Genetic variation of COI gene of *Hippa admirabilis* in Northern Sulawesi

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Abstract. Hippoid crab is crustacean species which inhabits sandy beach in tropical and subtropical area. One of them is *Hippa admirabilis*. Previously, this species was reported only in Sulawesi Island in Indonesia beside Taiwan and New Guinea. The aim of this study was to elucidate the genetic variation of *Hippa admirabilis* in Northern Sulawesi. Ten specimens were collected from two locations in the northern part of Sulawesi in 2016 which is Ogotumubu (Province of Central Sulawesi) and Gorontalo (Province of Gorontalo). The length of the amplified COI gene fragment is 596 bp. The obtained sequences are compared to our previous work in Genbank which is *Hippa admirabilis* from Banggai, Sulawesi. The phylogenetic tree was constructed to phylogeographic scenario based on Neighbor-Joining methods with Kimura 2-parameters models. The haplotype analysis was performed using DnaSP software. The phylogenetic tree shows that all of the *H. admirabilis* samples assembled into one clade. Five haplotypes of *Hippa admirabilis* was discovered in this study. There is one shared haplotype group with ten individuals from Gorontalo and Ogotumubu. The rest haplotype is exclusive belongs to each location. The nucleotide variation between Gorontalo, Ogotumubu, and Banggai (as reference sequence) was 8 nucleotide bases.

Keywords: genetic variation; haplotype; hippoid crab

1. Introduction

Hippoid crab is crustacean species which inhabits sandy beach in tropical and subtropical area. There are two families reported Hippidae and Albunidae in Indonesia, which comprises of several species. Family Hippidae has the characteristic of elongated carapace and telson also and pereiopod with the pointed end [1]. The Hippidae consists of seven species in Indonesia, one of them is *Hippa admirabilis*.

*Hippa admirabilis* has carapace covered with a transverse ridge which is numerous; frontal margin with 2 median lobes separated by small rounded lobe with lateral lobes greatly exceeding tips of median lobes. The lateral carapace surface of this species has a submarginal row of setose pits, around 49–51 in
numbers. Antennal flagellum composed of 1 or 2 articles. Pereopod I dactylus has sub-cylindrical shape. Pereopods II and III dactyls incurvar on dorsal margin [2].

*Hippa admirabilis* was firstly described from Papua New Guinea by Thallwitz in 1891 [3]. Later on, this species was reported in Taiwan and Indonesia (Ambon and Sulawesi Island) [4, 5]. Besides the occurrence and morphological information of this species, there is also information about its phylogenetic relationship with other Hippidae species based on COI gene [6]. So, the information of this species is comparatively impoverished, especially in genetic information. There is only one data about this species in Genbank, which is data of COI gene of *H. admirabilis* from Banggai Island, Sulawesi Province, Indonesia with accession number KR047031.1. Up to present, there had been no published results dealing with intraspecific genetic variation of hippoid crab in Indonesia. The intraspecific variation could be studied based on either nuclear or mitochondrial DNA (mtDNA). Mitochondrial DNA (mtDNA) shows a high level of polymorphism with an evolutionary rate that is 10 times faster than nuclear genes and is subjected to maternal inheritance [7]. Mitochondrial genes such as cytochrome c oxidase subunit I (COI) are popular markers used for the study of crustaceans at the species and population levels [8]. COI was used in this study because it is known as more variable than 16S rRNA gene e.g. in crayfish and shrimp [9, 10]. Also, COI gene appears to have a better phylogenetic signal than any other mitochondrial gene [11]. These properties make the mitochondrial COI gene an ideal molecular marker to identify genetic variation in natural populations [12]. Due to these reasons and availability in gene library, we used COI as a genetic marker in this study. Therefore in this study, we would like to elucidate genetic variation of *Hippa admirabilis* in Northern Sulawesi.

2. **Materials and methods**

2.1. **Materials**

The samples which were used in this study were from Dr. Achmad Farajallah collection. These samples were collected by Dr. Achmad Farajallah and Fahri, S.Si M.Si from two location of Northern Sulawesia area, which is Ogotumubu and Gorontalo area (figure 1) on July, 2016. As many as ten specimen were used in this study. Each specimen were named with format: scientific name_sampling site_specimen number, ie. *H. admirabilis* _Ogutumubu_ _3_, means scientific name of this specimen is *H. admirabilis*, collected from Ogutumubu area and its specimen number is 3.

![Figure 1. Map of Sulawesi Island indicating sampling sites of *H. admirabilis* in red circles.](image-url)
2.2. **DNA extraction**
Muscle tissues were obtained by cutting the pereiopods using sterile scissors to avoid DNA contamination from parasites or such. The muscle tissues, then, placed into 1.5 mL tubes, after that we added sterile aquadest until it was submerged, to remove the alcohol from the tissue by soaking the tissue for 20–30 minutes. Next, the samples were centrifuged with a speed of 10,000 rpm for 1 minute and the water was removed. The genomic DNA from the tissues was extracted using a DNA extraction kit (GENEAID Genomic DNA Mini Kit (Tissue) and conducted according to manufacturer protocol.

2.3. **COI gene amplification and visualization**
Amplification of COI gene fragment was performed in vitro using Polymerase Chain Reaction (PCR) with Biometra Thermo Cycle. Amplification of COI gene fragment was conducted using primer AF 215 (forward) 5’-TTC AAC AAA TCA TAA AGA TAT TGG-3’ and AF 216 (reverse) 5’-TAA ACT TCA GGG TGA CCA AAA AAT CA3’ [6] with GoTaq® Green Mastermix. This pair of primers amplified the upper part of COI gene, near its 5’ ends (figure 2).

![Figure 2. Primer binding site of forward primer AF215 and reverse primer AF216 in COI gene.](image)

PCR condition was set under the following condition: pre-denaturation 94°C (3 min), followed by 30 cycles of denaturation 94°C (1 min); annealing 55°C (1 min); and elongation 72°C (1 min), then post-elongation 72°C (2 min), and finalization 15°C (4 min). The obtained amplicons then migrated in polyacrylamide gel 6% with 180 volts for 55 min then visualized using silver staining method [13].

2.4. **Data analysis**
Amplicons from PCR were sent to 1st Base Company to be sequenced using Sanger method [14] using Applied Biosystem Big Dye® terminator kit V.3.1. The chromatograms were edited and analyzed using software MEGA 7 [15]. Alignment to obtain homolog sequence was performed using ClustalW which was embedded in MEGA 7. The standard option was chosen during alignment. Molecular identification using nucleotide comparison method with BLAST-N feature embedded in Genbank. Nucleotide composition, mutation point, nucleotide variation, haplotype diversity, were calculated using DNASP 5 [16]. The statistical method used to construct the phylogenetic tree is Neighbor Joining method with nucleotide substitution model of Kimura-2 Parameter with 1000 bootstrap using MEGA7. Many scientists used Neighbor-Joining method with Kimura-2-Parameter as a substitution model in analyzing the diversity pattern of COI gene [17, 18]. Genetic distance was performed using MEGA7, and calculated by considering all codon positions, disregarding all positions containing gaps or missing data, and we using Kimura-2 parameter as a model. In this analysis, we used *H. admirabilis* from Banggai (KR047031.1) as in-group together with our samples sequences (10 sequences) and for the outgroup were *Hippa adactyla* (KR047033.1), *Hippa ovalis* (KR047034.1), and *Emerita emeritus* (KR047035.1).

3. **Result and discussion**
The verification of our sequences was done with BLAST-N in Genbank, which compares with the published sequence of *Hippa admirabilis* from Banggai with an accession number of KR047031.1 [6]. The result shows similarity >99.6% of our sequences and the published sequence. The average
composition of our sequences are containing G+C content: 0.355 and A+T content: 0.64. From a total 596 bp COI gene which successfully amplified, there are 588 bp invariable/conserve nucleotides and 8 bp variable nucleotides (table 1). Two of the 8 nucleotide variations occur due to transversion, while the rest occur due to transition. Based on the nucleotide variation, 4 haplotypes were determined (table 2) with haplotype diversity value (Hd): 0.491. This shows the diversity of Hippa admirabilis of Northern Sulawesi is rather low.

Table 1. Nucleotide variation in COI gene of Hippa admirabilis.

| No | Haplotype | Nucleotide Position |
|----|-----------|---------------------|
|    |           | 123     195     225     291     303     432     492     595 |
| 1  | H-1       | T       C       A       G       G       A       G       G   |
| 2  | H-2       | C       T       G       .       A       T       A       .   |
| 3  | H-3       | C       T       .       .       A       T       A       .   |
| 4  | H-4       | .       .       .       A       .       .       .       T   |

Table 2. List of haplotypes of Hippa admirabilis in Northern Sulawesi.

| No | Haplotype | Sequence Name                          |
|----|-----------|---------------------------------------|
|    |           | H. admirabilis_Ogotumubu_3            |
|    |           | H. admirabilis_Ogotumubu_8            |
|    |           | H. admirabilis_Ogotumubu_13           |
|    |           | H. admirabilis_Ogotumubu_21           |
| 1  | H-1       | H. admirabilis_Gorontalo_1            |
|    |           | H. admirabilis_Gorontalo_3            |
|    |           | H. admirabilis_Gorontalo_5            |
|    |           | H. admirabilis_Gorontalo_4            |
| 2  | H-2       | H. admirabilis_Gorontalo_2            |
| 3  | H-3       | H. admirabilis_Ogotumubu_14           |
| 4  | H-4       | KR047031.1_Hippa_admirabilis_Banggai  |

Haplotype H-1 was regarded as a common haplotype in this study, due to almost all samples were included in this group. This haplotype was most similar to H-4, because there are only two variable nucleotides between these haplotypes. Meanwhile, H-1 most distinctive from H-2 due to six variable nucleotides between them.

The results of the genetic distance calculation are presented in the form of a data matrix (table 3). The genetic distance of COI gene fragments between Hippa admirabilis in Northern Sulawesi study sites is ranging from 0.00 – 0.014. Genetic distance could be influenced by multiple factors, such as geographical conditions, coverage of migrations areas, environmental characteristics, and genetic structure [19].

The phylogenetic tree (figure 3), shows there’s no grouping between species from Ogotumubu or Gorontalo. Intraspecies ingroup of Hippa admirabilis from each location have close relationship and forming one clade. This result could be fathomed caused by closeness of sampling area which still located in Tomini Bay with Banggai as center of Tomini Bay also, there is no barrier in Tomini Bay, Northern Sulawesi. Hippa admirabilis has close kinship with the outgroup species (H. adactyla, H. ovalis and Emerita emeritus) because they belongs in Family Hippidae. Larva of Family Hippidae is pelagic with long pelagic larval phase [20] which follows the sea current. Genetically mixed clade in phylogenetic tree are caused by several factors such as environmental condition, changes in haplotypes, random mating and connectivity between areas [21]. Indonesia is a biodiversity hotspot which to be believed has similar hippoid crab species number with Taiwan, Philippines, and Australia, because those area were located in Indo West Pacific area [2].
Table 3. Genetic distance of hippoid crab (Hippidae) using Kimura 2-Parameter model.

|    | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1  | 0.010 |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 2  | 0.010 | 0.000 |     |     |     |     |     |     |     |     |     |     |     |     |
| 3  | 0.000 | 0.010 | 0.000 |     |     |     |     |     |     |     |     |     |     |     |
| 4  | 0.000 | 0.010 | 0.000 | 0.000 |     |     |     |     |     |     |     |     |     |     |
| 5  | 0.000 | 0.010 | 0.000 | 0.000 | 0.000 |     |     |     |     |     |     |     |     |     |
| 6  | 0.000 | 0.010 | 0.000 | 0.000 | 0.000 | 0.000 |     |     |     |     |     |     |     |     |
| 7  | 0.000 | 0.010 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |     |     |     |     |     |     |     |
| 8  | 0.000 | 0.010 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |     |     |     |     |     |     |
| 9  | 0.008 | 0.002 | 0.008 | 0.008 | 0.008 | 0.008 | 0.008 | 0.008 | 0.008 |     |     |     |     |     |
| 10 | 0.000 | 0.010 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |     |     |     |     |
| 11 | 0.003 | 0.014 | 0.003 | 0.003 | 0.003 | 0.003 | 0.003 | 0.003 | 0.003 | 0.003 | 0.012 | 0.003 |     |     |
| 12 | 0.182 | 0.185 | 0.182 | 0.182 | 0.182 | 0.182 | 0.182 | 0.182 | 0.182 | 0.182 | 0.182 | 0.182 | 0.188 | 0.211 |
| 13 | 0.192 | 0.185 | 0.192 | 0.192 | 0.192 | 0.192 | 0.192 | 0.192 | 0.192 | 0.192 | 0.192 | 0.192 | 0.188 | 0.211 |
| 14 | 0.224 | 0.229 | 0.224 | 0.224 | 0.224 | 0.224 | 0.224 | 0.224 | 0.224 | 0.224 | 0.224 | 0.224 | 0.224 | 0.250 | 0.238 |

Note: 1 = H. admirabilis_Gorontalo1; 2 = H. admirabilis_Gorontalo2; 3 = H. admirabilis_Gorontalo3; 4 = H. admirabilis_Gorontalo4; 5 = H. admirabilis_Gorontalo5; 6 = H. admirabilis_Ogotumubu3; 7 = H. admirabilis_Ogotumubu8; 8 = H. admirabilis_Ogotumubu13; 9 = H. admirabilis_Ogotumubu14; 10 = H. admirabilis_Ogotumubu21; 11 = KR047031.1_H. admirabilis_Banggai; 12 = KR047033.1_Hippa adactyla; 13 = KR047034.1_Hippa ovalis; 14 = KR047035.1_Emerita emeritus

Figure 3. Phylogenetic tree of *Hippa admirabilis* based on COI gene fragment.

4. Conclusion
There are total 8 nucleotide variation in Northern Sulawesi *H. admirabilis*. These nucleotide variation forming four haplotypes of *H. admirabilis*, with H-1 regarded as common haplotypes. While other haplotypes were regarded as unique haplotype in respected area (Gorontalo, Ogotumubu, and Banggai).
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