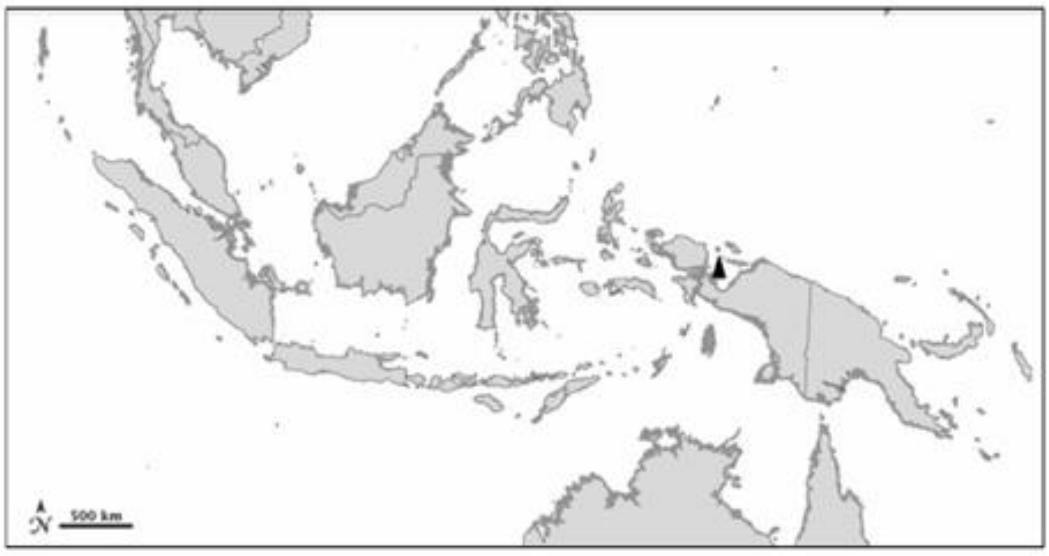

Figure 1. The Location of Cenderawasih Bay (black triangle) in West Papua Province, Indonesia (Toha et al 2016).

Table 1

DNA sequence of *Panulirus versicolor* from Genbank

Location		*Accession number*	*Reference*
Sri Lanka	SL	KF548586	Senevirathna & Munasinghe (2013)
Sri Lanka	SL	KF548585	Senevirathna & Munasinghe (2013)
Sri Lanka	SL	KF548584	Senevirathna & Munasinghe (2013)
Sri Lanka	SL	KF548583	Senevirathna & Munasinghe (2013)
China	Chi	JN591366	Li et al (2011)
India	In	JQ229882	Jeena et al (2011)
Ryukyu Japan	RJ	AB244283	Chow et al (2005)
Persian Gulf and Oman Sea	PG	KT001513	Samadi et al (2015)
Persian Gulf and Oman Sea	PG	KT001512	Samadi et al (2015)
South Africa (South-west Indian Ocean)	Af	KX275386	Singh et al (2016)
Palau Island (North Pacific Ocean)	PI	AF339472	Ptacek et al (2001)

DNA extraction, isolation, and amplification. Extraction of genomic DNA from all samples was performed using the KIT method: Genomic DNA Mini Kit Animal Tissue (GENE AID). Amplification of mitochondrial cytochrome c oxidase subunit I gene (COI) was performed using primary LCO1490: 5'-ggtcaacaaatcataaagatattgg-3 'and HCO2198: 5'-taaacttcagggtgaccaaaaaatca-3' (Folmer et al 1994). Making of PCR master mix was performed by adding ddH2O 14 µL, LCOI and HCO2 primers 2.5 µL each, DMSO 1 µL, Go Taq Green 25 µL and 5 µL DNA extract. Amplification was done at the final volume of 50 µL. The PCR process includes pre-denaturation at 94°C for 3 minutes for 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and extension stage at 72°C for 45 seconds. The PCR result was then performed by electrophoresis process using 1% agarose gel with 50 mLTris Borate EDTA (TBE).

Data analysis. Bi-directional sequencing was done by First Base CO (Malaysia) using Big Dye © terminator chemistry (Perkin Elmer). Online Identification of species uses available data at National Center for Biotechnology Information (NCBI) with the Basic Local Alignment Search Tool (BLAST) method. The sequenced DNA was aligned using MEGA 6.06 software (Tamura et al 2011). Analysis of nucleotide diversity (π), haplotype (h), polymorphic DNA using DnaSP 5.1 (Rozas et al 2003) was performed. We compared the results of the Cenderawasih Bay with the available data in the Genbank for the Indian Ocean and Pacific Ocean with the Maximum Likelihood Method (Lemey et al 2009), Kimura-2 model parameters and bootstrap test 1000 × using MEGA 6.06 (Tamura et al 2011). The reconstruction of haplotype network uses Network 5.0.

Results and Discussion

Genetic characteristics. The length of the amplified fragments of *P. versicolor* lobster COI gene from Cenderawasih Bay using primer LCO1490 and HCO2198 is 750 bp (base pairs) (Figure 2). The primary use of LCO1490 and HCO2198 is based on the study of Folmer et al (1994), which describes DNA primers for polymerase chain reaction (PCR) COI gene from 11 invertebrate phyla. The results show that primer pairs HCO2198 and LCO1490 consistently reinforce the fragment 710 bp CO I throughout the invertebrate series which produce an informative sequence for phylogenetic analysis of species and higher taxonomic level (Folmer et al 1994). Several studies use the same primers as shown by Senevirathna & Munasinghe (2014), Ptacek et al (2001), Inoue et al (2007). The length of the COI gene fragments shows different results even in usage of the same primers. Senevirathna & Munasinghe (2014), find that the length of COI gene fragments from 27 samples of *Panulirus homarus* lobsters are 658 bp and 650 bp length of COI

gene fragment from 22 taxa of genus *Panulirus* (Ptacek et al 2001). The differences in the length of the amplified DNA fragments are due to the primary primer used, primer base composition, primer length size, DNA quality found, food, ancestry and environment (Shizuka & Lyon 2008).

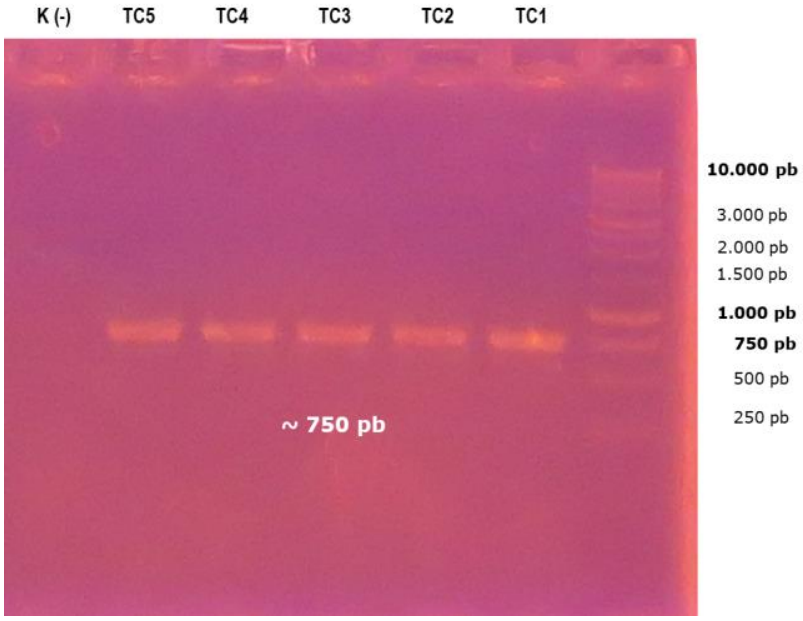

Figure 2. Electrophoresis results of Lobster *Panulirus versicolor*. K (-) = control.

All *P. versicolor* samples from Cenderawasih Bay were identified in GenBank, using the BLAST method. The samples were identified as *P. versicolor* with Query Cover value of 100%, E-value of 0.0 and Identity Value of 99-100% (Table 2). Based on the results of BLAST analysis, it can be concluded that *P. versicolor* DNA sequences have a high degree of similarity to the DNA sequences available in Genbank. According to Claverie & Notredame (2003), if the value of E-value <0.4 then the DNA sequence has a similarity or high homology.

Table 2

Nucleotide sequence identifying through BLAST analysis

Sample code	*Species outcome*	*BLAST*			
		Access code of NCBI	*Query cover (%)*	*E-value*	*Identity value (%)*
TC1	*P. versicolor*	*gi|564282695|KF548586.1*	100	0.0	100
TC2	*P. versicolor*	*gi|949175589|KT001513.1*	100	0.0	99
TC3	*P. versicolor*	*gi|949175589|KT001513.1*	100	0.0	100
TC4	*P. versicolor*	*gi|564282691|KF548584.1*	100	0.0	99
TC5	*P. versicolor*	*gi|1177257049|KX275386.1*	100	0.0	99

Genetic diversity. The analysis showed five individuals of *P. versicolor* lobster to have a diversity haplotype (Hd) = 1.000 (variance = 0.01600; sd = 0.126) and nucleotides (Pi) 0.00841. There are twelve nucleotide mutations and twelve identified polymorphic sites. The average number of nucleotide differences, k: 4,800. There are 2 categories of haplotype diversity values (Hd) which are ≥0 and <0.5 in the low category, while >0.5 and ≤1 are in the high category (Hobbs et al 2013). In addition, according to Nei (1987), haplotype diversity (Hd) 0.1-0.4 is considered as low category, (Hd) 0.5-0.7 medium category and (Hd) 0.8-2.00 high category. Based on these categories, the diversity of the *P. versicolor* haplotype in Cendrawasih Bay is categorized as high.

The mean nucleotide composition of the control areas is 27.71% adenine, 31.31%, thymine, 23.33% cytosine and guanine 17.65%. This result is consistent with

several previous studies reporting on the control areas of the mitochondrial genome full of adenin and timine in many invertebrates, including crustaceans (Diniz et al 2005; Ptacek et al Abdullah et al 2013; Abdullah et al 2014). The ratio of transition/transversion rate is k_1 = 1000 (purines) and k_2 = 672.16 (pyrimidines). Maximum Composite Likelihood Estimate of the pattern of nucleotide transitional substitution (A-G 21.45; G-A 33.67; T-C 19.06 and C-T 25.58) and transversional substitutions (A-T 0.04; T-A 0.03, A-C; C-A 0.03, T-G 0.02; G-T 0.04; C-G 0.02; G-C 0.03). The analysis involved five sequences of nucleotides from Cenderawasih Bay. The results show that transition substitution is higher than in transversion substitution. It is generally assumed that the transition rate to transversions is higher in the animal genome, probably as a result of the underlying mutation chemistry (Suresh et al 2012).

Phylogeny and relatedness. We assess the phylogenetic relationship of *P. versicolor* using a variety of nucleotide sequences in the specific area of the mitochondrial genome (mtDNA) gene (COI) with Maximum Likelihood method. The result of the phylogenetic tree reconstruction of *P. versicolor* shows tree topology structure divided into clause I (a subclade), II and III. Clade I forms a monophyletic clade to be categorized in a large group called Tarsier Tarsier-Complex. Clade II forms a monophyletic clade in small groups. Clade III consists of 1 individual forming a monophyletic clade. Our research shows that *P. versicolor* of Cenderawasih Bay is monophyletic with some individuals from Palau Island (North Pacific Ocean), Ryukyu Japan, China, Sri Lanka, Persian Gulf and Oman Sea, India, South Africa (South-west Indian Ocean). They are all mutually related to each other, this is supported by paired distance analysis and phylogenetic tree reconstruction using Maximum Likelihood (Figure 3).

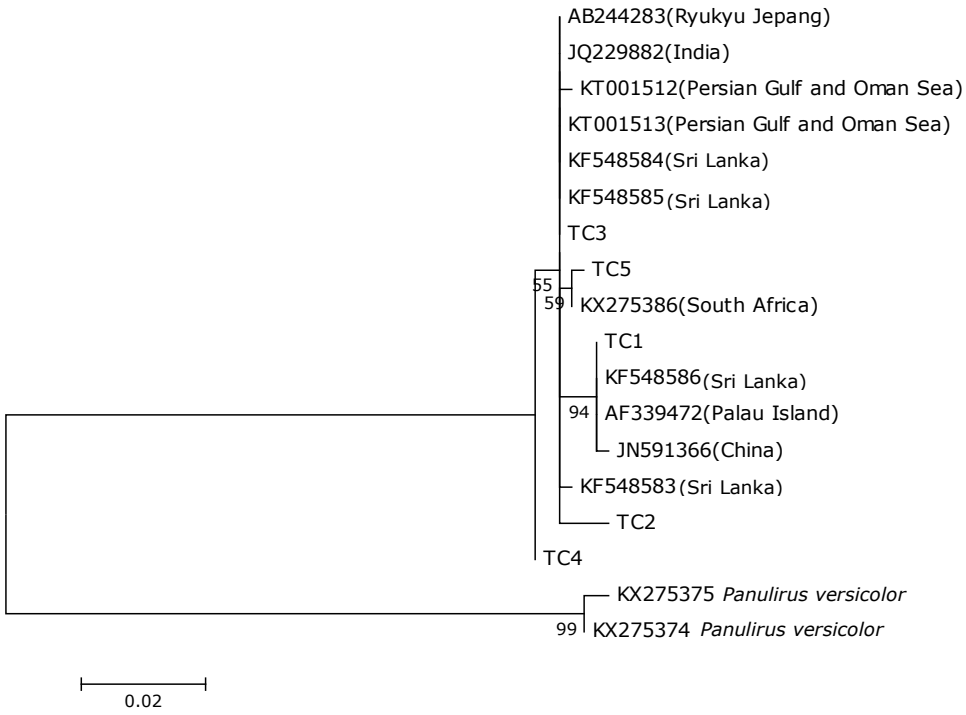

Figure 3. The reconstructed phylogenetic tree of *Panulirus versicolor* from Cenderawasih Bay and Several Regions in the Indian and Pacific Oceans.

The current pattern in the Pacific Ocean allows the widespread of lobster phyllosoma larvae between regions. Our results shows that some individuals of *P. versicolor* from Cendrawasih Bay are closely related to some individuals of *P. versicolor* from Sri Lanka, the Persian Gulf and Oman Sea, Ryukyu Japan and India (P-distance 0.000), and *P. versicolor* from Sri Lanka and Palau Island (North Pacific Ocean) (P-distance 0.000) (Table 3).

Table 3

Pairwise distance analysis of *Panulirus versicolor*

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*
TC1[1]																
TC2[1]	0.014															
TC3[1]	0.006	0.008														
TC4[1]	0.010	0.012	0.004													
TC5[1]	0.010	0.012	0.004	0.008												
KF548586[2]	0.000	0.014	0.006	0.010	0.010											
KF548585[2]	0.006	0.008	0.000	0.004	0.004	0.006										
KF548584[2]	0.006	0.008	0.000	0.004	0.004	0.006	0.000									
KF548583[2]	0.008	0.010	0.002	0.006	0.006	0.008	0.002	0.002								
KT001513[3]	0.006	0.008	0.000	0.004	0.004	0.006	0.000	0.000	0.002							
KT001512[3]	0.008	0.010	0.002	0.006	0.006	0.008	0.002	0.002	0.004	0.002						
AB244283[4]	0.006	0.008	0.000	0.004	0.004	0.006	0.000	0.000	0.002	0.000	0.002					
AF339472[5]	0.000	0.014	0.006	0.010	0.010	0.000	0.006	0.006	0.008	0.006	0.008	0.006				
JQ229882[6]	0.006	0.008	0.000	0.004	0.004	0.006	0.000	0.000	0.002	0.000	0.002	0.000	0.006			
JN591366[7]	0.002	0.016	0.008	0.012	0.012	0.002	0.008	0.008	0.010	0.008	0.010	0.008	0.002	0.008		
KX275386[8]	0.008	0.010	0.002	0.006	0.002	0.008	0.002	0.002	0.004	0.002	0.004	0.002	0.008	0.002	0.010	

Notes: Cenderawasih Bay (TC)[1]; SL[2]; PG[3]; RJ[4]; PI[5]; In[6]; Chi[7]; Af[8].

The reconstruction of a haplotype network with Network 5.0 shows that there is 9 haplotype with the highest frequency of 6 individuals. One haplotype consists of 3 individuals, while the other haplotype consists of 1 individual respectively (Table 4). The distribution of the haplotypes of *P. versicolor* in the Cenderawasih Bay and the region around the Indian and Pacific Oceans indicates that everything is closely related in a relative way and no sample area is genetically isolated one from other (Figure 4). Similar results were reported by Inoue et al (2007), that there is no genetic difference between the *P. japonicus* lobster populations in Japan.

Table 4

Distribution of *Panulirus versicolor* haplotype

Haplotype	
Type	*Code (ind.)*
Hap_1 (6 ind)	AB244283;KF548585;KF548584;KT001513;JQ229882;TC3
Hap_2 (3 ind)	KF548586;AF339472;TC1
Hap_3 (1 ind)	KF548583
Hap_4 (1 ind)	KX275386
Hap_5 (1 ind)	KT001512
Hap_6 (1 ind)	JN591366
Hap_7 (1 ind)	TC2
Hap_8 (1 ind)	TC4
Hap_9 (1 ind)	TC5

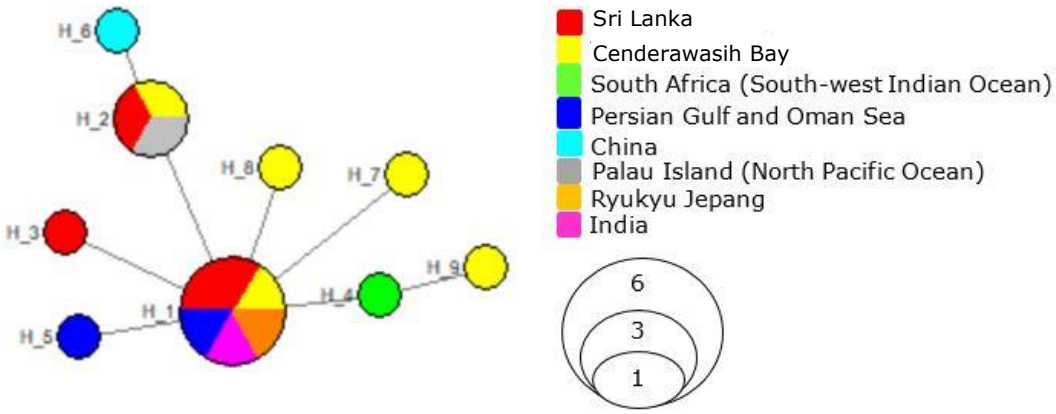

Figure 4. The haplotype network of *Panulirus versicolor*.

The genus *Panulirus* is widely spread, which is supported by long and plantonic larval period (Rogers & Harpending 1992). The phyllosoma larvae period is estimated to last for 6 months (Chow et al 2011). Abdullah et al (2013) explain that the *Panulirus* lobster is widely spread from the origin region and allows for a stock to occur in new areas. In addition, Palero et al (2008) also explain that long periods of planktonic larvae such as phyllosoma larvae for crustaceans can be found in the wider geographical districts.

On the other hand, the distribution patterns of phyllosoma larvae are strongly influenced by physical factors of the waters and geographical formations. Bradbury et al (2008) explain that an important factor affecting the transportation and mixing of larvae in waters is the force of water movement and the length of the larval period. The current tends to create barrier and direction from the spread of lobster phyllosoma larvae (Riginos et al 2011; Abdullah et al 2014). In addition, the pattern distribution of the larvae of lobster phyllosoma in a sea form is less open demographically. Furthermore, they are geographically isolated due to the presence of the barrier such as geographic

distance. As shown by Kennington et al (2006), that the barrier has caused a significant difference in allele frequencies between different locations.

Conclusions. Several individuals of *P. versicolor* from Cenderawasih Bay are closely related to individuals from several regions of the Indian and Pacific Oceans. This is supported by the P-distance value of 0.000-0.014 and the phylogenetic tree topology which indicates a monophyletic clade. As well as the haplotype distribution which showed that sample areas genetically are not isolated one from other.

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Bayu Pranata, University of Brawijaya, Graduate School of Fisheries and Marine Sciences, Indonesia, Malang, 65145, e-mail: pr4n4t4b4yu05@gmail.com
Mohamad Fadjar, University of Brawijaya, Faculty of Fisheries and Marine Science, Department of Aquaculture, Indonesia, Malang, 65145, e-mail: f4dj4r_02@ub.ac.id
Feni Iranawati, University of Brawijaya, Faculty of Fisheries and Marine Science, Department of Marine Science, Indonesia, Malang, 65145, e-mail: dzimi2012@gmail.com; fe.iranawati@yahoo.com
Abdul Hamid Toha, University of Papua, Fisheries Department, Indonesia, Papua Barat, Manokwari, 98314, Jl. Gunung Salju Amban, e-mail: hamid.toha@gmail.com
Jeni, University of Papua, Biology Department, Indonesia, Papua Barat, Manokwari, 98314, Jl. Gunung Salju Amban, e-mail: jenisaja@gmail.com

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