

Transoceanic Disperse of the White-lipped Island Pit Viper (*Cryptelytrops insularis*; Kramer,1997) from Sundaland to Lesser Sunda, Indonesia

Bagus Priambodo^{1,2,3}, Fu-Guo Robert Liu², Nia Kurniawan¹

¹Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Malang

²Department of Life Sciences, National Central University, Taiwan

³Department of Biology, Faculty of Mathematics and Natural Sciences, State University of Malang

Abstract

White-lipped island pit viper (*Cryptelytrops insularis*) is one of the most distributed Viperidae in Indonesia, especially in eastern part of Sundaland and Lesser Sunda. To investigate the evolutionary history and the dispersal pattern of *C. insularis*, we collected 23 samples from 11 localities. Four simultaneous genes composing two mitochondrial genes (16S rRNA & ND4) and two nuclear genes (71βFIB & 3ITBP) have been successfully amplified and sequenced. Bayesian inference was performed to reconstruct the phylogeny tree. Furthermore, time divergence and the population demography analyses were estimated. The phylogeny tree of *C. insularis* exhibits monophyletic group, with four geographically structured lineages. The time divergence estimation indicated that *C. insularis* evolved at approximately 7 million years ago (mya). Population demography was inferred by Bayesian Skyline Plot analysis, it shows that the population increased constantly from the past to recent time. The evolutionary history of *C. insularis* can be explained by a pattern of the time divergences estimation that indicating movement from West (Java) to East (Lesser Sunda). We expected that the dispersal factor of *C. insularis* into many different islands (in Lesser Sunda) is caused by the animal helped and also oceanic rafting which could be the stepping stones to another island.

Keywords: *Cryptelytrops insularis*, dispersal patterns, phylogeny, population demography, time divergence

INTRODUCTION

Indonesia was one of some biggest hotspot biodiversity in the world, especially the Sundaland and Wallacea area [1]. There are 5 large islands, Sumatra, Java, Kalimantan, Sulawesi, Papua New Guinea, and around 17.000 small islands. [2]. These islands have unique geographical history leading to unique biological evolution.

During Pliocene approximately 5 million years ago (mya) and Pleistocene (12000 years ago) Epoch, there were several sea level fluctuations due to the glaciations [3]. At that time, the Sundaland area including Sumatra, Java, and Borneo was in a changing phase, and also the unity of Sulawesi and the appearance of Lesser Sunda were formed due to the collisions of Philipines and Australian plates [4]. These periods were suggested to affect recent animal compositions [3]. The lowering of sea level caused the emergence of some landbridges for dispersal. In contrast, the raising of sea level will inundate some islands and coastal plain, and restrict the dispersals of some fauna [5]. During the period of isolation, organisms would evolve independently such as snake [6].

Insular species was very interesting to study the evolution and biogeography because they are separated by islands [7]. *Cryptelytrops insularis* (*Trimeresurus insularis*) or white-lipped island pit viper was one of the insular species that distributed in Java, Bali, Adonara, Alor, Flores, Komodo, Lombok, Padar, Rinca, Romang, Roti, Sumba, Sumbawa, Timor, and Wetar [8,9]. Based on the Reptile Database this species has type locality from Timor Island [8]. This species was well-distributed in Java, Bali (Sundaland) and Lesser Sunda, but in fact between Sundaland and Lesser Sunda are never been connected. Thus, we raised a question, how can *C. insularis* dispersed into those islands?

Robust phylogeny on the genus level of *Trimeresurus* (previous scientific genus name) were done by Maholtra & Thorpe [10], Creer et al [11], Maholtra & Thorpe [12], Creer et al [13], and Maholtra et al [14], but intra-species level on each species still unclear. Based on molecular assessment using Cyt B David et al [15] showed that *C. insularis* has a close relationship with *Cryptelytrops fasciatus* from Tanahjampea Island, South Sulawesi. Interestingly, there is another viper whose distribution overlapped with *C. insularis*, namely *Cryptelytrops albolabris*. But, the relationship is closer to *C. fasciatus* than *C. albolabris* which have similar morphology [14]. We hypothesize that geographical history might influence their relationships as well. Therefore,

* Correspondence address:

Bagus Priambodo

Email : priambodo.fmipa@um.ac.id

Address : Dept. Biology, State University of Malang, Jl.

Semarang 5 Malang 65145

using more genes and more samples we have aimed to evaluate the phylogeny and biogeography of white-lipped island pit viper, and also to estimate their dispersal patterns.

MATERIALS AND METHODS

Sample collection

We collected a total of 23 samples including one outgroup (*Cryptelytrops albolabris*) and two samples from Genbank (NCBI) [16]. Sample collections were conducted during May 2016 - January 2017, in Java, Bali, and Lombok Islands. Visual Encounter Survey (VES) method was used to collect the sample by looking around the tracking area [17]. Additional sequences were obtained from Genbank NCBI (Supplementary 1). Tissues collection was taken from muscle, tail tips, and liver then preserved into 1.5 mL tubes containing 98% ethanol or lysis buffer (Tris, EDTA, and SDS). Specimen's whole body were preserved into 70% ethanol and stored in the room temperature.

Molecular protocols

DNA extraction was conducted by QIAGEN kit (QIAamp DNA Minikit, Hilden, Germany) and following their protocol, also by manual procedure with phenol and chloroform. Tissue digestion conducted with 330 μ L STE buffer (cell lysis buffer) (10 mM Tris-HCl pH 5.0, 0.1 NaCl, 1 mM EDTA), 40 μ L 20% SDS, and 20 μ L of 20 mg.mL⁻¹ Proteinase K [18]. DNA pellet was dissolved with 50 μ L ddH₂O, then kept in -20°C freezer for later use.

We used two mitochondrial genes (16S rRNA and ND4) and two nuclear genes (71 β FIB and 3ITBP). These genes were succeeded to amplify Asian pit viper and generated well-resolved phylogeny [13]. Primers used were shown in Supplementary 2. PCR mixture composition was followed by the 2x Taq DNA Polymerase Master mix RED reaction composition (BIONOVAS, Toronto, Ontario). Thermal cycles used were: pre-denaturation at 94°C for 1-minute, then followed by 40 cycles of 30 seconds denaturation at 94°C, 1-3 minutes annealing at 50-57°C and 1-minute extension at 72°C. then 10-15 minutes final extension at 72°C. We have a difficulty in the nuclear genes amplification, the PCR products were amplified with an unstable successful rate. In order to increase the sensitivity, a nested PCR approach was applied [19]. PCR products were cleaned up with PCR Advanced Clean-Up System (VIOGENE). These four genes were completely sequenced from both directions with the following primers in

Biomedical Research Center at Academia Sinica and Mission Biotech (Taiwan), using ABI PRISM Model 3730 Version 3.2.

Sequences analyses

Sequences were aligned by Clustal W and modified implemented in MEGA 7 [20], then double-checked manually with FinchTV 1.4.0 [21]. Phylogenetic trees were reconstructed using Bayesian inference (BI), with the best fit model of GTR+I+G (general time-reversible + Proportion invariable sites + gamma distribution) was selected with Akaike information criterion (AIC) by jModeltest 2.1.10 [22,23]. The parameter for Proportion invariable was 0.6583 and for gamma/shape was 0.7690. BI was estimated using MrBayes 3.2.3 with two runs of four Monte Carlo Markov chains for 100.000.000 generations and a tree was sampled every 100 generations [24]. The consensus topology was summarized with burn-in first 25% trees.

Time divergence was estimated using BEAST v1.8.4 [25]. The parameters were set up in BEAUti v1.8.4 as GTR substitution model, empirical base frequencies, 6 number of gamma categories, random local clock model, Coalescent; constant size tree prior. At the Prior column, fossil records calibration used are: (i). *Cryptelytrops insularis* divergence around 8.5 million years ago (mya), (ii). *Cryptelytrops albolabris* divergence around 7.15 mya [26]. For the alpha parameter used is gamma distribution; Initial value/shape=0.6583), and for the Pinvariable the parameter used is lognormal distribution with Initial value/mean=0.6583, stdev=0.32915) then using 200.000.000 chain length. The BEAST was performed with two independent runs. The BEAST log result was checked by TRACER 1.6 [27] estimations to check the validity of convergence and effective sample size (ESS). Two BEAST tree files then combined by the LogCombiner v1.8.4, then the combined tress would summarize by TreeAnnonator v.1.8.4. then the time-calibrated tree was visualized and modified by FigTree v.1.4.3.

Bayesian skyline plot (BSP) was performed by BEAST v.1.8.4 [25] to estimates changes in effective population size through the time. The analysis was conducted at all individuals which belong to *Cryptelytrops insularis* (22 individuals). The parameter for the tree prior was used the Coalescent; Bayesian Skyline, and for the time calibration used was 7.15 mya for *C. insularis* [26]. The BEAST file was double generated in

3,000,000 chain length. The BSP result was analyzed and visualized by TRACER 1.6 [27].

RESULTS AND DISCUSSION

Phylogenetic tree and genetic distances

To determine the phylogeny of 22 individuals in *Cryptelytrops insularis*, Bayesian inference tree was constructed based on 3575 base pairs sequences from 4 simultaneous genes (16S rRNA, ND4, 71βFIB, and 3ITBP).

Our phylogenetic tree based on 4 simultaneous genes showed a monophyletic group with four clades (Fig. 1). Clade A containing *C. insularis* samples from Lesser Sunda area such as Lombok and Timor, while Clade E consisting samples from Sundaland such as Java and Bali. In detail, Clade B consisted of *C. insularis* from East Java, Clade C are composed of samples from Bali Island, and Clade E consisted of samples from East Java. Most of the posterior probability on the nodes were weakly supported by Bayesian Inference, however, there were some nodes received high supports such as Clade C and the common ancestor of Clade B, C, and D.

The genetic distances among clades are varied between individuals (Table 1), but there are no significant genetic differences. The highest genetic differences were 0.009 and 0.008 which showed on samples from East Java (sample no. 1)

with the sample from Bondowoso, East Java (sample no. 22), and samples from Bondowoso, East Java (sample no. 22) with the sample from Tanah Lot, Bali (sample no. 56), respectively.

Divergence times estimation

Divergence time estimation indicated that *C. insularis* was younger than *C. albolabris* (Fig. 2). Most ancient *C. insularis* evolved around 7 mya (node a), then followed by first diversification on the node b, around 6.7 mya in Sundaland area (Java and Bali). Samples from Lesser Sunda (node c) diverge around 4 mya. Based on our estimations we expected that there is an eastward movement of *C. insularis*.

Population demography (Bayesian skyline plot)

To estimate the effective population size through the time, we performed the Bayesian skyline plot (BSP) on Figure 3. BSP analysis on *C. insularis* population showed increased constantly from the past to recent time. At the first diversification of *C. insularis* around 7-5 mya, the effective population size was increased slightly. Moreover, around 5-3 mya the population size was increased significantly. Then around 3 mya to recent time, the population size estimation showed the most stable with slightly increase.

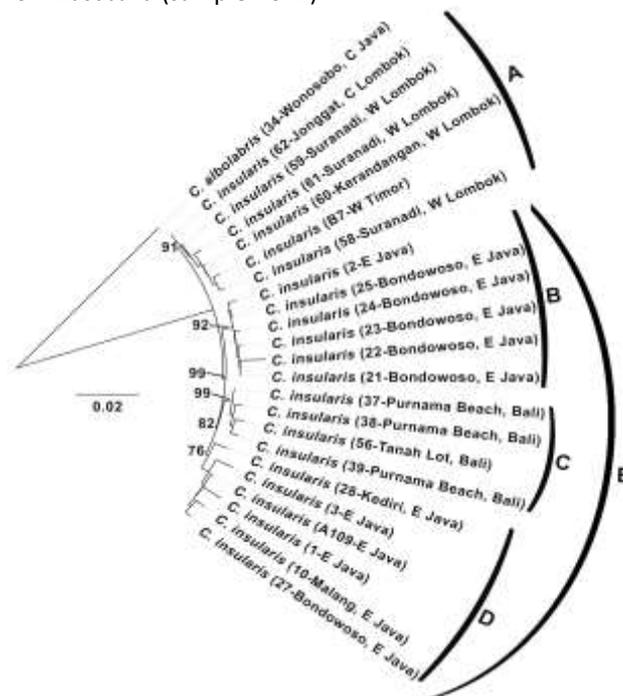


Figure 1. Bayesian phylogeny semi circular-tree based on 4 simultaneous genes (16S rRNA, ND4, 71βFIB & 3ITBP). *C. albolabris* was used as an outgroup. The number on the nodes represent the Bayesian posterior probability (pp). Sample number and locality were showed in parentheses. (C: Central, E: East, W: West).

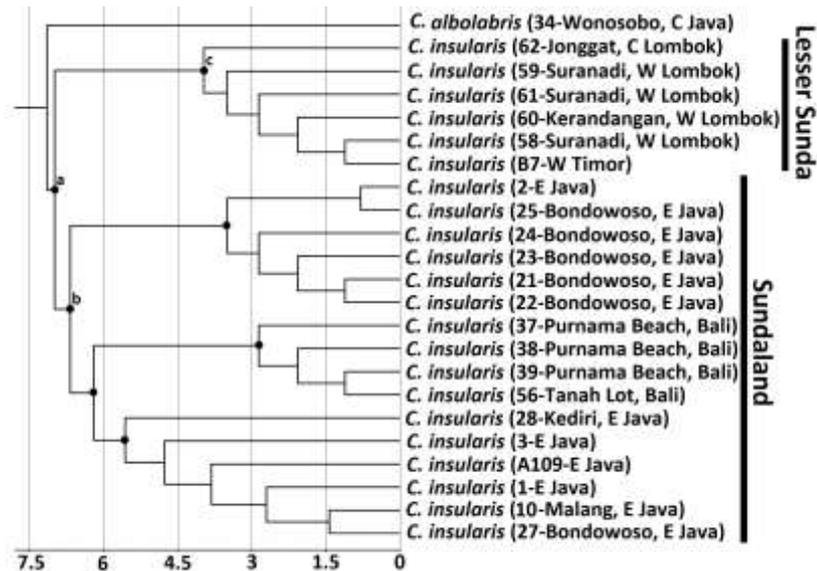


Figure 2. Time-calibrated tree based on 4 simultaneous genes (16S rRNA, ND4, 71βFIB & 3ITBP). Sample number and locality were showed in parentheses. The scaled line below the trees represents the time in million years ago. The alphabets on the nodes represent the divergence time which explained in the results section (C: Central, E: East, W: West).

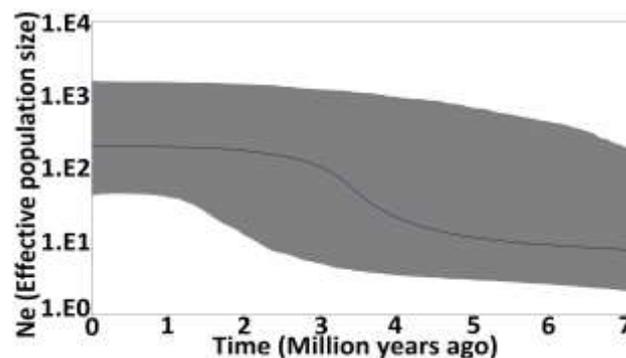


Figure 3. Bayesian Skyline Plot illustrating effective population size (N_e) through the time on *Cryptelytrops insularis*. Grey solid color represent 95% posterior probability range. Blackline represents the median population size.

Historical biogeography and population demography of *Cryptelytrops insularis*

Java and Bali emerged to the surface by 10 mya and then followed by Lombok and Sumbawa by 5 mya [28]. The emergence of younger islands to the east, starting from Bali to Lombok, Sumbawa, Flores, and Wetar were corresponding to the relationship in our phylogeny tree [29]. Seems, our time divergences analysis also demonstrate the same condition, which samples from Java-Bali was oldest then followed by Lesser Sunda's sample.

Timor was emerged around 2 mya, as the result of the collision between Australian continental margin and the Banda volcanic arc. [3,30-32]. These reports strengthen our findings, that sample from Timor is the youngest one than another which is included in Lesser Sunda.

During Miocene Epoch, warm temperature, humid climate, and rainforest habitat were

predominated thus provided organisms suitable habitats [34]. However, started from Pliocene (around 3 mya) to recent the temperature was cold, even extremely cold [35,36]. In such conditions, some organisms might experience population fluctuation and even extinction [37]. But, *C. insularis* population are constantly increased. We suggested that this species has a strong survival rate to adapt in several conditions and habitat, especially cold temperature [35].

Dispersion probability

Java and Bali's islands have experience of being connected by the shallow sea during Pleistocene [3]. Java and Bali shared a lot of well-distributed fauna such as avians and mammals [38]. Other evidence showed in freshwater fishes, a total of 227 species spread in Java and Bali [39].

Cryptelytrops insularis dispersal pattern
(Priambodo et al)

Table 1. Genetic distances with the selected parameters based on jModeltest. Evolutionary model= GTR+I+G, Proportion of invariable sites (I) = 0,6583 and Gamma distribution shape parameter = 0,7690. The number in bold showed the most significant genetic differences. Sample number and locality were showed in parentheses. (C: Central, E: East, W: West).

	1	2	3	4	5	6	7	8	9	10	11	12
1 <i>Cryptelytrops insularis</i> (1-E Java)	-											
2 <i>C. insularis</i> (2-E Java)	0.004	-										
3 <i>C. insularis</i> (3-E Java)	0.003	0.003	-									
4 <i>C. insularis</i> (10-Malang, E Java)	0.003	0.004	0.002	-								
5 <i>C. insularis</i> (21-Bondowoso, E Java)	0.004	0.001	0.003	0.003	-							
6 <i>C. insularis</i> (22-Bondowoso, E Java)	0.009	0.004	0.005	0.006	0.003	-						
7 <i>C. insularis</i> (23-Bondowoso, E Java)	0.005	0.001	0.003	0.004	0.000	0.004	-					
8 <i>C. insularis</i> (24-Bondowoso, E Java)	0.005	0.001	0.003	0.003	0.000	0.004	0.000	-				
9 <i>C. insularis</i> (25-Bondowoso, E Java)	0.005	0.000	0.003	0.003	0.000	0.004	0.000	0.000	-			
10 <i>C. insularis</i> (27-Bondowoso, E Java)	0.003	0.002	0.002	0.002	0.001	0.004	0.001	0.001	0.001	-		
11 <i>C. insularis</i> (21-Kediri, E Java)	0.004	0.003	0.003	0.003	0.003	0.006	0.003	0.002	0.002	0.003	-	
12 <i>C. insularis</i> (21-Purnama Beach, Bali)	0.004	0.003	0.003	0.004	0.002	0.006	0.003	0.002	0.002	0.003	0.002	-
13 <i>C. insularis</i> (38-Purnama Beach, Bali)	0.005	0.004	0.003	0.004	0.004	0.006	0.004	0.004	0.004	0.003	0.003	0.001
14 <i>C. insularis</i> (39-Purnama Beach, Bali)	0.003	0.004	0.002	0.003	0.003	0.006	0.004	0.004	0.004	0.002	0.003	0.001
15 <i>C. insularis</i> (56-Tanah Lot, Bali)	0.004	0.004	0.003	0.004	0.004	0.008	0.005	0.005	0.004	0.004	0.005	0.002
16 <i>C. insularis</i> (58-Suranadi, W Lombok)	0.005	0.003	0.003	0.005	0.002	0.006	0.003	0.003	0.002	0.004	0.003	0.003
17 <i>C. insularis</i> (59-Suranadi, W Lombok)	0.004	0.002	0.002	0.004	0.001	0.006	0.002	0.002	0.001	0.003	0.003	0.003
18 <i>C. insularis</i> (60-Kerandangan, W Lombok)	0.004	0.003	0.003	0.004	0.003	0.006	0.003	0.003	0.002	0.004	0.004	0.003
19 <i>C. insularis</i> (61-Suranadi, W Lombok)	0.005	0.003	0.003	0.005	0.003	0.007	0.003	0.003	0.003	0.004	0.004	0.003
20 <i>C. insularis</i> (62-Jonggat, C Lombok)	0.004	0.002	0.002	0.003	0.001	0.006	0.002	0.002	0.001	0.003	0.003	0.002
21 <i>C. insularis</i> (A109-E Java)	0.005	0.002	0.000	0.001	0.002	0.002	0.003	0.002	0.002	0.002	0.001	0.002
22 <i>C. insularis</i> (B7-W Timor)	0.006	0.003	0.003	0.004	0.003	0.002	0.003	0.003	0.002	0.004	0.005	0.003
23 <i>Cryptelytrops albolabris</i> (34-Wonosobo, C Java)	0.052	0.052	0.053	0.067	0.057	0.072	0.063	0.064	0.062	0.067	0.066	0.067

Table 1. Continue

	13	14	15	16	17	18	19	20	21	22	23
13 <i>C. insularis</i> (38-Purnama Beach, Bali)	-										
14 <i>C. insularis</i> (39-Purnama Beach, Bali)	0.002	-									
15 <i>C. insularis</i> (56-Tanah Lot, Bali)	0.002	0.002	-								
16 <i>C. insularis</i> (58-Suranadi, W Lombok)	0.003	0.003	0.004	-							
17 <i>C. insularis</i> (59-Suranadi, W Lombok)	0.003	0.003	0.003	0.001	-						
18 <i>C. insularis</i> (60-Kerandangan, W Lombok)	0.003	0.003	0.003	0.001	0.001	-					
19 <i>C. insularis</i> (61-Suranadi, W Lombok)	0.003	0.004	0.004	0.002	0.002	0.001	-				
20 <i>C. insularis</i> (62-Jonggat, C Lombok)	0.003	0.003	0.003	0.001	0.000	0.001	0.002	-			
21 <i>C. insularis</i> (A109-E Java)	0.003	0.002	0.003	0.003	0.002	0.001	0.003	0.001	-		
22 <i>C. insularis</i> (B7-W Timor)	0.003	0.003	0.002	0.001	0.001	0.001	0.001	0.001	0.002	-	
23 <i>Cryptelytrops albolabris</i> (34-Wonosobo, C Java)	0.067	0.053	0.053	0.051	0.052	0.050	0.051	0.058	0.060	0.042	-

The existence of landbridge between Java and Bali might play an important role in *C. insularis* dispersal [3].

Between Bali and Lombok, there is a deep strait which is known as fauna discontinuation, called by Wallace's line [38]. But this Wallace line has no effect on *C. insularis*, this species is well distributed eastward to Timor, despite being separated by inter-island barriers [40]. Therefore, we suggested that there is an intervention of another animal, which influenced the movement of *C. insularis* from Bali to Lombok.

Komaki et al suggested that there are two possibilities for terrestrial animal transoceanic dispersal, ocean flow or carry by other animals with high dispersal ability (birds or human) [41]. However, at that time (around 4 mya) humans did not exist yet [42]. Therefore, we convinced that the dispersal pattern from Bali to Lombok was helped by birds. *C. insularis* categorized in the Viperidae Family which usually less-mobile compared to other families [9]. This behavior could also enhance the chance for birds to catch this species then moved to another place.

Zoologists explained that insects, mammals, and reptiles have been demonstrated the transoceanic dispersal by rafting [43-45]. Some floating stuff such as macroalgae, pumice, wood, and vegetation could be a substrate for the snake to hold up [41,46]. The ocean drift usually following the ocean throughflow and winds [46].

The eastward distribution of *C. insularis* from Lombok until Timor is more likely to be helped by ocean flow from the Pacific Ocean [28]. Water flows from the north (Makassar Strait) head eastward from Lombok to Timor. Then the throughflow circled Timor (to the south of Timor) and turned west toward the Indian Ocean [28,47,48]. Another viper species which exhibit a similar distribution is *Cryptelytrops albolabris* [8]. However, there is no report or genetic data of *C. albolabris* from Lesser Sunda.

CONCLUSION

Our phylogeny showed monophyletic group, Sundaland and Lesser Sunda were geographically separated with independent common ancestor. *Cryptelytrops insularis* started to evolve around 7 million years ago, then followed by first diversification around 6.7 mya in Sundaland area (Java and Bali). Samples from Lesser Sunda diverge around 4 mya. The *C. insularis* transoceanic dispersal are observed with eastward movement. We tend to believe that, the overseas dispersal is helped by other animal

and rafting by the ocean drift. We still don't have complete samples representing all the islands in the Lesser Sunda. Therefore, more samples from other localities were needed for further research to explore the biogeography of *C. insularis*.

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Supplementary 1. List of the sample used in this study

Species	Code*	Locality	Accession number			
			16S	ND4	71βFIB	3ITBP
<i>C. insularis</i>	1	E Java	MG561477	MG561528	MG561620	MG561575
<i>C. insularis</i>	2	E Java	MG561478	MG561529	MG561621	MG561576
<i>C. insularis</i>	3	E Java	MG561479	MG561530	MG561622	MG561577
<i>C. insularis</i>	10	Malang, E Java	MG561486	MG561537	-	MG561584
<i>C. insularis</i>	21	Bondowoso, E Java	MG561497	MG561548	MG561632	MG561594
<i>C. insularis</i>	22	Bondowoso, E Java	MG561498	MG561549	-	-
<i>C. insularis</i>	23	Bondowoso, E Java	MG561499	MG561550	-	MG561595
<i>C. insularis</i>	24	Bondowoso, E Java	MG561500	MG561551	-	MG561596
<i>C. insularis</i>	25	Bondowoso, E Java	MG561501	MG561552	-	MG561597
<i>C. insularis</i>	27	Bondowoso, E Java	MG561503	MG561554	-	MG561599
<i>C. insularis</i>	28	Kediri, E Java	MG561504	MG561555	-	MG561600
<i>C. insularis</i>	37	Purnama beach, Bali	MG561513	MG561564	-	MG561608
<i>C. insularis</i>	38	Purnama beach, Bali	MG561514	MG561565	-	MG561609
<i>C. insularis</i>	39	Purnama beach, Bali	MG561515	MG561566	MG561638	MG561610
<i>C. insularis</i>	56	Tanah Lot, Bali	MG561518	MG561568	MG561639	MG561613
<i>C. insularis</i>	58	Suranadi, W Lombok	MG561520	MG561570	MG561641	MG561615
<i>C. insularis</i>	59	Suranadi, W Lombok	MG561521	MG561571	MG561642	MG561616
<i>C. insularis</i>	60	Suranadi, W Lombok	MG561522	MG561572	MG561643	MG561617
<i>C. insularis</i>	61	Suranadi, W Lombok	MG561523	MG561573	MG561644	MG561618
<i>C. insularis</i>	62	Jonggat, C Lombok	MG561524	MG561574	MG561645	MG561619
<i>C. insularis</i>	A109/A96	E Java/Indo	AY352738	AY352833	GQ428347	-
<i>C. insularis</i>	B7	W Timor	AY059550	AY059586	DQ116978	DQ117500
<i>C. albolabris</i>	34	Wonosobo, C Java	MG561510	MG561561	MG561636	MG561606

*The code start with a number were obtained in this study. Whereas the code start with alphabet was obtained from Genbank. (C: Central, E: East, W: West)

Supplementary 2. Primers and sources used in this study

Genes	Primers	Primer sequences	References
16S	16Sf4	5'- CTTGTACCTTTGCATCATGGT-3'	This study
	16Sr2	5'-CCGGTCTGAACCTCAGATCACGT-3'	This study
ND4	ND4	5'-CACCTATGACTACCAAAGCTCATGTAGAAGC-3'	Arevalo et al [49]
	LEU	5'-CATTACTTTTACTTGGATTGCACCA-3'	Arevalo et al [49]
71βFIB	ND4-R	5'-TTACCAGATTGAAGAGTTAGCAGGTCTT-3'	This study
	TRIMFIB-B17U	5'-AGAGACAATGATGGATGGTAAG-3'	Creer et al [13]
	TRIMFIB-B17L	5'-CCTTTGGGATCTGGGTGTA-3'	Creer et al [13]
3ITBP	71βFIB-2F	5'-TATAGCACCTGCAAAACG-3'	This study (Nested PCR)
	71βFIB-2R	5'-CTTGCTTACTTACTATGTG-3'	This study (Nested PCR)
	TRIMTBPI3F	5'- CCTTTACCAGGAACCACACC-3'	Creer et al [13]
3ITBP	TRIMTBPI3R	5'-GAAGGGCAATGGTTTTAG-3'	Creer et al [13]
	3ITBP-2F	5'-CAGGTTTACTCTGCTCACA-3'	This study (Nested PCR)
	3ITBP-2R	5'-AATGGACTCCAGAAGATG-3'	This study (Nested PCR)