DNA barcoding of sea turtles (Dermochelyidae and Cheloniidae) and its protocol using different tissues quality: implication to conservation managers

H H Madduppa1*, S Bahri2, B Subhan1, N P Anggraini1, H Ohoiulun3, T Abdillah3, D Arafat1, P Santoso1 and I M Sangadji4

1Department of Marine Science and Technology, Faculty of Fishery and Marine Science, Bogor Agricultural University, Bogor, Indonesia
2Department of Marine Science, Faculty of Fishery and Marine Science, University of Teuku Umar, Aceh, Indonesia
3WWF-Indonesia Inner Banda Arc Subseascape, Langgur, Indonesia
4Balai Kawasan Konservasi Perairan Nasional (BKKPN) Kupang, Kupang, Indonesia

*E-mail: hawis@apps.ipb.ac.id

Abstract. Conservation effort of sea turtles faces several challenges, e.g. habitat destruction, eggs and turtle meat consumptions, or production of souvenirs (e.g. turtle bodies). In their duties, conservation officers may find sea turtles in different condition (e.g. body fragments, eggs, pieces of meat, etc.), usually it is difficult to identify due to incomplete morphology. Therefore, the use of DNA barcoding becomes an alternative for for identification at species level, contribute taxonomic and biodiversity research. This study was conducted to develop a protocol suitable for identifying sea turtles from different tissue samples conditions using DNA barcoding. A total of 16 tissue samples in different condition (fresh, dead-body, smoked-meat) were collected. A protocol was developed to enable identification of tissue samples of different quality or condition. A 719 bp control region fragment was analyzed. The high percentage similarity was confirmed in GenBank CO1 sequence with 99%-100%. Four sea turtle species were identified among the samples, i.e. Chelonia mydas (4 samples), Lepidochelys olivacea (9 samples), Eretmochelys imbricata (2 samples) and Dermochelys coriacea (1 samples). This study was successfully amplified by using DNA target of control region and therefore, will be beneficial for conservation management of sea turtles in Indonesia.

Keywords: conservation genetics, coral triangle, DNA forensic, marine conservation, molecular identification

1. Introduction

Seven species of sea turtles in the world belonged to two families i.e. Dermochelyidae and Cheloniidae. These include Dermochelyidae (Leatherback Dermochelys coriacea) and Cheloniidae (the loggerhead Caretta caretta, the green Chelonia mydas, the hawksbill Eretmochelys imbricata, the Kemp’s Ridley Lepidochelys kempii, the Olive Ridley Lepidochelys olivacea, Flatback Natator depressus and Leatherback (Dermochelys coriacea) [1, 2]. Five out of seven identified sea turtles species are distributed in Indonesia [3]. However, many of sea turtles are currently facing different threats. The disruption of turtle habitat is one of population decline. Also, humans look for eggs and turtle meat to be consumed and make souvenirs that make the decline of turtle populations [4]. For example, people collecting eggs and meat of sea turtle is found in some areas such as Pangumbahan, Sukabumi, where
in this location, the most widely hunted is the egg of *Chelonia mydas*. While in Kei Island, some people also consume turtle meat from *Dermochelys coriacea*.

The mitochondrial DNA (mtDNA) Barcoding is widely used in identifying different species, such as sea cucumber [5], groupers [6], sharks [7-9], rays [10], soft coral [11, 12], gastropods [13], and tuna [14-16]; which contributes to taxonomic and biodiversity research. DNA barcoding methods have been used in conservation works, e.g. identification of fish eggs or larvae or analysis for stomach contents [17]. One of advantages of DNA barcoding over traditional species condition is that the method can be used for animals with no complete morphology, such as headless animals or just fraction of its body or even a small piece of tissue samples [18]. DNA barcoding can help identify species captured illegally from tissue samples [19]. DNA barcoding is applicable to any condition of animals, such as pieces of flesh, eggs, shells, and bones that are consumed or sold illegally [19]. In turtle conservation, officers may find the condition of the turtles is not necessarily alive. Hence, conventional identification of the turtles become difficult. This study was conducted to develop a species identification protocol suitable for any condition of seaturtles, and to reconstruct phylogenetic trees of the identified samples, and develop genetic database of four important sea turtles in Indonesia.

2. Materials and Methods

2.1. Tissue sample collection and preservation

A total of 16 tissue samples from different locations were collected. Fresh tissue samples were collected from Padang (West Sumatera), Pengumbahan (West Java), Kapoposang (South Sulawesi), Kupang (East Nusa Tenggara), dead turtle-tissue samples were collected from Teluk Cendrawasih (Papua) and Tidung Island (Seribu Island), and one tissue smoked-meat sample was collected from Kei Island. The tissues were preserved in tube 1.5 mL with ethanol of 96%.

2.2. Extraction, PCR, electrophoresis and data analysis

The tissue samples were extracted by using GSYNC extraction kit for tissue and blood and 10% Chelex Solution. A fragment of mitochondrial D-loop control region was amplified using the following primer set: LTEi9 (GGG AAT AAT CAA AAG AGA AGG) and H950 (GTC TCG GAT TTA GGG GTT TG) [18]. The target for this identification used mtDNA with locus Dloop. Polymerase Chain Reaction (PCR) was conducted in 25 µL reaction volume containing 1 µL template DNA, 12.5 µL Kapa Master Mix, 1.25 µL of each primer (10 mM), and 9 µL ddH2O. PCR conditions were: initial denaturation at 95°C for 3 min, followed by 38 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min. The final extension step was conducted at 72°C for 1 min. The quality of PCR products was assessed by 1% agarose gel electrophoresis and ethidium bromide staining and visualized using UV transilluminator. All good PCR products were sent to sequencing facility at First Base, Malaysia. Sequences were aligned and edited in Mega 6 [20]. A Neighbour-Joining (NJ) tree was constructed in Mega 6 [20] based on Kimura 2-parameter model, and 1,000 bootstrap replicates.

3. Results and Discussion

3.1. Protocol optimation

Protocol optimization was developed for tissue sampling, preservation, extraction and amplification (table 1). The fresh tissue samples was collected from flipper of sea turtles, following [21, 22] protocol. The tissue samples were taken from the surface near flipper for the adult of sea turtles and from the flipper with the smooth skin for small sea turtles. The tissue samples were collected using scalpel and took gently into 7-8 mm sizes. The dead-tissue and smoked-dead samples were collected from any part of available pieces of meat. All tissues were preserved in ethanol of 96% although they can also be preserved in 50%-70% isopropanol or use of NaCl [21].
Table 1. Protocol optimization for tissue sampling, preservation, extraction and amplification.

| Tissue type      | Tissue sampling                  | Preservation   | Extraction                         | Amplification                        |
|------------------|----------------------------------|----------------|------------------------------------|--------------------------------------|
| Fresh            | Nail of skin of flipper          | Ethanol 96%    | Follow normal protocol: 10% Chelex Solution | H: 95°C for 30 s                    |
|                  |                                  |                |                                     | A: 50°C for 30 s                     |
|                  |                                  |                |                                     | E: 72°C for 1 m                     |
| Dead-tissue      | Nail of flipper or any part of body | Ethanol 96%    | Heat incubation for night over, Extraction kit 10% Chelex Solution | H: 95°C for 30 s                    |
|                  |                                  |                |                                     | A: 50°C for 30 s                     |
|                  |                                  |                |                                     | E: 72°C for 1 m                     |
| Smoked-dead      | Any part of body (meat and soft bone) | Ethanol 96%    | Heat incubation for overnight, Extraction Kit for Blood and Tissue | H: 95°C for 30 s                    |
|                  |                                  |                |                                     | A: 50°C for 30 s                     |
|                  |                                  |                |                                     | E: 72°C for 1 m                     |

The genetic analysis was performed using mtDNA with target Control Region D-Loop [18]. Two different methods of tissue extraction were applied, i.e. 10% Chelex Solution [23] and GSYNC Extraction Kit for Blood and Tissue, by following company’s protocol. For the first method, the temperature to tissue lysis was 95°C for about 2-3 hours. The chelex solution was used for fresh tissue and dead tissue. For the second method (GSYNC Extraction Kit for Blood and Tissue) was used for smoked-dead samples with temperature to lysis tissue is 60°C for about 8 hours. The process of PCR in this research used primer from [18] with a modify protocol. Our protocol for PCR was conducted in 25 µL reaction volume containing 1 µL template DNA, 12.5 µL Kapa Master Mix, 1.25 µL of each primer (10 mM), and 9 µL ddH2O. PCR conditions were: initial denaturation at 95°C for 3 min, followed by 37 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min. The final extension step was conducted at 72°C for 1 min. If we get low band DNA, we can use the 3 µL template DNA.

3.2. Phylogenetic reconstruction

A total of 16 tissue samples from different quality of tissue was successfully amplified by using DNA target of control region. A 719 bp control region fragment was analyzed. The high percentage similarity was confirmed in GenBank CO1 sequence with 99%-100% (table 2). Three seaturtle species was confirmed including *Chelonia mydas* (4 samples), *Lepidochelys olivacea* (9 samples), *Eretmochelys imbricata* (2 samples) and *Dermochelys coriacea* (1 samples).

Table 2. Percentage similarity of targeted CO1 sequence confirmed in GenBank.

| No | ID number               | bp  | Species ident       | % of BLAST |
|----|-------------------------|-----|---------------------|------------|
| 1  | 2516695_02a_LTEI9       | 719 | Dermochelys coriacea| 99%        |
| 2  | 14. che_PGB_33_PB_H950  | 719 | Chelonia mydas      | 100%       |
| 3  | 16.che_Padang_1_PB_LTEI9| 719 | Chelonia mydas      | 100%       |
| 4  | 13.che_PGB_16_PB_LTEI9  | 719 | Chelonia mydas      | 100%       |
| 5  | 17.che_PDG_2_PB_LTEI9   | 719 | Chelonia mydas      | 100%       |
| 6  | 02.che_TC_43_PB_H950    | 719 | Lepidochelys olivacea| 100%     |
| 7  | 23.che_KPP_06_PB_LTEI9  | 719 | Lepidochelys olivacea| 100%     |
| 8  | 29.che_KPG_PB_H950      | 719 | Lepidochelys olivacea| 100%     |
| 9  | 1997576_KPG_19_H950     | 719 | Lepidochelys olivacea| 99%      |
| 10 | 1997578_KPG_2_H950      | 719 | Lepidochelys olivacea| 100%     |
| 11 | 03.che_TC_14_PB_LTEI9   | 719 | Lepidochelys olivacea| 100%     |
| 12 | 04.che_TC_24_PB_LTEI9   | 719 | Lepidochelys olivacea| 100%     |
| 13 | 24.che_KPP_07_PB_LTEI9  | 719 | Lepidochelys olivacea| 100%     |
| 14 | 26.che_KPP_08_PB_LTEI9  | 719 | Lepidochelys olivacea| 100%     |
| 15 | ITK_TID_SIK_40_LTEI9_F  | 719 | Eretmochelys imbricata| 100%     |
| 16 | ITK_TID_SIK_03_LTEI9_F  | 719 | Eretmochelys imbricata| 99%      |
The distance matrix was calculated by Kimura 2-Parameters. The average value from differentiation between Codon or nucleotide will use for distance matrix [24]. Nucleotide divergence among species was ranging from 0.142 for *Eretmochelys imbricata* and *Lepidochelys olivacea* to 0.242 for *Dermochelys coriacea* and *Eretmochelys imbricata* (table 3). The genetic distance showed differences between population where the population was had differences value. The differences were likely influenced by several factors, such as genetic drift and natural selection [25].

Table 3. Inter-specific genetic distance matrix (LO: *Lepidochelys olivacea*, CM: *Chelonia mydas*, DC: *Dermochelys coriacea*, EI: *Eretmochelys imbricata*).

| Species | LO  | CM | DC  | EI  |
|---------|-----|----|-----|-----|
| LO      | -   | 0.165 | -   | -   |
| CM      | 0.165 | -   | 0.213 | 0.242 |
| DC      | 0.213 | 0.263 | -   | -   |
| EI      | 0.142 | 0.165 | 0.242 | -   |

The phylogenetic tree have a four clade which represents each species: clade one for *Lepidochelys olivacea*, clade two for *Eretmochelys imbricata*, clade three for *Chelonia mydas* and clade four for *Dermochelys coriacea* (figure 1). The control region target can discriminate all the species and made two clades between Cheloniidae and Dermochelyidae (figure 1). *Chelonia mydas* and *Lepidochelys olivacea* were identified as close related because of they from same family, Cheloniidae. *Dermochelys coriacea* was from family Dermochelyidae [26-27].

Figure 1. The phylogenetic tree using Neighbour-Joining from 16 sequences and three additional sequences from genbank for four seaturtle species, with 1000x bootstrap, and the value of the tree is indicated on the node of the branches.

DNA barcoding promises to be a powerful tool for species identification and other conservation in marine turtles [28]. Commonly, DNA barcoding used the COI target. The COI target was proposed to
be a good candidate for barcoding animal species [29], for example on sharks [7-9], grouper [6], and tuna [14-16] have been successfully applied. Others researches have suggested that loci might also serve as a basic for species identification, such as control region. Sometimes, control region sequences have been used for wildlife forensic [30]. Furthermore, control region is more variable than COI to genetic population [31]. Characteristic species control region sequences are can identify seaturtle species using DNA barcoding. This method can also be used during the fieldwork when identifying lost nest and animal stranded [2]. DNA barcoding can be used for forensic litigation when turtles eggs or meat only available materials [2]. This turtle identification protocol can be used by law enforcement officers, especially in customs and quarantine bodies at airports and ports. With this protocol, the law enforcers can identify quickly with molecular techniques, especially parts of turtles that have been cut off and are difficult to identify morphologically.

4. Conclusion

This study has developed protocols to enable identification of tissue samples of different quality or condition. From 719 bp control region fragment analyzed showed high percentage similarity to GenBank COI sequence with 99%-100%. Four seaturtle species were identified among the samples, i.e. *Chelonia mydas* (4 samples), *Lepidochelys olivacea* (9 samples), *Eretmochelys imbricata* (2 samples) and *Dermochelys coriacea* (1 samples). This study was successfully amplified different tissue samples sources by using DNA target of control region and therefore, will be beneficial for conservation management of sea turtles in Indonesia.

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