TROPICAL LIFE SCIENCES RESEARCH

Genetic Diversity of the Haliotis diversicolor squamata from Southern Coastal Java (Banten, Pangandaran and Alas Purwo) and Bali Based on Mitochondrial CO1 Sequences

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Highlights

- The morphology of the abalone *Haliotis squamata* and *Haliotis diversicolor* squamata are very similar.
- The results of genetic identification in Haliotis squamata and Haliotis diversicolor squamata species using COI genes from mitochondria show an average difference in genetic distance of 16%.
- From the results of this study, we propose that the abalone from the southern waters of Java and Bali be separated species, *H. squamata* Reeve 1846.

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Genetic Diversity of the *Haliotis diversicolor squamata* from Southern Coastal Java (Banten, Pangandaran and Alas Purwo) and Bali Based on Mitochondrial CO1 Sequences

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Abstract.: The morphology of *Haliotis* genus is very difficult to distinguish because of its high similarity. Therefore, an identification tool such as genetics is needed to distinguish the genus. This study describes molecular characters of a species of *Haliotis* (Mollusca: Gastropoda: Haliotidae) from the southern Java and Bali waters, Indonesia, that is currently considered to be a subspecies of another occurring in China, Taiwan and Japan. DNA of *Haliotis* specimens collected from southern Java and Bali waters was extracted from epipodial tissues using a DNeasy® Blood and Tissue Kit, and partial CO1 genes amplified using AB-CO1DivF and AB-CO1DivR primers. Genetic distances were determined by Kimura 2-parameter, and phylogenetic trees constructed using the Neighbor-joining method in MEGA 5.0 software. Based on the genetic distance using the COI mtDNA gene as a barcoding species, the difference was 15.9%–16.7% with *H. diversicolor superteksta* from China, *H. diversicolor* from Taiwan (7.73%–9.12%), and *H. diversicolor* from Japan (7.80%–9.20%), because of boundary for determining species groups based on of 4% and mollusks at 4.8%, then *H. diversicolor squamata* spread in the southern islands of Java and Bali is worthy of being separated into its own species. From the results of this study, we propose that abalone from the south of Java and Bali waters became a separate species, *H. squamata* Reeve 1846. While percentage difference in interpopulation genetic distance from Java and Bali about 0.000%–0.011% or 0.0%–0.11%, averaging 0.60%.

Keywords: CO1, *Haliotis diversicolor squamata*, Java, Bali, Indonesia

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INTRODUCTION

Abalone (Haliotidae) are represented by a single genus, *Haliotis*, with more than 58 recent species described (Geiger 1999). Seven species are reported from Indonesian waters (Dharma 1988; Geiger 1999); they typically occur in rocky habitats and waters with strong wave breaks (Dharma 2009; Setyono 2006).

Identification of species has traditionally been based on morphological characteristics, such as shell colour pattern and size, in addition to other body characteristics such as the number of open holes, shell sculpture and the colour of the body and tentacles (Dharma 1988; 2009; Soelistyowati *et al*. 2013). However, the similarity in many of these characters and their states between species has contributed to taxonomic confusion. By way of example, the World Register of Marine Species (WoRMS) currently considers two species, *H. diversicolor squamata* from Indonesia and *H. diversicolor* from China, Taiwan and Japan to be subspecies of *H. diversicolor* Reeve 1846, a species previously reported from Indonesia as *H. squamata* (Dharma 2009).

Accurate species identification is fundamental to studies on biodiversity, population dynamics and conservation (Halt *et al*. 2009). Identifications based on molecular genetics are generally more accurate and the process more time efficient than traditional morphological identification (Hebert *et al*. 2003), given morphological characters can vary in different environmental conditions (Tissot 1988).

Molecular genetic analysis provides a tool to both critique the validity of characters and their states used to differentiate species with similar morphologies (species *complexes*) and evaluate phylogenies (Arlyza *et al*. 2013), and to analyse the distributions and degree of connectedness between populations (Gruenthal & Burton 2005). The molecular marker used in this study, mitochondrial cytochrome oxidase 1 (CO1), is highly polymorphic between species (Solihin 1994). As CO1 provides information on nucleotide base sequence changes between species, it is commonly used to “Barcode DNA” and because of its low variability (1%–2%), it is ideal for determining the identity and genetic relationships between species (Zein & Prawiradilaga 2013). CO1 sequence data are now available for a wide variety of invertebrate taxa (Hubert *et al*. 2015; Borsa *et al*. 2002; Hutama *et al*. 2017). Several studies have already used CO1 to differentiate *Haliotis* species (An *et al*. 2005, Gruenthal & Burton 2005; Wang *et al*. 2004), and to describe connectivity between their populations (Gruenthal & Burton 2008).

Our objective is to resolve the systematic status of one species of *Haliotis* that occurs in southern Java (Banten, Pangandaran and Alas Purwo) and Bali waters that has been variously attributed to *H. diversicolor* Reeve 1846, *H. squamata* Reeve 1846 and *H. diversicolor squamata* Reeve 1846, using mitochondrial CO1 gene sequences.

MATERIALS AND METHODS

Abalone (25 individuals) were collected from the intertidal to 10 m depth off the coast southern from Java Island, Indonesia (IJ-B-W: Binuanguen-Wild, IJ-P-W: Pangandaran-Wild, and IJ-AP-W: Alas Purwo-Wild), and Bali Island, Indonesia (IB-N-W: Negara-Wild) (Fig. 1 and Table 1). Tissue samples of 0.2–0.4 g were extracted from the epipodium (between the foot and mantle) and placed into absolute ethanol. Shells were identified following Dharma (1988). Morphological and molecular analysis were conducted at the Laboratory of Molecular Biology at the Research Centre for Biological Resources and Biotechnology (RCBRB), and Integrated Laboratory of the Department of Biology, Bogor Agricultural University, Indonesia.

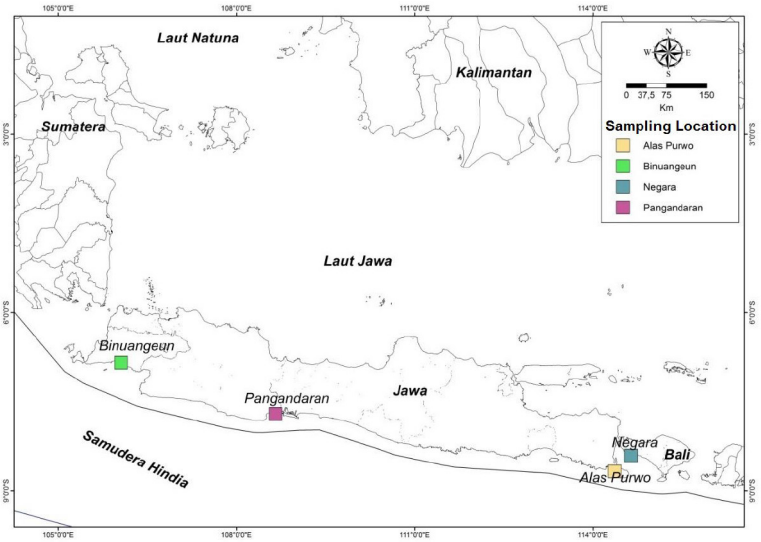


Figure 1: Survey sites, southern coastal Java and Bali waters.

Table 1: Information on sample data in the field.

Region	Sampling location	Sources	Code	N	Coordinates
Java Island, Indonesia	Binuangeun, Banten	Wild	IJ-B-W	5	06º49',87,4"S; 105º53'630"E
	Pangandaran, West Java	Wild	IJ-P-W	5	07º42'18,8"S; 108º39' 38,1"E
	Alas Purwo, East Java	Wild	IJ-AP-W	9	08º40'20,4"S; 114º22'6,24"E
Bali Island, Indonesia	Negara, Bali	Wild	IB-N-W	6	08º24'25,92"S; 114º38'22,25"E

Isolation of Total DNA

Tissue samples were rinsed in low EDTA TE buffer (10 mM Tris, pH 8.0 using HCL 1 mM EDTA) to remove absolute ethanol. DNA was extracted using a commercial kit from Dneasy® Blood and Tissue Kit no 69504 (50) (Qiagen, Germany) following the manufacturer instructions with some modification (the sample was first crushed, then dried).

Polymerase Chain Reaction Amplification, Sequencing and Data Analysis

The target gene was amplified using polymerase chain reaction and specific forward [AB-CO1DivF (5'-TGA TCT GGA CTA GTC GGA ACC GC-3')] and reverse [AB-CO1DivR (5'-GAT GTA TTA AAA TTA CGG TCG GT-3')] primers designed for the partial CO1 gene of *H. diversicolor supertexta*, of length 1272 bp, using Primer3 (http://bio-info.ut.ee/primer3-0.4.0/primer3) (Rozen & Skaletsky 2000), producing a 581 bp partial sequence. The total volume of PCR reaction was 25 µl, consisting of 9.8 µl of ddH_2O, 4 µl of Q5 buffer, 5 µl of Q5 enhancer, 1 µl of dNTP, 1 µl of forward primer, 1 µl of reverse primer, 3 µl of DNA template, and 0.2 µ of Taq Hot Start Q5.

CO1 amplification began with an initial pre-denaturation at 95°C for 3 min, followed by denaturation for 35 cycles at 95°C for 45 s, annealing at 56°C for 45 sec, elongation at 72°C for 1 min, and a final cycle of extension at 72°C for 6 min. PCR products were purified by electrophoresis through a 1.2% agarose gel using a 1 × TBE (Tris-borate-EDTA acid) buffer. A single band of PCR product was sequenced by a sequencing service (1st BASE www.base-asia.com) Malaysia.

Sequence data were corrected and aligned using MEGA 5.0 software (Tamura *et al.* 2011), with the best sequences from each location subjected to an NCBI BLAST sequence similarity search. A phylogenetic tree was constructed using the Neighbour-joining method based on the Kimura 2-parameter model, validated using 1,000 bootstrap replicates. GenBank sequences for *H. diversicolor* superteksta (access code AY319443) from China (Wang *et al.* 2004), *H. diversicolor* from Taiwan (access code KX853618–KX853621) and *H. diversicolor* from Japan (KX853541–KX853545) were used as outgroups (Hsu & Gwo 2017). All sequences of species H. diversicolor squamata obtained in this study have been posted on GenBank (MK562704–MK562729). The diversity of haplotypes (haploid type) uses the DNAsp ver program. 510.01 and Network ver 5.

RESULTS AND DISCUSSION

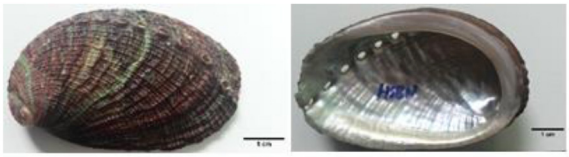
Shell Morphology

Southern coast Java and Bali *Haliotis* shell morphology (Fig. 2a) was consistent with *H. squamata* (Dharma 1988), in that the shell was oval, had a thick texture and wavy surface (Fig. 2b), an exterior reddish-brown colour, 6–8 rounded and

slightly prominent open respiratory holes, black edges of body, black epipodium and tentacle, and a yellow shell covered by a portion of the foot (Fig. 3a). Japanese specimens attributed to *H. diversicolor* differ slightly in having an oval reddish-coloured shell with thin texture (Geiger 1999) (Fig. 3b). As abalone shell colour can be influenced by diet (Liu *et al*. 2009), and growth can be affected by food, habitat and water temperature (Mcshane *et al*. 1994; Tissot 1988), such differences in shell characteristics do not necessarily indicate that species differ. This is where CO1 sequence data assists in resolving systematic confusion (Arlyza *et al.* 2013; Borsa 2002; Hebert *et al*. 2004; Kong & Qi 2009).

(a) (b)

Figure 2: *H. diversicolor squamata* (a) actively creaping and (b) shell surface.



(a)

(b)

Figure 3: (a) Shell of *H. diversicolor squamata* and (b) shell of *H. diversicolor*.

Genetic Diversity of Small Abalone from Indonesia

Based on the COI gene on small abalone of *H. diversicolor squamata* from southern coast Java and Bali waters, Indonesia produced a sequence length (581 bp) with a total of 25 individuals, with three locations for southern coastal Java (Banten,

Pangandaran and Alas Purwo) and one location for Bali waters (Negara) (Fig. 1). Hsu and Gwo (2017) have also reported that there are seven individuals in the COI *H. diversicolor squamata* gene sequence from Bali, Indonesia in their publication with (access code KX853841–KX853846) in the GenBank-NCBI but they do not have specific location.

A phylogenetic tree with *H. diversicolor squamata* and an outgroup (*H. diversicolor supertexta* and *H. diversicolor*) depicts two main clusters: (1) *H. diversicolor squamata* (I) and (2) *H. diversicolor supertexta* and *H. diversicolor* (II) (Fig. 4).

Six haplotypes of *H. diversicolor squamata* are apparent: (1) two individuals from Negara Island Bali (IB-N-W_1 and IB-N-W_5); (2) individuals Negara Island Bali (IB-N-W_2); (3) three individuals from Island Bali (IB-N-W 3, 4, and 6); (4) individuals Island Bali (IB-W_1); and (5) six individuals Island Bali (IB-W_2, 3, 4, 5, 6, and 7); (6) nineteen individuals Island Java: Banten group (IJ-B-W_1, 2, 3, 4, and 5), Alas Purwo group (IJ-BW-W_1, 2, 3, 4, 5, 6, 7, 8, and 9) and Pangandaran group (IJ-BW-W_1, 2, 3, 4, and 5) (Fig. 4). The occurrence of six haplotypes indicates populations of abalone in our four sampled locations have experienced genetic divergence. Similar divergence has been reported for *H. marmorata* from the coast of Senegal (Wormhoudt *et al*. 2009). Relationships between four species and two subspecies of abalone in Korea were also close (An *et al*. 2005). Phylogenetic results of the *H. diversicolor* spp. complex from China, Taiwan and Japan separate from samples from Indonesia (Hsu & Gwo 2017).

CO1 gene variations exceeding 4% indicate isolated reproduction, but differences below 2% indicate conspecific taxa (Ratnasingham & Hebert 2003). Feng *et al.* (2010) revealed that the difference in threshold for molluscs was 4.8%. While between-species (*H. diversicolor supertexta* from China, *H. diversicolor* from Taiwan and *H. diversicolor* from Japan) and *H. diversicolor squamata* genetic distances were very significant, 15.9%–16.7%, 7.73%–9.12% and 7.80%–9.20%, respectively (Table 2). Within species *H. diversicolor squamata* from Negara, Bali genetic distances were insignificant (< 1%), about 0.003–0.009 or 0.3%–0.9% averaging 0.005% or 0.50% (Table 2). Within population from Negara, Bali (this research) and sample data of Bali population from Hsu and Gwo (2017) showed genetic distance were insignificant (<1%), about 0.000–0.013 or 0.0%–0.13%, averaging 0.71% (Table 2). Within species *H. diversicolor squamata* from Java (Banten, Pangandaran and Alas Purwo) about 0.00%–0.000% or 0.0%–0.0%, averaging 0.00% (Table 2), while percentage difference in interpopulation genetic distance from Java and Bali about 0.000%–0.011% or 0.0%–0.11%, averaging 0.60% (Table 2). Lack of variability in CO1 from four southern coastal Java and Bali waters locations indicates a population of common origin. Significant differences in genetic distance (about 20%) have also been reported between other taxa in the Asia-Pacific region (An *et al*. 2005).

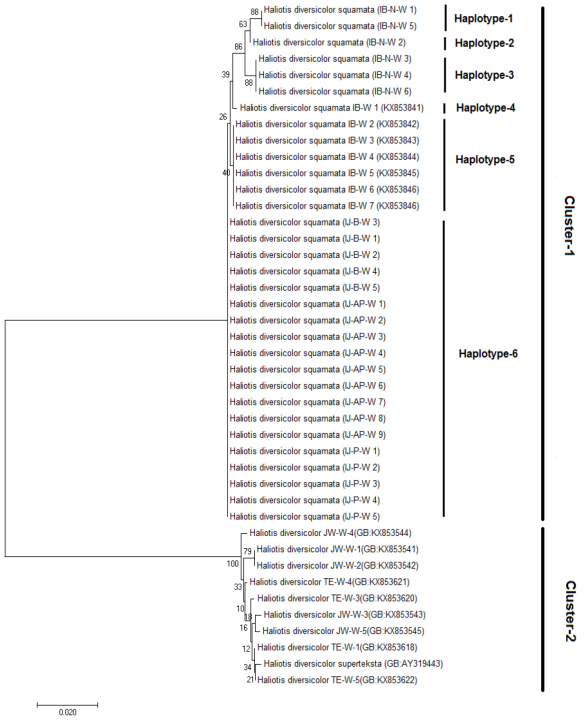


Figure 4: Reconstruction of phylogenetic tree.

Table 2: Genetic distances of Haliotis CO1 gene based on Kimura 2-parameter model.

Population	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]
[1] Binuangeun-Banten (IJ-B-W)	0.00*							
[2] Pangandaran-West Java (IJ-P-W)	0.00	0.00*						
[3] Alas Purwo-East Java (IJ-AP-W)	0.00	0.00	0.00*					
[4] Negara-Bali (IB-N-W)	0.67	0.87	0.93	0.50*				
[5] Bali (KX853841–KX853845)	0.12	0.21	0.21	0.71	0.53*			
[6] China (AY319443)	15.9	15.9	15.9	16.7	16.2	0.00*		
[7] Taiwan (KX853618–KX853621)	8.72	8.72	7.73	9.12	8.47	0.32	0.30*	
[8] Japan (KX853541–KX853545)	8.81	8.81	7.80	9.20	8.54	0.54	0.48	0.44*

Note: * Genetic distance between individuals in the population (intraspecies)

Haplotype Diversity

Nucleotide sequences of our *Haliotis* elaborated with revelead six haplotypes: One haplotype (haplotype 1) from southern coastal Java and five haplotypes from Bali. Five haplotypes from Bali (lenght 544 bp) are apparent: three haplotypes from this research (haplotype 2, haplotype 3 and haplotype 4) and two haplotypes (haplotype 5 and haplotype 6) from Hsu and Gwo (2017) (Fig. 5). Three haplotypes from Bali in this research is distinct from Java. However, two haplotypes from Bali by Hsu and Gwo (2017) is close with Java in this research (Fig. 5). This is due to difference the sampling location. Our *Haliotis* were collected from the western Bali and Hsu and Gwo (2017) were collected from the southern Bali.

If the sequence length is enlargered earlier (581 bp), the results of Java population can be distinguished more clearly with Bali population. The specific nucleotide sites are found in the population of Binuangeun-Banten with the position of sites 41 and 56. Additional specific nucleotide sites have been reported for other *Haliotis* taxa throughout the Asia-Pacific region (An *et al*. 2005). The presence of these specific nucleotides might be due to physiological and genetic adaptations influenced by environmental factors, such as habitat, food availability and temperature (Wang *et al*. 2004).

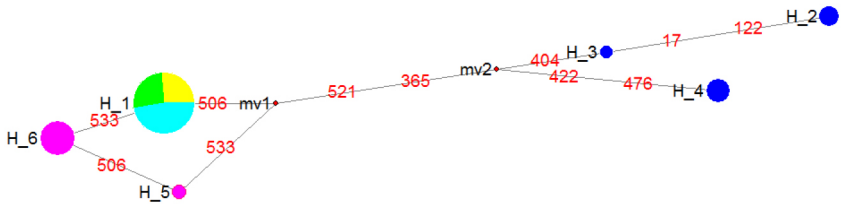


Figure 5: Haplotype network for six haplotype of small abalone (*H. diversicolor squamata*).

CONCLUSION

Species was consistent in morphology with *H. diversicolor squamata*. Based on the genetic distance using the COI mtDNA gene as a barcoding species the difference was 15.9%–16.7% with *H. diversicolor superteksta* from China, *H. diversicolor* from Taiwan (7.73%–9.12%), and *H. diversicolor* from Japan (7.80%–9.20%), because of boundary for determining species groups based on of 4% and mollusks at 4.8%, then *H. diversicolor squamata* spread in the southern islands of Java and Bali is worthy of being separated into its own species. From the results of this study, we propose that abalone from the waters south of Java and Bali became a separate species, *H. squamata* Reeve 1846.

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