Characterization of whole mitochondrial genome
Amyda cartilaginea from Dharmasraya, West Sumatera

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Abstract. Previous data of the whole mitochondrial genome of soft-shelled turtle that it’s available in a public repository: Dogania subplana, Pelochelys cantorii, Chitra indica, Trionyx triunguis, Apalone spinifera, Rafetus swinhoei, and Pelodiscus sinensis. The first two species have natural distribution in Indonesia. Amyda cartilaginea is a species of soft-shelled turtle that is abundant in Indonesia. However, the whole mitochondrial genome data of A. cartilaginea is not available. This study aims to characterize the partial mitochondrial genome and analyze the phylogenetic position of A. cartilaginea. We reported almost complete mitochondrial genome of A. cartilaginea that caught from Batanghari river in Dharmasraya District, West Sumatera. The amplification of mitochondrial DNA fragments was performed using several primers designed following mitochondrial gene organization and sequence of D. subplana (Accession No AF366350). We sequenced half of the whole mitochondrial genome (7757 bp, 48%). The gene organization of the mitochondrial genome of A. cartilaginea was identic with D. subplana and P. cantorii. The phylogenetic tree analysis based on 16SrRNA revealed that the position of A. cartilaginea clustered with another soft-shelled turtle. Further study is needed to make a complete sequence of the mitochondrial genome of A. cartilaginea, with special focus on the control region to be applied to sustainable wild population management.

Keywords: A. cartilaginea; mitochondrial DNA; soft-shelled turtle; 16SrRNA

1. Introduction

Soft-shelled turtles belong to the order Testudines, which have morphologically unique and they are grouped into family Trionychidae. The soft-shelled turtles (Trionychidae) are highly aquatic because it has a derivative morphological character that has evolved and finally can adapt to an aquatic environment. In addition, they have several characteristic features that can distinguish them from other families include having a much reduce bony shell and leathery skin covering the shell surface, the body shape is flattened, and webbed toes [1, 2]. Soft-shelled turtles (Trionychidae) are divided into two subfamilies, namely Trionychinae and Cyclanorbinae with 31 species and 13 genera [3]: Trionyx, Chitra, Pelochelys, Amyda, Nilssonia, Dogania, Palea, Rafetus, Apalone, Pelodiscus, Lissemys,
Cycloderma and Cyclanorbis [4]. They are geographically widespread occurring in Africa, Asia (including New Guinea), the Mediterranean, and North America [5, 6].

Four species of soft-shelled turtle spread naturally in Indonesia, that is Dogania subplana, Pelochelys cantorii, Chitra indica, and Amyda cartilaginea. In addition, there is one introduced turtle is Pelodiscus sinensis [7]. Most of the soft-shelled turtles live in fresh water such as rivers, lakes, swamps, and puddles both in tropical and subtropical areas [8]. A. cartilaginea is a member of the family Trionychidae which is also known as the Asian soft-shelled turtle because it is spread in most Asian countries including India, Myanmar, Laos, Vietnam, Cambodia, Thailand, and Indonesia [5, 9]. In Indonesia, A. cartilaginea can be found in Kalimantan, Sumatra, Java, Lombok, and recently reported distribution in Sulawesi [10, 11]. Although A. cartilaginea is distributed in Indonesia, the genetic characteristics data of this species is still limited. On the other hand, there is a phenomenon of overharvesting due to a high increase in market demand (food consumption, traditional medicine, and aquarium ornament). Therefore, the DNA sequence data generated from this study has the potential for conservation actions and population monitoring for sustainable wild harvest.

One of the ways to know the genetic characteristics of a species can be done by using mitochondrial DNA (mtDNA). mtDNA data analysis is moderately easy to compare with the nuclear genome because the mtDNA has a conserved gene sequenced, lack of introns and no recombination, therefore the mtDNA is very popularly used. The nucleotide substitution rate in mtDNA is high [12] and thus provides a rich source of variable characters. In a public repository, several species of soft-shelled turtles have the data on the characterization of the whole mitochondrial genome, include Dogania subplana, Pelochelys cantorii, Chitra indica, Trionyx triunguis, Apalone spinifera, Rafetus swinhoei, and Pelodiscus sinensis. In this study, we characterized a partial mitochondrial genome of A. cartilaginea captured from Dharmasraya, West Sumatra. This study aims to analyze the phylogeny position of A. cartilaginea with other soft-shelled turtles based on 16S rRNA in mitochondrial whole-genome data.

2. Material and method

2.1. Sample collection and mitochondrial DNA extraction

The A. cartilaginea samples were captured from the Batanghari river in the Dharmasraya District, West Sumatra Province, Indonesia (figure 1). The tissue sample of A. cartilaginea was stored in 70% ethanol. DNA extraction from tissue samples was carried out using Genomic DNA mini kit: Geneaid NR8329 GT 300. The extraction process followed the procedure set provided in the kit.

Figure 1. The sample of A. cartilaginea from Batanghari river.
2.2. Mitochondrial DNA amplification and sequencing

Mitochondrial DNA amplification was performed using PCR techniques. The total volume of the PCR reaction was 30 μL consisting of 13.4 μL of nuclease-free water, 0.3 μL of forward primer, 0.3 μL of reverse primer, 1 μL of template DNA, and 15 μL of Promega Go Taq Green. Amplification was performed using several primers (table 1) designed by following the mitochondrial genome of *D. subplana* from the National Center for Biotechnology Information (NCBI) database with an accession number AF66350. PCR conditions were: predenaturation (94 °C, 5 minutes), denaturation (94 °C, 1 minute), annealing (53-55 °C, 1 minute), extension (72 °C, 1 minute), and post extension (72 °C, 2 minutes). Amplification products were separated using electrophoresis on 6% polyacrylamide gel (PAGE) at 200 V for 45 minutes. Amplikon was visualized with Diamond Nucleic Acid Dye [13].

PCR sequencing and determination of nucleotide sequences used Malaysia's first-base sequencing service from PT Genetika Science, Indonesia.

**Table 1.** List of primers used for amplification and sequencing of the mitochondrial genome in *D. subplana* (No ACC GeneBank AF366350; [22]).

| No. | Target Gene | Code | F/R | PCR Primer 5′------3′ |
|-----|-------------|------|-----|-----------------------|
| 1   | 12S         | AF591| F   | AGATTTGGTCTCTAATCTTAATGTTAAC |
|     | 12S         | AF592| R   | GCACCTTCCGGTACACCTTAC   |
| 2   | 12S         | AF593| F   | CCGCCCGTCACCCCCCACTCAAC |
|     | 16S         | AF594| R   | CCTCGTTTAGCCCTTTCTATA   |
| 3   | COI         | AF601| F   | CCTAAACCCCAAAAAATAAGA   |
|     | COI         | AF602| R   | TATGATGGGCTCATACAAARAA  |
| 4   | COII        | AF603| F   | GTTTGGAATAATCTCCACGTA   |
|     | COII        | AF604| R   | GTGTTGGGAATTATGTAYGAGTC  |
| 5   | COII        | AF605| F   | ATCGTYCTAATYACYATTGCAC   |
|     | ATP6        | AF606| F   | GTATTTGGTGTTTGTTGGTGCGTA  |
| 6   | ATP6        | AF607| F   | CACACCAACYACACACACTATC   |
|     | COIII       | AF608| F   | AAYTGAAYTGAATGATTGGGAAG  |
| 7   | ND4         | AF609| F   | CCAACCAACACACATATGATA    |
|     | ND4         | AF610| R   | GTGTGTTGAGTACTTCTCCACATT |
| 8   | ND5         | AF613| F   | CCGCCACAAATATCCCACATT   |
|     | ND5         | AF614| R   | TATTTGGGCTGATTTAATGAGA   |
| 9   | ND6         | AF615| F   | CCGCAGCCTATAGCCTACTAGAT  |
|     | CYTB        | AF616| R   | GCTTGTTAGTGTCATTAGTACT   |
| 10  | ND6         | AF617| F   | TAATAAGGAGAAGGACCAGAAG   |
|     | CYTB        | AF618| R   | TGCTTCTCGATCTGATTART     |

F: forward, R: reverse, A: Adenine, G: Guanine, C: Cytosine, T: Timine (nucleotide base), R: nucleotide code for Adenine or Guanine.

2.3. Bioinformatic analysis

The nucleotide bases of the mitochondrial genome of *A. cartilaginea* Dharmasraya were edited and aligned using the MEGA 7.0 program. Protein coding genes and ribosomal RNA were searched using BLAST search. The relationship analysis was reconstructed using the 16SrRNA gene from 8 species in the family Trionychidae and one species from the family Geomidae (*Cuora trifasciata*) was selected as the outgroup. All species used in the reconstruction of phylogeny trees were downloaded from the National Center for Biotechnology Information (NCBI) database (except for *A. cartilaginea* which was used in this study) (table 2). 16SrRNA gene sequences from 9 species were aligned using Clustal W alignment on MEGA 7.0 [14]. Phylogeny tree was inferred using the Neighbor-joining method [15], with a bootstrap values of 1000 replicates [16]. The evolutionary distances were computed using the Kimura 2-parameter method [17].
Table 2. Mitochondrial genome information was used in this study.

| Accession Number | Species                  | Origin     | Reference |
|------------------|--------------------------|------------|-----------|
| Ingroup          |                          |            |           |
| NC_002780        | Amyda cartilaginea       | Indonesia  | This study|
| NC_015825        | Dogania subplana         | Indonesia  | [22]      |
| NC_026028        | Pelochelys cantorii      | China      | [23]      |
| NC_012833        | Chitrea indica           | China      | [19]      |
| NC_017901        | Trionyx trianguloides    | Saudi Arabia| [24]    |
| NC_021371        | Apalone spinifera        | China      | [19]      |
| NC_006132        | Pelodiscus sinensis      | China      | [19]      |
| Outgroup         |                          |            |           |
| NC_022857        | Cuora trifasciata        | China      | [31]      |

3. Results

3.1. Gene organization in the mitochondrial DNA of A. cartilaginea Dharmasraya
The A. cartilaginea Dharmasraya mitogenome was amplified using several primers designed following mitochondrial gene organization and sequence of D. subplana (Accession No AF366350) and the results of the alignment of the nucleotide base sequences were obtained 7757 bp from the total estimated length of the mitochondrial genome of 17,000 bp. Thus, this study has obtained data as much as 48% of the whole mitochondrial genome in A. cartilaginea. Based on the alignment results, this study was able to characterize 2 ribosomal RNA (rRNA)-coding genes, 5 transfer RNA (tRNA)-coding genes, and 8 protein-coding genes. The structural organization of the partial mitochondrial genome of A. cartilaginea Dharmasraya is shown in table 3.

Table 3. Mitochondrial genome organization in A. cartilaginea Dharmasraya.

| Gene    | Position | Size (bp) |
|---------|----------|-----------|
| 12S rRNA| 1-966    | 966       |
| tRNA-Val| 967-1036 | 70        |
| 16S rRNA| 2037-2017| 981       |
| COI     | 2018-2774| 757       |
| tRNA-Ser| 2775-2845| 71        |
| tRNA-Asp| 2846-2914| 69        |
| COII    | 2915-3269| 355       |
| ATP6    | 3270-3658| 389       |
| COIII   | 3659-4442| 784       |
| tRNA-Gly| 4443-4472| 30        |
| ND4     | 4473-5644| 1172      |
| ND5     | 5645-6154| 510       |
| ND6     | 6155-6679| 525       |
| tRNA-Glu| 6680-6750| 71        |
| CytB    | 6751-7757| 1007      |

3.2. Phylogenetic analysis
The phylogenetic tree produced by Neighbor-joining analysis (figure 2) using the 16SrRNA gene (this region is highly conserved), and bootstrap support is presented together on the tree. The phylogenetic tree constructed by 16SrRNA place A. cartilaginea Dharmasraya within other soft-shelled groups. The analysis results were divided into two clusters, that is the family Trionychidae and family Geomidae (C. trifasciata) as outgroups. The family Trionychidae cluster is divided into two large clusters, that is...
cluster 1 consisting of *A. cartilaginea*, *D. subplana*, *R. swinhoei*, *P. sinensis*, and *A. spinifera*. While cluster 2 consists of *T. triunguis*, *P. cantorii*, and *C. indica*. These results are consistent with previous studies by Le et al. [18] and Li et al. [19]. The result also showed that *A. cartilaginea* is a sister to *D. subplana* with bootstrap values 67% and these results are consistent with previous studies from Le et al. [18], Li et al. [19] and Yu et al. [20] which are placed *A. cartilaginea* as a sister to *D. subplana* with different bootstrap values.

![Figure 2](image)

**Figure 2.** Phylogenetic tree by the neighbor-joining method, based on the nucleotide sequences of 16SrRNA of 8 Trionychidae species and one outgroup. Node numbers showed the values of bootstrap support.

4. Discussion
The mitochondrial genome in vertebrates consists of a circular DNA sequence of 16-18 kb containing 22 transfer RNA (tRNA)-coding genes, 2 ribosomal RNA (rRNA)-coding genes, 13 peptide coding genes and control region [21]. In this study, the nucleotide bases of *A. cartilaginea* Dharmasraya obtained 7,757 bp of the estimated whole mitochondrial genome of 17,000 bp, which is about 48%. The total length of the mitochondrial whole genome in Trionychidae is about 16,000–17,000 bp. Based on data from the repository, that several species of family Trionychidae have been recorded to have the complete whole mitochondrial genome include: *D. subplana* (17 289 bp) [22], *P. cantorii* (17 499 bp) [23], *C. indica* (16 726 bp) [19], *T. triunguis* (16 590 bp) [24], *A. spinifera* (16 749 bp) [19], *R. swinhoei* (16 990 bp) [19], and *P. sinensis* (17 364 bp) [19]. The gene organization in *A. cartilaginea* is the same as the gene organization in other species of the family Trionychidae. Based on organization genes that arrangement *A. cartilaginea* that is identical with *D. subplana* and *P. cantorii*. However, in this study only 15 genes from *A. cartilaginea* species were obtained. For this reason, further study is needed to obtain the whole mitochondrial genome in *A. cartilaginea*.

The phylogenetic relationships of soft-shelled turtles have been studied for decades. Comprehensive phylogenetic analysis of soft-shelled turtles first presented by Meylan [1] using skeletal morphology.
The result of Meylan’s phylogenetic divide the family Trionychidae into Trionychinae and Cyclanorbinae. Since the mtDNA popular, phylogenetic studies using mtDNA have been carried out to generate new insights into the phylogenetic relationships of soft-shelled turtles [6, 18, 25, 26, 27]. Phylogenetic analysis of this study generally agrees with Engstrom et al. [6]. Based on the results in this study, phylogenetic analysis constructed by 16SrRNA placed A. cartilaginea Dharmasraya within other Trionychidae groups. The trees showed that Trionyx, Pelochelys and Chitra is a sister taxa supported by strong bootstrap values. This result is consistent with Engstrom et al. [6], Le et al. [18], Li et al. [19] and they also showed the strong support of bootstrap values. In addition, these species have a larger body size [6] and are also known as giant soft-shelled turtles. The result also showed that Apalone is a sister genus to Amyda, Dogania, Rafetus, and Pelodiscus.

A. cartilaginea has the closest relationship to D. subplana compared to other Trionychidae species. Amyda and Dogania are soft-shelled turtles that spread naturally in Indonesia, these two species are difficult to distinguish in detail [28], however A. cartilaginea is often found in the downstream area of the river while D. subplana is more often found in the upstream river. A. cartilaginea is one of the soft-shelled turtles that are abundant in Indonesia, but its existence is now difficult to find due to overharvesting. Soft-shelled turtles of A. cartilaginea are generally used for consumption, traditional medicinal ingredients and also as aquarium ornament [29]. In 2004, CITES (Convention on International Trade on Endangered Species of Wild Flora and Fauna) has stated that with the excessive use of A. cartilaginea in several countries where it is distributed, the population of A. cartilaginea has been decreased. So that, in 2005 A. cartilaginea was registered as animals in Appendix II. Furthermore, in 2010 IUCN (International Union for Conservation Nature) also gave a “vulnerable” status [30].

5. Conclusion
We provide the partial mitochondrial genome of A. cartilaginea that caught from Dharmasraya West Sumatera with a sequence length of 7757 bp which contains 2 ribosomal RNA (rRNA)-coding genes, 5 transfer RNA (tRNA)-coding genes, and 8 protein-coding genes. The organization of the mitochondrial genome of A. cartilaginea is identic with D. subplana and P. cantorii. Further study is needed to make a complete sequence of the mitochondrial genome of A. cartilaginea, with special focus on the control region to be applied to sustainable wild population management.

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