

Meta-analysis of Indonesian *Octopus laqueus*; Kaneko and Kubodera 2005 (Cephalopoda: Octopodidae) using Mt-DNA COI as Genetic Marker

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Abstract

Octopus laqueus was first discovered not long ago in 2005 in the Ryuku Islands, Japan. Its geographical distribution and molecular identification are therefore still rarely. Nucleotide sequences based on mt-DNA COI for *O. laqueus* that have been uploaded in the GenBank until before this study was carried out were only six sequences. Since DNA barcoding of mt-DNA COI has some advantageous characteristics, this study aimed to analyse the genetic difference of Indonesian *O. laqueus* to the data available in the GenBank. Samples were collected in 2019 - 2020 from Karimunjawa (n=16) and Bangka-Belitung (n=2). The mt-DNA COI was extracted using 10% chelex methods, PCR amplified using Folmer's primer and sequenced in Sanger methods. Pairwise alignment and genetic distance were carried out in MEGA-X, whereas the phylogenetic tree was reconstructed using Bayesian methods. BLAST identification resulted in 685 bp with a range of 92,07-99,24 percentages of identity. The genetic mean pair-wise distances within-clade were 0,002 and 0,006, whilst the distance between the clade was 0.0883. Combining the suggestion with the ITF current, it is concluded that *O. laqueus* taken from Karimunjawa raised from the same species as those in Malaysia (MN711655) and Japan (AB302176). Specimens from Bangka-Belitung were suggested came from different species, as they were separated into the second clade by 8.83%. One single sample from Japan (AB430543) which laid outside the two clades by 11.63%-11.38% was also suggested to represent a different species. Overall, this study opens to various further studies on *O. laqueus* using other loci of genetic markers.

Keywords: *Octopus laqueus*, mt-DNA COI, meta-analysis, Indonesia, Malaysia, Japan

INTRODUCTION

As one of the newly discovered macrobenthic species, *O. laqueus* is a tropical organism that lives on sand and coral reef substrates of shallow waters of 0 to 18 m, the size may reach up to 48 mm mantle length. Commonly found scattered in Okinawa Island, Japan and northern Great Barrier Reef, Australia (Kaneko and Kubodera, 2005), it is confirmed by Ikeda (2009) that this nocturnal species is widespread in the Indo-Pacific (Ikeda, 2009). Research on *O. laqueus* has not been done much when compared to its congeneric. Data on nucleotide sequences that have been collected in GenBank (ncbi.nlm.nih.gov) for *O. laqueus* until before

this study was carried out only 6 sequences consisting of 3 sequences of Cytochrom Oxidase sub-unit I (COI) locus (Kaneko *et al.*, 2011; Rosli *et al.*, Unpublished; Fukui and Furuya, unpublished), 1 sequence of COIII locus (Kaneko *et al.*, 2011), 1 sequence of 12S ribosomal RNA locus (Fukui and Furuya, unpublished) and 1 sequence 18S ribosomal RNA locus (Fukui and Furuya, unpublished). Sample data for other species such as *O. vulgaris* has reached 33,671 sequences, *O. cyanea* reaches 298 sequences and *O. aegina* has 65 sequences.

Mitochondrial DNA (mt-DNA) is a widely used marker in genetic studies since it possesses several advantageous

characteristics and present in high numbers in the cell, also thereby easy to isolate (Zhang and Hewitt, 1996; Kaneko *et al.*, 2011). Aside of it, DNA barcoding of mt-DNA COI is useful for species identification and phylogeographic studies within a single species (Hui *et al.*, 2016). This study therefore, aimed to analyse Indonesian *O. laqueus* using mt-DNA COI, compare it with available data from the GenBank and register its presence geographically in Indonesia as part of the Indo-Pacific malacological region.

MATERIALS AND METHODS

This study using samples of *O. laqueus* collected from 2019 to 2020 by local fishermen from Karimunjawa (n=16) and Bangka-Belitung (n=2) Indonesia (Figure 1). The fishing ground location was confirmed by the local fishermen to ensure that samples were taken within the area of Karimunjawa and Bangka-Belitung. Samples in the form of tentacles tip were preserved in 96% ethanol, sent to the laboratory, renewed with fresh ethanol upon arrival and stored at 4 °C.

The mt-DNA COI was extracted using 10% chelex (Walsh *et al.*, 1991). The universal primer (forward) LCO1490: 5'- GGT CAA CAA ATC ATA AAG ATA TTG G -3' and (reverse) HCO2198: 3'- TAA ACT TCA GGG TGA CCA AAA AAT CA -5' describe by Folmer *et*

al. (1994) were used for the amplification of the mt-DNA COI fragment. PCR amplification was performed in 25 µl reaction mixture containing 1 µl DNA template, 0.5 µl of each primer at 10 mM, 12.5 µl of Bioline master mix and 10.5 µl ddH₂O. The following temperature profile was used: denaturation 94 °C for 5 min, 35 cycles of denaturation 94 °C for 1 min, annealing 56 °C for 1 min, extension 72 °C for 1.5 min and final extension was conducted at 72 °C for 1.5 min (Kholilah *et al.*, 2021). PCR products were visualised using 1% agarose gel stained with Biotium® gel red stain. The successfully PCR products were sent to a DNA Sequencing facility to get sequenced using Sanger methods.

The sequences were edited using MEGAX (Kumar *et al.*, 2018) i.e. correct representation of nucleotides was checked based on the chromatogram peaks. Species identification was performed using the identification system for mt-DNA COI from NCBI (www.ncbi.nlm.nih.gov) with BLAST (Basic Local Alignment Search Tools). The identified samples were then compared with another samples of *O. laqueus* were retrieved from GenBank (BOLD Systems, <http://v3.boldsystems.org/>), i.e., from Japan (n=2, accession no. AB302176 and AB430543, Kaneko *et al.*, 2011) and Malaysia (n=1, accession no. MN711655, Rosli *et al.*, unpublsh).

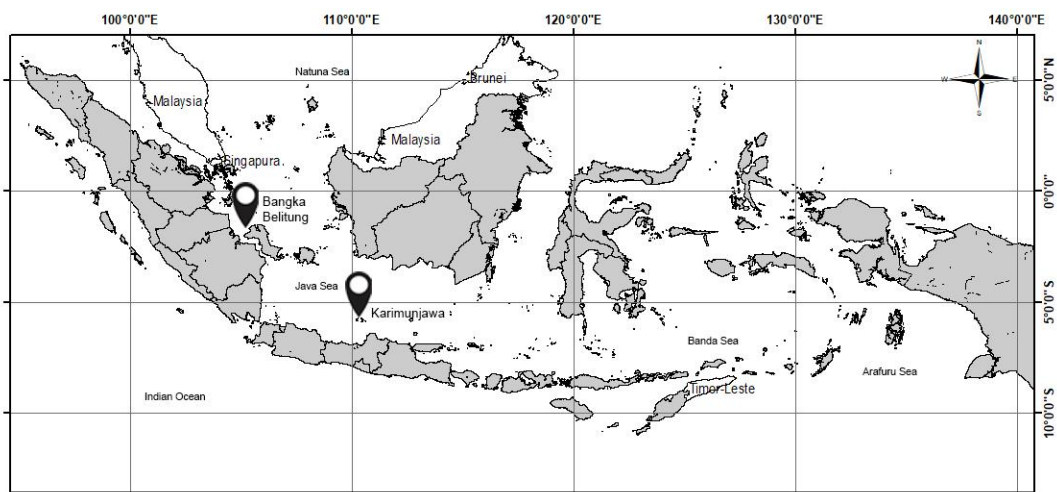


Figure 1. Sampling location of *O. laqueus* in Karimunjawa and Bangka-Belitung, Indonesia waters (Kholilah *et al.*, 2021)

Multiple and pairwise alignments was carried out using Muscle in the software MEGAX (Kumar *et al.*, 2018). The phylogenetic

tree was reconstructed using Bayesian method with BEAST2 package programme (Drummond *et al.*, 2007). The best fit model of

molecular evolution for each portioning data was determined using jModeltest program (Darriba *et al.*, 2012) and HKY (Hasegawa Kishino & Yano) was selected as the best model. The Markov Chain Monte Carlo (MCMC) analysis with a random starting tree was run for 10 million generations. The genetic pairwise distance also calculated using MEGAX (Kumar *et al.*, 2018).

RESULTS AND DISCUSSION

Samples from Karimunjawa (n= 16) and Bangka-Belitung (n= 2) were obtained from local fishermen using a spear-gun looking for octopus as fishing bait (Table 1). This type of octopus is not an export commodity, so it is not caught in large quantities. All samples can be well amplified (Figure 2). The resulting band be in the range of 700 bp, which is in accordance with the study of Carlini & Graves (1999) targeting the mt-DNA COI locus carried out in the Cephalopod class.

Each sample of *O. laqueus* from Karimunjawa (n= 16) and Bangka-Belitung (n= 2) resulted in 685 bp sequences. BLAST identification revealed a range of 92,07- 99,24 percentages of identity (Table 1). Of all, 16 specimens in Table 1 have been registered on GenBank with accession number LC552307-LC552342 and verified as *O. laqueus*, whilst the last two specimens from Bangka-Belitung is yet with accession number (MW559642 and MW559643). Leray *et al.* (2013) stated that the

minimum percentage of identity needed to declare species similarity is 97 %, in other words 3% dissimilarity is a different species. In this case, samples from Bangka-Belitung performed a similarity below 97%, suggesting that they were a different species. However, these low percentages for Bangka-Belitung specimens probably happened due to the limited comparison sequences available at the GenBank.

Research by Hwang *et al.* (2016), which was carried out using morphological and phylogenetic examinations on well-known cephalopods in Southeast Asia including Thailand, Indonesia, Vietnam and China have been identified three orders, four families and nine genera. Out of those, for the Octopoda order they only found out two species, i.e., *O. vulgaris* and *O. minor*. Although *O. laqueus* was previously reported by Kaneko and Kubodera (2005) only in Japan and Australia, it turns out that this species is also found in Malaysia (Rosli *et al.*, Unpublished) as well as in Indonesia, to be precise in Karimunjawa and Bangka-Belitung.

The phylogenetic tree *O. laqueus* produced 2 clades with the first clade being the samples originated from Bangka-Belitung and the second clade being those from Karimunjawa, Japan and Malaysia. Meanwhile, one single sample which was also from Japan laid separately from the two formed clades (Figure 3).

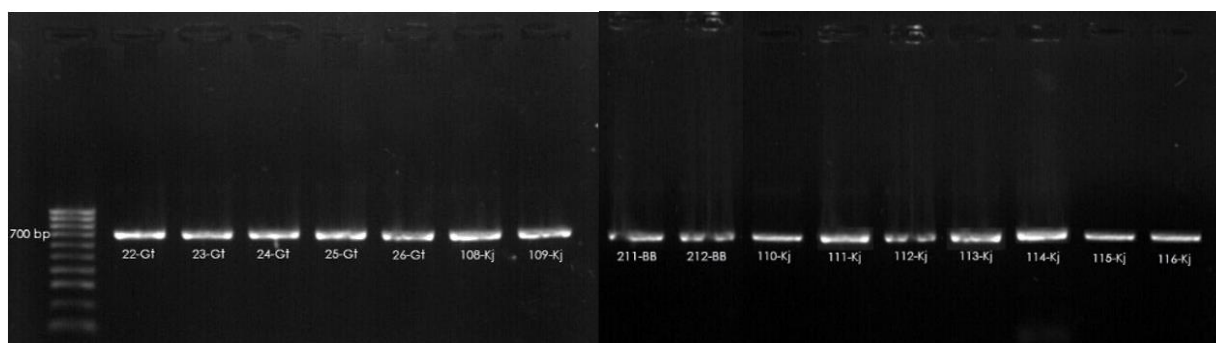


Figure 2. Electrophoresis of mt-DNA COI of *O. laqueus* from Karimunjawa and Bangka-Belitung, Indones

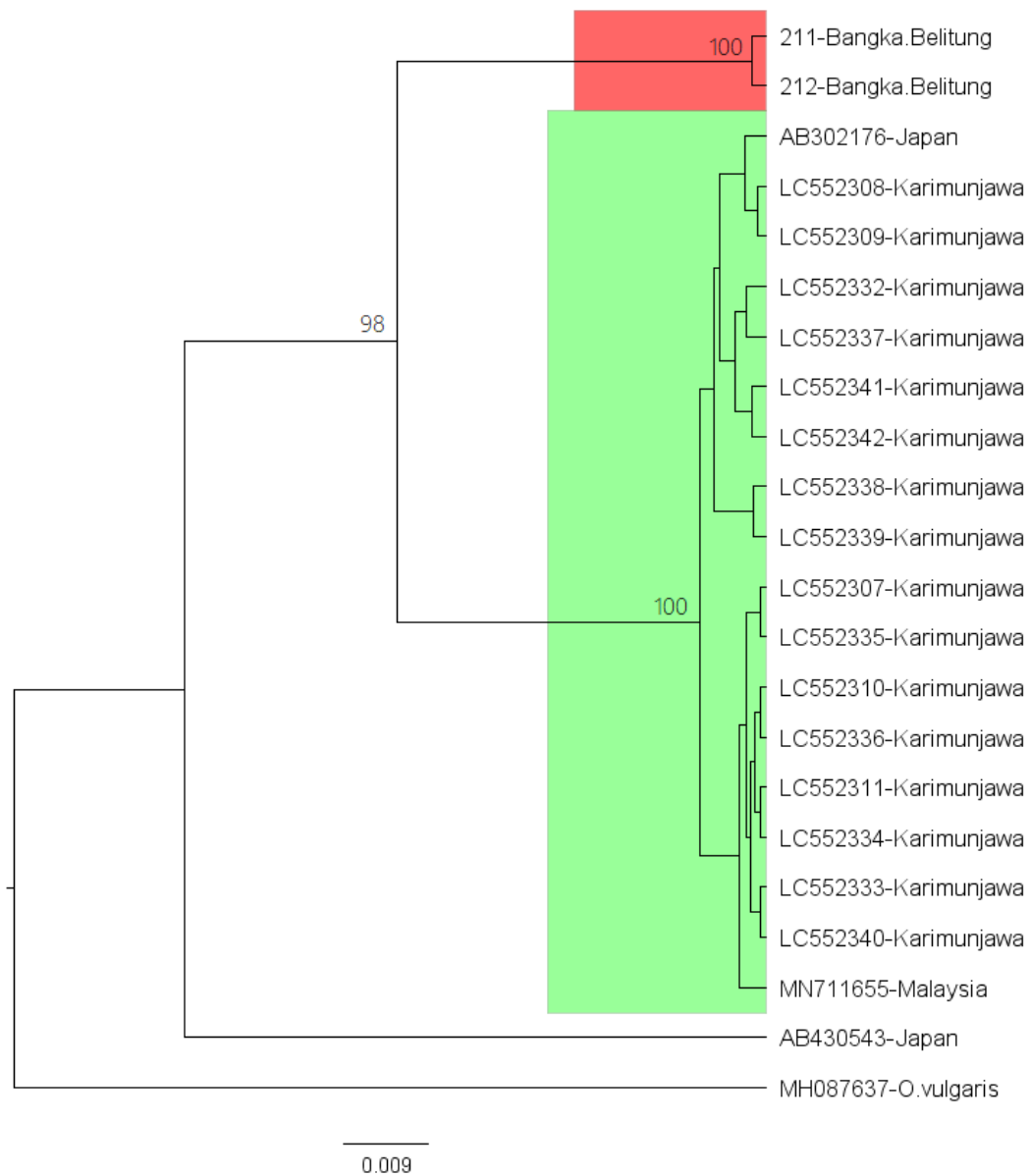


Figure 3. Phylogenetic tree of *O. laqueus* from Karimunjawa and Bangka-Belitung, Indonesia in compilation with *O. laqueus* from the GenBank (Japan and Malaysia), analysed using Bayesian methods and *O. vulgaris* as the outgroup.

The distribution of *O. laqueus* populations may be influenced by geography and oceanography. Using a map provided in the work of Susanto & Song (2015) (Figure 4), the Indonesian seas, with their complex coastline geometry and narrow passages, provide the only pathway for low latitude Pacific water to flow into the Indian Ocean, this is known as the Indonesian ThroughFlow (ITF). ITF may cause *O. laqueus* larvae to be distributed by the current flow from Japanese waters to the Indonesian waters. This was also

suggested to the larvae of *O. cyanea* which were planktonic and have been carried away in the water column for 1-2 months before settling (Reberinary & Benbow, 2012). This causes very wide geographical distribution, reaching hundreds of kilometers with the help of currents (Murphy *et al.*, 2002; Casu *et al.*, 2002). Moreover, specimens from Bangka-Belitung made up the second clade suggesting that they were originated from different species. It is however difficult to simply explain that although Bangka-Belitung laid

within an area that was passed by the main ITF (Figure 3), they developed a different population, or even a different species. Furthermore, a single sample with accession number AB430543 originating from Japan is also separated from the two clades, confirming that it may be a completely different species. Veron *et al.* (2009) stated that the main barrier genetic connectivity pathway in marine organisms is currents.

The genetic distance between *O. laqueus* individuals ranged from 0.000-0.124 (Table 2). The lowest distance is in the sample

from Karimunjawa (0.000), while the highest distance is in the sample from Japan (AB430543-Japan; 0.124). This is inconsistent with the phylogenetic tree (Figure 3), in which that particular sample from Japan creates its own line apart from the two clades.

The genetic mean pair-wise distances within-clade of *O. laqueus* were 0,006 (0,06 %) and 0,002 (0,02 %) and the distance between-clade is 0,0883 (8,83 %). The genetic mean pair-wise distances between-clade of *O. laqueus* is 22 times higher than the main pair-wise distance within-clade.

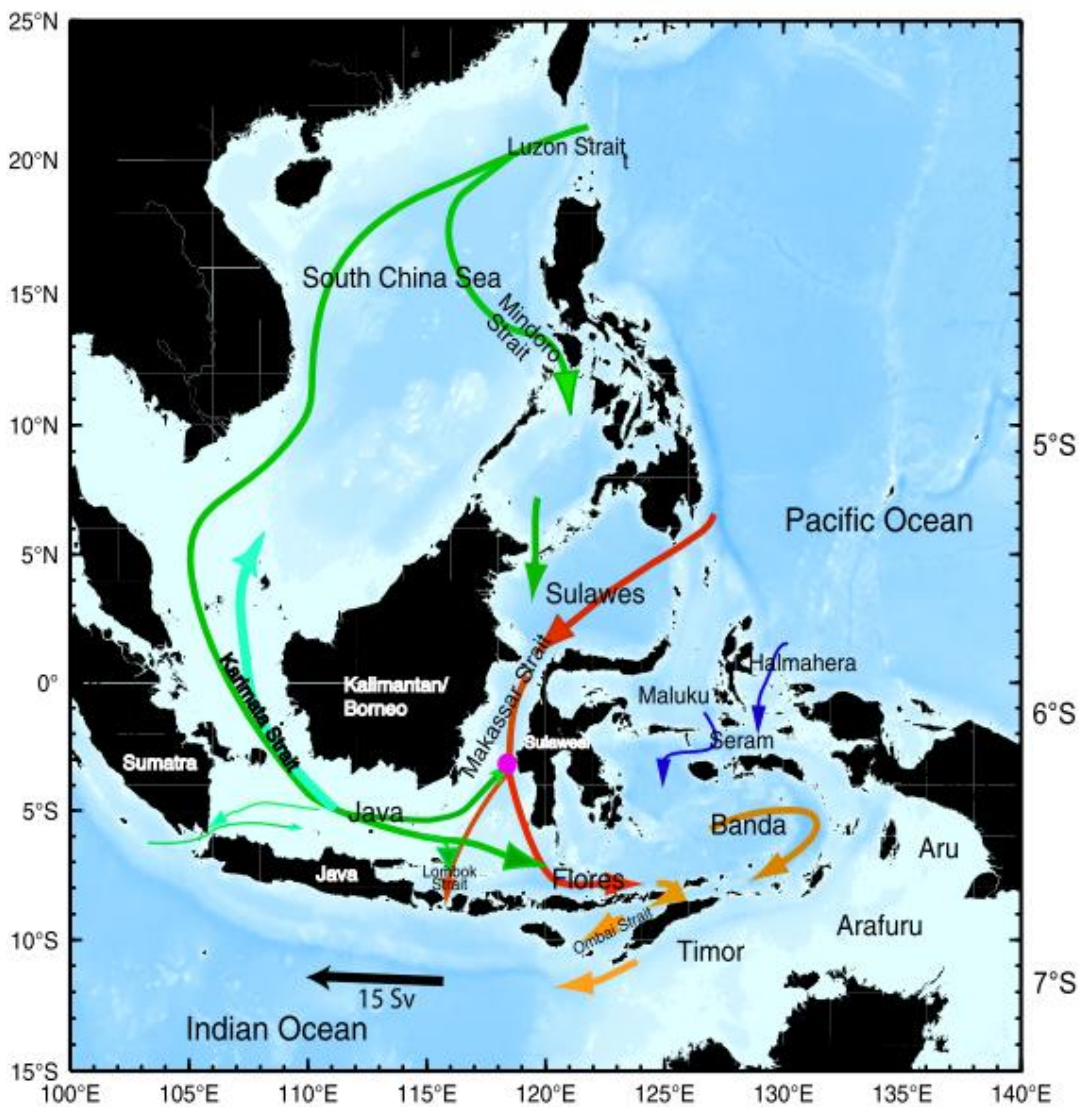


Figure 4. Indonesia throughflow pathways (ITF, green and red lines) in the Indo-Pacific region (Susanto & Song, 2015).

Table 1. BLAST identification of *O. laqueus* from Karimunjawa and Bangka Belitung, Indonesia

No	Sample ID	BLAST result	Accession number	Sequence length (Bp)	Ident (%)	Query Cover (%)
1.	22_Gt	<i>O. laqueus</i>	LC552307	685	99	91
2.	23_Gt	<i>O. laqueus</i>	LC552308	685	99	91
3.	24_Gt	<i>O. laqueus</i>	LC552309	685	99	91
4.	25_Gt	<i>O. laqueus</i>	LC552310	685	99	91
5.	26_Gt	<i>O. laqueus</i>	LC552311	685	99	91
6.	108_Kj	<i>O. laqueus</i>	LC552332	685	98.99	92
7.	109_Kj	<i>O. laqueus</i>	LC552333	685	99.09	91
8.	110_Kj	<i>O. laqueus</i>	LC552334	685	99.09	92
9.	111_Kj	<i>O. laqueus</i>	LC552335	685	99.09	91
10.	112_Kj	<i>O. laqueus</i>	LC552336	685	99.09	92
11.	113_Kj	<i>O. laqueus</i>	LC552337	685	99.24	89
12.	114_Kj	<i>O. laqueus</i>	LC552338	685	98.78	92
13.	115_Kj	<i>O. laqueus</i>	LC552339	685	98.94	92
14.	116_Kj	<i>O. laqueus</i>	LC552340	685	99.09	92
15.	117_Kj	<i>O. laqueus</i>	LC552341	685	98.78	91
16.	118_Kj	<i>O. laqueus</i>	LC552342	685	99.09	92
17.	211_BB	<i>O. laqueus</i>	MW559642	685	92.07	95
18.	212_BB	<i>O. laqueus</i>	MW559643	685	92.07	95

Note: Gt= Genting, Karimunjawa; Kj= Karimunjawa; BB= Bangka Belitung

Table 2. Genetic pair-wise distance between individuals of *O. laqueus*

No		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	LC552342-Karimunjawa																					
2	LC552341-Karimunjawa	0.003																				
3	LC552340-Karimunjawa	0.006	0.009																			
4	LC552339-Karimunjawa	0.008	0.011	0.008																		
5	LC552338-Karimunjawa	0.009	0.012	0.009	0.002																	
6	LC552336-Karimunjawa	0.006	0.009	0.000	0.008	0.009																
7	LC552335-Karimunjawa	0.006	0.009	0.000	0.008	0.009	0.000															
8	LC552334-Karimunjawa	0.006	0.009	0.000	0.008	0.009	0.000	0.000														
9	LC552333-Karimunjawa	0.006	0.009	0.000	0.008	0.009	0.000	0.000	0.000													
10	LC552332-Karimunjawa	0.005	0.008	0.008	0.009	0.011	0.008	0.008	0.008	0.008												
11	LC552311-Karimunjawa	0.006	0.009	0.000	0.008	0.009	0.000	0.000	0.000	0.008	0.008											
12	LC552310-Karimunjawa	0.006	0.009	0.000	0.008	0.009	0.000	0.000	0.000	0.000	0.008	0.000										
13	LC552309-Karimunjawa	0.006	0.009	0.006	0.008	0.009	0.006	0.006	0.006	0.006	0.008	0.006	0.006									
14	LC552308-Karimunjawa	0.006	0.009	0.006	0.008	0.009	0.006	0.006	0.006	0.006	0.008	0.006	0.006	0.000								
15	LC552307-Karimunjawa	0.006	0.009	0.000	0.008	0.009	0.000	0.000	0.000	0.000	0.008	0.000	0.000	0.006	0.006							
16	LC552337-Karimunjawa	0.002	0.005	0.005	0.006	0.008	0.005	0.005	0.005	0.005	0.003	0.005	0.005	0.005	0.005	0.005						
17	211-Bangka,Belitung	0.090	0.088	0.086	0.088	0.090	0.086	0.086	0.086	0.086	0.091	0.086	0.086	0.086	0.086	0.088						
18	212-Bangka,Belitung	0.091	0.090	0.088	0.090	0.091	0.088	0.088	0.088	0.088	0.093	0.088	0.088	0.088	0.088	0.090	0.002					
19	MN711655-Malaysia	0.008	0.011	0.002	0.009	0.011	0.002	0.002	0.002	0.002	0.009	0.002	0.002	0.008	0.008	0.002	0.006	0.088	0.090			
20	AB430543-Japan	0.116	0.118	0.116	0.122	0.124	0.116	0.116	0.116	0.116	0.114	0.116	0.116	0.112	0.112	0.116	0.114	0.113	0.115	0.118		
21	AB302176-Japan	0.009	0.012	0.009	0.011	0.012	0.009	0.009	0.009	0.009	0.011	0.009	0.009	0.003	0.003	0.009	0.008	0.090	0.091	0.011	0.114	

Table 3. Mean pair-wise distances of within-clade and between-clade of *O. laqueus*

	Mean pair-wise distances within-clade	Mean pair-wise distances between-clade	
		Clade I	Clade II
Clade I	0.006	-	-
Clade II	0.002	0.0883	-
AB430543-Japan		0.1163	0.1138

The genetic distance between clade-1 and clade-2 in Table 3 is 8.83%, indicating that samples of *O. laqueus* from either clades arises from two different species. Plaisance *et al.* (2009) applied a species-level criterion of 5% similarity to distinguish the number of unidentified decapod species and referred it as OTU (Operational Taxonomic Unit). Since the Japan single specimen (AB430543-Japan) also separated from the two clades by 11,63-11,38 %, it is then catagorised as different species too.

CONCLUSION

By using mtDNA COI as genetic marker, it is concluded that *O. laqueus* taken from Karimunjawa came from the same populations to those in Malaysia (MN711655) and Japan (AB302176). Whereas specimens from Bangka-Belitung were suggested as different species, since they were separated to form the second clade. Meanwhile, one single sample from Japan (AB430543) which was not even laid within the two clades also indicated to represent a different species; or perhaps, other loci may be more appropriate to elaborate this occurrence.

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