DNA barcoding as a method for species identification: case study in *Ahaetulla* snake

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**Abstract.** Species identification using molecular analyses has recently been developed, one of which is DNA barcoding method. The purpose of this study was to apply the DNA barcoding method to identify *Ahaetulla* snake which previously had not been identified molecularly. The specimens used came from Sumatra, Java, Bali, Lombok and additional samples obtained from Genbank. Cytochrome B gene was extracted and amplified to obtain DNA barcode. Phylogenetic analysis using ML method was used to determine the kinship relationship of the *Ahaetulla* snake. Morphological analyses were added to confirm the results of molecular identification. The results of molecular analysis using DNA barcoding method showed the presence of two *Ahaetulla* species in this study, there are *A. prasina* and *A. mycterizans*. This difference is indicated by the formation of two separate main clades, clade I contains *A. prasina* and clade II contains *A. mycterizans*. The results of the morphological analyses also showed differences in the character of the two different clade species. Thus, molecular analysis results agree with morphological analyses. It can be concluded that the DNA barcoding method can be used as a valid method to identify *Ahaetulla* snake species.

1. **Introduction**

Traditional method for species determination is by identifying morphological characters compare to the other species. However, this traditional method fails to discriminate some species due to several conditions, for example, species that having various external body colorations especially when specimens are not fresh [1], museum preserved species [2] and samples showing phenotypic plasticity [3].

Nowadays, DNA barcode attracts the taxonomies attention as the latest system in the identification of almost all fauna species, both interspecific and intraspecific, quickly and accurately. DNA barcoding uses small regions of mitochondrial DNA (mtDNA) that used as a barcode to amplify a gene. Various primers set of mitochondrial gene for barcoding are available in published studies [4]. With DNA barcode, identification by taxonomies will be easier. This identification technique fully supports the improvement of animal classification as well as helps to sort out any ambiguity at the species level. However, DNA barcoding with the support of traditional taxonomy has the capability to identify species complexes within populations [5].

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The COI gene is a part of mtDNA which is commonly used as a barcode to identify species, however, the cyt b gene is also part of mtDNA which has a high mutation rate, therefore it also can be used as a barcode. The variation in the Cyt B sequence causes this gene to be widely used to compare species in the same genus or family. The uniqueness of the Cyt B gene sequence is that there are parts that are conserved in species level, so it can be used for grouping based on animal species or for determining kinship relationships between animal species [6]. The results of the study by Laopichienpong et al. [7] showed that the use of the Cyt B gene was more effective for the identification of Ahaetulla snakes than the COI gene.

In this study, Cyt B gene was isolated from Ahaetulla snake that widely distributed in Indonesia. There are at least three species distributed in this country, A. prasina, A. mycterizans, and A. fasciolata [8]. Although it is widely distributed with vulnerable population, this snake is also threatened by human killed and traded. Faith and Williams [9] argued that the most significant contribution of DNA barcoding to conservation efforts is its role in improving and speeding up phylogenetic diversity assessments. Previous study by Leo et al.[10] showed that there are no significant morphological differences among A. prasina of Java and Sulawesi. Therefore, it is necessary to do research about identification of Ahaetulla snake using molecular analyses.

This study aimed to hold molecular analyses by using Cyt B gene as barcode in Ahaetulla snake of Indonesia. Morphological analyses also added to confirm the validity of molecular analyses.

2. Materials and methods

2.1. Samples collection

All samples were collected from 10 localities along Sumatra, Java, Bali, and Lombok Island (Figure 1). The total of 10 samples including of 3 samples of Sumatra, 5 samples of Java, a sample of Bali and a sample of Lombok. Four samples were taken from Genbank used as outgroup (www.ncbi.nlm.nih.gov). There are A. prasina, A. mycterizans, Chrysopelea ornata, and Dendrelapis pictus.

Figure 1. Distribution area of Ahaetulla Genus in Indonesia used in this study edited by QGIS software. Source of map: www.naturalearthdata.com.
Table 1. List of specimens used as in group and outgroup in the phylogenetic analysis.

| Specimens       | Locality              | Genbank Accession no. | Source    |
|-----------------|-----------------------|-----------------------|-----------|
| Ahaetulla sp.   | Lombok, NTB           |                       | This study|
| Ahaetulla sp.   | Sukawati, Bali        |                       | This study|
| Ahaetulla sp.   | Banyuwangi, East Java |                       | This study|
| Ahaetulla sp.   | Malang, East Java     |                       | This study|
| Ahaetulla sp.   | Nusakambangan, Central Java |                   | This study|
| Ahaetulla sp.   | Bandung, West Java    |                       | This study|
| Ahaetulla sp.   | Pandeglang, Banten    |                       | This study|
| Ahaetulla sp.   | Padang, West Sumatra  |                       | This study|
| Ahaetulla sp.   | Bukittinggi, West Sumatra |                 | This study|
| Ahaetulla sp.   | Medan, North Sumatra  |                       | This study|
| A. prasina      | Thailand              | LC105637              | [7]       |
| A. mycterizans  | Thailand              | KX660437              | [11]      |
| Chrysopelea     |                       |                      | [12]      |
| ornata          |                       |                      |           |
| Dendrelaphis    |                       | KY700863              | [13]      |
| pictus          |                       |                      |           |
| Malang, East Java |                  |                      |           |

2.2. DNA extraction

Cyt B fragments was amplified by PCR using Forward – L14910 (5’-GAC CTG TGA TMT GAA AAC CAY CGT TGT -3’) and Reverse – H16064 (5’- CTT TGG TTT ACA AGA ACA ATG CTT TA -3’) primer [14]. The cycle of PCR was set for pre denaturation 940 for 7 mins then followed by 40 cycles of denaturation 940 C 30 seconds, annealing 460 C 30 seconds, extension 720 C 60 seconds, and post extension 720 7 mins. [14]. The amplification product was sequenced using the same primer as PCR amplification.

2.3. Phylogenetic analyses

Cyt B sequences were contiged by Sequencher (Gene codes, Ann Arbor, Michigan, USA). The sequences were aligned using MEGA7 by Clustal W method and genetic distance was calculated as uncorrected pairwise distance [15]. Phylogenetic tree was inferred using maximum likelihood (ML) method. For ML, tree was constructed by RAxML using automatically bootstrap option by RAxML. Bootstrap value equal or more than 70% was defined as significant value [16].

3. Result and Discussion

3.1. DNA barcoding analyses

The results of the genetic distance analysis showed that there were two identified species based on the genetic comparisons with A. prasina and A. mycterizans sequences from Genbank. The genetic distance between A. prasina and A. mycterizans is about >6%. According to Jeong [17], Cyt B sequences in reptiles that have a genetic distance >5% are considered as different species. The results of this genetic distance have identified that A. prasina and A. mycterizans in this study have >6% genetic distance. The next analyses is the construction of phylogenetic trees to determine the relationship among Genus Ahaetulla in Indonesia.
Table 2. Genetic distances among Genus *Ahaetulla* based on Cyt B sequences.

| No | Samples                        | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  |
|----|--------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1  | *A. prasina*_NS                 |     |     |     |     |     |     |     |     |     |     |     |     |     |
| 2  | *A. prasina*_WS2               | 1.02|     |     |     |     |     |     |     |     |     |     |     |     |
| 3  | *A. prasina*_WS1               | 1.21| 0.18|     |     |     |     |     |     |     |     |     |     |     |
| 4  | *A. prasina*_WJ                | 1.98| 1.69| 1.88|     |     |     |     |     |     |     |     |     |     |
| 5  | *A. prasina*_EJ1               | 2.28| 2.27| 2.46| 2.08|     |     |     |     |     |     |     |     |     |
| 6  | *A. prasina*_EJ2               | 2.37| 2.37| 2.56| 2.18| 0.65|     |     |     |     |     |     |     |     |
| 7  | *A. prasina*_CJ                | 2.39| 2.39| 2.59| 2.19| 0.67| 0.02|     |     |     |     |     |     |     |
| 8  | *A. prasina*_BA                | 2.86| 3.15| 3.34| 2.47| 1.50| 1.78| 1.77|     |     |     |     |     |     |
| 9  | *A. prasina*_Thailand          | 4.56| 4.45| 4.45| 4.86| 4.98| 6.28| 6.26| 5.28|     |     |     |     |     |
| 10 | *A. mycterizans*_Genbank       | 6.48| 6.15| 6.15| 6.95| 6.92| 6.39| 6.37| 6.596.44|     |     |     |     |     |
| 11 | *A. mycterizans*_BN            | 7.56| 7.54| 7.54| 7.12| 7.24| 7.24| 7.26| 7.137.42| 5.7 |     |     |     |     |
| 12 | *A. mycterizans*_LO            | 7.78| 7.76| 7.77| 7.34| 7.47| 7.47| 7.45| 7.257.64| 5.71| 0.74|     |     |     |
| 13 | *C. ornata*                    | 13.75| 13.75| 13.87| 13.76|13.90|13.90|13.92|13.76|13.73|13.97|13.92|13.67|     |
| 14 | *D. pictus*                    | 12.94|12.94|13.82|12.80|12.96|13.96|12.98|13.08|12.8|12.04|12.51|23.14|14.99|     |

The results of phylogenetic analysis show that there are two separated main clades with high reliability values (ML = 100). The separation of those two clades was based on the two *Ahaetulla* species found in this study. Based on clustering with *Ahaetulla* sequences from Genbank, two *Ahaetulla* species in this study are *A. prasina* and *A. mycterizans*. Clade I is consist of *A. prasina* of Sumatra, Java, Bali, and Thailand, while clade II is consist of *A. mycterizans* of Banten and Lombok. In the clade of *A. prasina*, there two subclades was formed (ML=95). Subclade I in *A. prasina* (ML=98) contains *Ahaetulla* of Indonesia (Sumatra and West Java, Central Java, East Java, and Bali), while subclade II only contains *A. prasina* of Thailand. This result indicates that Cyt B gene of *A. prasina* of Indonesia significantly different with *A. prasina* of Thailand. The presence of geographical barrier like Hindia Ocean made the snake of Indonesia can’t easily migrate to Thailand or vice versa.

In *A. prasina* of Indonesia clade, there are 2 subclades formed based on their localities (ML=99). Subclade I consist of *A. prasina* of Sumatra and West Java, while subclade II consist of *A. prasina* of Central Java, East Java, and Bali. The clustering joining of *A. prasina* of Sumatra and West Java is predicted because of the integration of the mainland of Sumatra and Java during glacial period. At that time, it was estimated that there were many species migrations which made it easier for species from Java and Sumatra to move each other [18]. This makes a pattern of adaptation which then affects the genetic structure of the species. Likewise, the Bali island which once joined with the mainland of Java allowed species migration from Java to Bali or vice versa. The island is part of the mainland of Sundaland that was once connected to become a major mainland in the past [19].
Figure 2. Phylogenetic tree of *Ahaetulla* Genus in Indonesia based on Cyt-B gene. Tree was constructed by ML analysis. Nodal supports represent ML bootstrap value.

Within *A. mycterizans* clade, 2 subclades were formed (ML=78) consisting of *A. mycterizans* sequence from Genbank and *A. mycterizans* of Lombok and Banten. *A. mycterizans* of Lombok and Banten formed a clade with significant bootstrap (ML=100) although Lombok is located in Lesser Sunda and Banten in Sundaland. This analyse indicates that *A. mycterizans* has high similarity Cyt B sequence with *A. mycterizans* of Banten. It can be predicted that *A. mycterizans* of Lombok could be originating from Sundaland. We refer to Inger [20] which proposed that fauna in Wallace's line, including Lesser Sunda region, originated from Sundaland region.

This result of molecular identification adds evidence that molecular analyses with Cyt-B gene as barcode can be used as alternative method for identifying species.

3.2. Morphological analyses

To confirm the identification result, we also add morphological identification. The specimens used are representative of *A. prasina* and *A. mycterizans* used in this study. The results of morphological identification are shown in Figure 3 and Table 2. As illustrated in Figure 3, the differences between *A. mycterizans* and *A. prasina* are: 1) *A. mycterizans* eyes is bigger than *A. prasina*, 2) upper surface of snout is convex in *A. mycterizans* but flat or even depressed in *A. prasina*. The differences between *A. mycterizans* and *A. prasina* not illustrated in Figure 3 and not mentioned in Table 4 are: 1) *A. mycterizans* has anal entire, while *A. prasina* has anal divided. 2) *A. mycterizans* has paler grey longitudinal lines in venter than *A. prasina*. 
Figure 3. Morphological comparison between *Ahaetulla* Genus of Indonesia: A) *A. prasina* of Bali (BA), B) *A. mycterizans* of Banten (BN).

Table 3. The morphological comparison between *A. prasina* and *A. mycterizans* identified in this study based on morphometry and meristic measurement. Morphometry measurement in cm.

| Specimen          | Locality | Sex | SVL | TaL | TL | TaL/TL | VEN | DSR | SC  |
|-------------------|----------|-----|-----|-----|----|--------|-----|-----|-----|
| *A. prasina*      | Bali     | F   | 43.2| 22.5| 65.7| 0.34   | 200 | 16:16:13 | 162 |
| *A. mycterizans*  | Banten   | F   | 75.0| 40.3| 115.3| 0.25  | 184 | 15:15:13 | 134 |

SVL: Snout-Vent Length; TaL: Tail Length; TL: Total Body Length (SVL + TL); DSR: Dorsal Scale Rows; VEN: Ventral Scale; SC: Sub caudal Scale

Based on the results of morphological identification, our specimen of Lombok agrees with five diagnostic morphological characters given for *A. mycterizans* by Miralles and David [21]: 1) ventral scale less than 200, 2) Anal entire, 3) Snout convex above, 5) venter with grey longitudinal lines. Moreover, our identification of *A. mycterizans* also agrees with recent studies that *A. mycterizans* eyes is wider than *A. prasina* and the number of ventral scales has a big influence in distinguishing *A. prasina* and *A. mycterizans* [22].

The morphological characters of *Ahaetulla* of East Java agree with diagnostics characters of *A. prasina* given by De Rooij [23] (who mentioned as *Dryophis prasinus*): 1. number of ventral scales 194-235 (206), 2. anal divided, 3. Snout more than twice the diameter of eye, 4. Sub caudal scales 151-207 (162). Therefore, we agree that there are 2 species found in this study based on morphological characters, *A. prasina* and *A. mycterizans*.

4. Conclusion
From those both analyses, it can be concluded that molecular analysis using Cyt B gene as a barcode can be used to identify species accurately because it shows appropriate result with morphological identification. Among animal taxonomists, the COI gene is the most often used for species identification. Based the results of this study, it can also be added that the Cyt B gene is also an effective gene used as a DNA barcode for species identification. Besides to be used for species identification, DNA barcoding method may also be used to analyse the biogeography of species in the distribution area.

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References

[1] Sriwattanarothai N, Steinke D, Ruenwongsa P, Hanner R and Panijpan B 2010 Molecular and morphological evidence supports the species status of the Mahachai fighter Betta sp. Mahachai and reveals new species of Betta from Thailand J Fish Biol 77 414-424

[2] Hebert P D, Zakharov E V, Prosser SW, Sones J E, McKeown J T, Mantle B and La Salle J 2013 A DNA ‘Barcode Blitz’: Rapid digitization and sequencing of a natural history collection PLoS One 8 e68535

[3] Weigand A M, Jochum A, Pfenninger M, Steinke D and Klussmann-Kolb A 2011 A new approach to an old conundrum—DNA barcoding sheds new light on phenotypic plasticity and morphological stasis in microsnails (Gastropoda, Pulmonata, Carychiidae) Mol. Ecol. Res. 11 255-265

[4] Intaz A, Nor SAM and Naim AM 2017 Review: Progress and potential of DNA barcoding for species identification of fish species Biodiversitas 10 1394-1405

[5] Iwatsuki Y, Tanaka F and Allen GR 2015 Lutjanus xanthopinnis, a new species of snapper (Pisces: Lutjanidae) from the Indo-west Pacific, with a redescription of Lutjanus madras (Valenciennes 1831) J Ocean Sci Found 17 22-42

[6] Stuart J A 2009 Mitochondrial DNA Methods and Protocol 2nd Edition (Canada: Humana Press)

[7] Laopichienpong N, Muangmai N, Supikamolseni A, Twilp Stuart J A 2015 Assessment of snake DNA barcodes based on mitochondrial COI and Cytb genes revealed multiple putative cryptic species in Thailand Genes 2016

[8] Das I 2015 A field Guide to The Reptiles of South-East Asia (London: Bloomsbury Publishing)

[9] Faith D P and Williams K J 2005 How large-scale DNA barcoding programs can boost biodiversity conservation planning: linking phylogenetic diversity (PD) analyses to the Barcode of Life Database (BOLD) In Australian Entomological Society’s 36th AGM and Scientific Conference/7th Invertebrate Biodiversity and Conservation Conference/Australian Systematics Society. Canberra, Australia. December 2005

[10] Leo S, Amarasinge T and Supriatna J 2015 Morphological variation of Ahaetulla prasina (Boie, 1827) (Squamata: Colubridae) in Java. The 1st Symposium on South East Asia Herpetology and Envenomation (SEASHE) “Species Diversity and Animal Bites in Emergency Medicine” in conjunction with The 4th Congress of Herpetological Society of Indonesia, University of Indonesia. August 2015

[11] Figueroa A, Mekelvy A D, Grismer L L, Bell C D and Lailvaux SP 2016 A Species Level Phylogeny of Extant Snakes with Description of a New Colubrid Subfamily and Genus PLoS ONE 11 9

[12] Alencar LRV, Quental TB, Graziottiin FG, Alfaro ML, Martins M et al. 2016 Diversification in vipers: phylogenetic relationships, time of divergence and shifts in speciation rates Molecular Phylogenetics and Evolution 105 50-62

[13] Nugraha F A D, Fatchiyah F, Smith E N, Kurniawan N 2018 Phylogenetic analysis of colubrid snakes based on 12S-rDNA reveals distinct lineages of Dendrelaphis pictus (Gmelin, 1789) populations in Sumatra and Java Biodiversitas 19 303 – 310

[14] Burbrink F T, Lawson R and Slowinski J B 2000 Mitochondrial DNA phylogeography of the polytypic north American rat snake (Elaphe obsoleta): a critique of the subspecies concept Evolution 54 2107–2118

[15] Kumar S, Stecher G and Tamura K 2016 MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets Mol. Biol. Evol. 33 1870-1874

[16] Drummon A J, Ho S Y, Phillips M J and Rambaut A 2006 Relaxed phylogenetics and dating with confidence PLoS Biol. 4: e88

[17] Jeong T A, Jun J, Han S, Kim H, Oh K and Kwak M 2013 DNA barcode reference data for korean herpetofauna and their applications Molecular Ecology Reseources 3 1019-1032
[18] Hall R 2012 Sundaland And Wallacea: Geology, Plate Tectonics and Palaeogeography Biotic Evolution and Environmental Change in Southeast Asia, eds D. J. Gower et al. (London: Cambridge University Press)

[19] Lohman D J, Bruyn M, Page T, Rintelen K, Hall R et al. 2011 Biogeography of the Indo-Australian Archipelago Annual Review of Ecology, Evolution, and Systematics 42 205-226

[20] Inger R F 2005 The frog fauna of the Indo-Malayan region as it applies to Wallace’s line. In: Wallace in Sarawak—150 years later. In: A. A. Tuen & I. Das, editors. Proceedings of an International Conference on Biogeography and Biodiversity. Institute of Biodiversity and Environmental Conservation, Universiti Malaysia Sarawak, Kota Samarahan pp:82-90

[21] Miralles A and David P 2010 First record of Ahaetulla mycterizans (Linnaeus, 1758) (Reptilia, Squamata, Colubridae) from Sumatra, Indonesia, with an expanded definition Zoosystema 32 449-456

[22] Baker N 2019 Some snake records from Gunung Arong Forest Reserve, Johor, Peninsular Malaysia Southeast Asia Vertebrate Records 001-006

[23] De Rooij N 1917 The Reptiles of the Indo-Australian Archipelago (Leiden: E.J. Brill)