Phylogenetic study of Sumatran *Microhyla heymonsi* Vogt, 1911 (Anura: Microhylidae) based on 16S rRNA mitochondrial gene

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Abstract. *Microhyla heymonsi* is a species that has a very wide distribution, ranging from Taiwan, China, India, Indochina, Peninsular Malaysia, to Sumatra. Species with a very wide distribution often contain cryptic species due to the geographical barrier resulting in reproductive isolation, gene mutation, and evolution. This study aims to determine the phylogenetic relationship between *M. heymonsi* species from Sumatra and outside Sumatra, as well as the value of genetic distance between the two groups of species. The genetic distance threshold as a species delimitation is 3 %. DNA sequences were obtained through the process of DNA barcoding and then phylogenetic studies were performed through analysis of Unweighted Pair Group Method with Arithmetic Mean, Neighborhood Joining, Maximum Likelihood, and Bayesian Inference. The results show that the *M. heymonsi* species from Sumatra form a large single clade on the phylogenetic tree of all analytical methods with significant bootstrap values. The genetic distance value of *M. heymonsi* Sumatra with *M. heymonsi* Singapore and Malaysia is still below 3 %. The genetic distance values between *M. heymonsi* Sumatra and *M. heymonsi* Thailand, Myanmar, Vietnam and China have exceeded 3 %.

Keywords: *Microhyla*, 16S rRNA, mitochondria, phylogenetics

1. Introduction

Genus *Microhyla* is a member of the Microhylidae which is known by its local name as the percil frog, referring to the main characteristic of its small body size [1]. *Microhyla* spreads from South Asia, East Asia, Indochina, Peninsular Malaysia, Philippines, Indonesia to Bali [2]. Research amount on the genus *Microhyla* in particular, and the order of Anura in general on the island of Sumatra is few and still done sporadically compared to other dispersal areas [3].

Sumatra Island is formerly a part of the Sundaland region, then began to separate during the early miocene, and separated perfectly during the mid-miocene. But in the Pleistocene period, the island of
Sumatra was once reunited with Peninsular Malaysia due to a decrease in sea level and the draining of basin [4]. Therefore, the fauna of Sumatra still has similarities with the Sunda fauna as a whole, but also allows the occurrence of speciation due to reproductive isolation [5]. Sumatra has been recorded as a home to approximately 90 species of Anura [6]. Seven of them are members of the Microhyla order, and one of them is *Microhyla heymonsi* [7].

*Microhyla heymonsi* (figure 1) is a species of the genus *Microhyla* that was first described by Vogt in 1911 in Formosa, Taiwan. This species can live at almost any height up to 800 asl, can be found mainly around river streams, secondary vegetation, rice fields, plantations, and abandoned land. Based on these characteristics, the existence of *M. heymonsi* in nature is still abundant and widely spread [8]. *Microhyla heymonsi* spreads from southern China, Taiwan Islands, Indochina countries, Peninsular Malaysia, and Sumatra as seen on figure 2. According to Mathew et al. [9] the spread of *M. heymonsi* was even recorded in Andaman Island, Nicobar Island, and Manipur in India. The wide dispersal area causes the species to be classified into wide-ranging species [10].

Species with a wide distribution often have members of the cryptic species [11]. The cryptic species is a term for two or more different species that are not yet known to be exact, or have not been correctly identified, so they are still classified as one common species, largely because of the indistinguishable morphological characteristics [12]. In the genus *Microhyla*, there have been found cases of cryptic species, named *Microhyla ornata* Dumeril & Bibron, 1841, proposed to be limited only to populations in the South Asian region, whereas for other areas the species are named *Microhyla fissipes* Boulenger, 1884 and *Microhyla okinavensis* Stejneger, 1901 [11]. The identification of cryptic species, which is morphologically indistinguishable, can be supported through additional methods, such as bioacoustics analysis, and the most commonly used molecular analysis via DNA barcoding [13].

Molecular analysis becomes one of the important methods in the identification of Anura species [14]. DNA sequences can be used as a tool for determining new species, by comparing the sequences of the new species candidate with the previously allegedly similar species [15]. 16S rRNA mitochondrial gene according to Vences, et.al. [15] can be a good marker and indicator of species identification according to their research when compared to other markers such as 5S, 12S or COI. This is due to the priming site of 16S rRNA gene is consistent and well-preserved, while for other markers such as COI, the priming site exhibit too high variations. In addition to GenBank data [16], the number of sequences in the Anura group most often derived from the 16S rRNA gene, then followed by the number of 12S rRNA genes and the last of the COI genes.

*M. heymonsi* is widely distributed with Sumatra as the southernmost dispersal region and the biogeographic factor of the island of Sumatra enabling the occurrence of reproductive isolation and speciation. The existence of prior research on species of the same genus

![Figure 1. Adult *Microhyla heymonsi* [17]](image1)

![Figure 2. Map of *Microhyla heymonsi* ‘s dispersal](image2)
indicate a case of cryptic species, the phylogenetic research of the existing *M. heymonsi* on Sumatra Island through analysis of 16S rNA mitochondrial gene with *M. heymonsi* from outside of Sumatra Island as a comparison. The purpose of this research is to find out how large the genetic distance and phylogenetic relationship between species *M. heymonsi* from Sumatra with *M. heymonsi* species from outside Sumatra island.

2. Materials and method

2.1. Molecular analysis

The workings of this research are broadly divided into two, namely molecular analysis and phylogenetic analysis. Molecular analysis begins with DNA isolation based on the phenol-chloroform method [18]. The DNA isolation starts with removal by adding Proteinase-K 10 mg/mL, 20 µL and SDS 10 %, 50 µL. The next stage is the extraction that will produce the supernatant. The formed supernatant is then precipitated, resulting in a DNA pellet. The DNA pellets are then dried on the aspirator and stored in suspension form in 1x TE 50 µL buffer. The next stage after the isolation is amplification. The amplification process begins with the addition of H3056 forward primer (5'-CTCCGGTCTGAACATCAGATGCTAGG-3') and reverse primer L2606 (5'-CTGACCGTGCAAAGGTAGCGTAATCACT-3'). The next stage is making PCR cocktail as much as 30 µL. PCR cocktails are then processed on a thermal cycler through 35 cycles. The amplified DNA was then electrophoresed to be visualized. Electrophoresis was carried out using 2 % agarose gel agar medium (4 g agarose in 1x TAE buffer 200 mL) at 100 V for 25 min. The electrophoresis results were visualized using a UV transilluminator. The last stage of molecular analysis is the sequencing process done using the Sanger Sequencing method and performed by 1st Base Asia Singapore.

2.2. Phylogenetic analysis

Sequences obtained are edited using the Chromas Pro program (Technelysium Pty Ltd., Tewntin, Australia). The edited sequences are then paralleled by 23 homologous sequences from the Genbank database using Clustal X in the Mega 7 program [19]. The altered sequences are then combined with the sequences from Genbank and saved as a FASTA file.

The phylogeny tree was constructed using the analysis of Unweighted Pair Group Method with Arithmetic Means (UPGMA), Neighboring Joining (NJ), Maximum Likelihood (ML), and Bayesian Infrence (BI). UPGMA and NJ analysis used the MEGA7 program with 1000 replications, p-distances model with transversional-transition substitution and gaps pairwise deletion. The DNA evolution model for ML and BI uses the General Time Reversible (GTR) with the gamma parameter (G) which is the best experiment on the Kakusan 3 program [20]. ML analysis based on Akike Information Criterion (AIC) with 1000 replication and 50 consenses using the Tree Finder program [21]. The BI analysis was evaluated using MrBayes 3.0b4 [22]. The genetic distance using the uncorrected p-distance model and the reference of the genetic spacing used as the species differentiator is 3 % [23]. Bootstrap values on tree branches believed to be valid are 70 % or more for NJ, UPGMA, and ML [24] analysis. In BI analysis, the value believed to be valid is 95 % or more [25].

3. Results

3.1. Phylogenetic relationship of Sumatran Microhyla heymonsi

The phylogeny tree (figure 3) based on bootstrap using BI, ML, NJ, and UPGMA analysis shows the existence of eight major groups of *Microhyla heymonsi*. Group 1 is *Microhyla heymonsi* from Sumatra Island cover Aceh, Medan, and Jambi with bootstrap value 100 %, 96 %, 97 %, and 93 %.
Figure 3. Phylogeny tree of Sumatran *Microhyla heymonsi*

Group 2 *M. heymonsi* from Singapore and Malaysia supported by bootstrap values of 99 %, 99 %, 100 %, and 99 %. Group 3 *M. heymonsi* species from parts of Thailand and Myanmar supported by bootstrap values of 64 %, 74 %, 98 %, and 99 %. Group 4 Species *M. heymonsi* from another parts of Thailand are specifically extracted from a specific location supported by bootstrap values of 89 %, 93 %, 99 %, and 99%. Group 5 Species *M. heymonsi* from Vietnam and China supported by 95 % bootstrap values , 79 %, 95 % and 77 %.

Group 6 Other species of the *Microhyla* genus except *M. berdmorei* supported by bootstrap values of 97 %, 79 %, 35 %, and 65 %. Group 7 *Microhyla berdmorei* supported by 100 % bootstrap value on BI analysis, 93 %, 97 %, and 97 %. Group 8 The outgroup species contains other species of the *Microhylidae* family that have close kinship to the *Microhyla* genus, *Kaloula pulchra* and *Kalophrynus pleurostigma*.

3.2. Genetic distance of *Microhyla heymonsi*

The genetic distance refers to the value of the uncorrected p-distances generated from the proportions and the arrangement of the nucleotide bases of each comparable sequence. The proportion is obtained by dividing the number of different nucleotides from the total number of overall nucleotide sites. The more differences in the proportions and arrangement of nucleotide sites between the sequences compared, the higher the value of uncorrected p-distances, which means the greater the genetic distance. The genetic distance value of all data tested can be seen in table 1.
Table 1. p-distances scores within group from all sample

| Species | Sumatra | Singapura | Malaysia | Thailand | Myanmar | Vietnam | China |
|---------|---------|-----------|----------|----------|---------|---------|-------|
| M. heymonsi Sumatra | 0.007 | 0.007 | 0.009 | 0.009 | 0.011 | | 0.011 |
| M. heymonsi Singapura | 0.028 | 0.002 | 0.009 | 0.009 | 0.012 | | 0.011 |
| M. heymonsi Malaysia | 0.028 | 0.002 | 0.009 | 0.009 | 0.011 | | 0.011 |
| M. heymonsi Thailand | 0.048 | 0.041 | 0.041 | 0.006 | 0.008 | | 0.007 |
| M. heymonsi Myanmar | 0.041 | 0.034 | 0.034 | 0.028 | | 0.009 | 0.009 |
| M. heymonsi Vietnam | 0.063 | 0.062 | 0.062 | 0.037 | 0.041 | | 0.007 |
| M. heymonsi China | 0.060 | 0.059 | 0.059 | 0.033 | 0.041 | | 0.023 |
| M. achatina | 0.102 | 0.103 | 0.103 | 0.082 | 0.094 | 0.083 | 0.078 |
| M. berdmorei | 0.121 | 0.121 | 0.121 | 0.112 | 0.114 | 0.108 | 0.110 |
| M. borneensis | 0.084 | 0.082 | 0.080 | 0.057 | 0.073 | 0.073 | 0.064 |
| M. malang | 0.082 | 0.076 | 0.076 | 0.052 | 0.064 | 0.064 | 0.057 |
| M. mantheyi | 0.097 | 0.098 | 0.098 | 0.077 | 0.084 | 0.082 | 0.071 |
| M. orientalis | 0.093 | 0.089 | 0.089 | 0.071 | 0.075 | 0.076 | 0.064 |
| Outgroup | 0.173 | 0.163 | 0.164 | 0.149 | 0.156 | 0.154 | 0.149 |

4. Discussion

4.1. Phylogenetic relationship of Sumatran Microhyla heymonsi

The Microhyla heymonsi species form one large clade of its own (clade 1-5) and are split apart from other Microhyla species supported by significant bootstrap values in all methods. Clade M. heymonsi is subdivided into several small groups based on the geographical location of the species locality. The M. heymonsi species from Sumatra form one group separately and supported by significant bootstrap values in all methods. The group of species is closely related to the group of M. heymonsi species from Singapore and Malaysia (Group 2), the kinship relationship is supported by high bootstrap values in all methods except UPGMA [26]. Group 3 consists of M. heymonsi species from parts of Thailand and Myanmar. Locality of Thai species in this group originates from the Thai region closer to Myanmar and Malaysia (Peninsular Malaysia) based on data in GenBank. Group 4 comprises part of the Thai population whose locality lies in the eastern region of Thailand and is closer to the Indochina region (Cambodia, Laos and Vietnam) and is supported by high bootstrap values in all methods except BI [27]. Group 5 is still closely related to group 4 supported by significant bootstrap values only on the ML method. Group 5 consists of M. heymonsi species from China and Vietnam that are suspected to be species of true M. heymonsi because of the location of these locations closest to the holotype location of M. heymonsi in Formosa, Taiwan [14].
4.2. Genetic distance of Microhyla heymonsi
Genetic distance is taken from the value of uncorrected p-distances obtained from MEGA7 software. The values are categorized by groups organized according to their locality performed on MEGA7 software. Comparison of the values of the genetic distance between the groups indicating whether the species of the group are still in the same taxa group, or may have originated from different taxa groups [19].

The genetic distance of M. heymonsi species of Sumatra is closest to the species group from Singapore and Malaysia, which is 0.28 or 2.8 %. This is in line with the geographical location of Sumatra Island adjacent to Singapore and Malaysia. The biogeographic history of the three regions is also appropriate and supported by the same reconstruction process of the Sunda region that occurred millions of years ago [5].

The genetic distance between the species of M. heymonsi from Sumatra and the Thai and Burmese groups was not significantly different, at 0.48 or 4.8 % and 0.41 or 4.1 %. This genetic distance has exceeded the distance set by [23] at 3% but not exceeding the range set by [27] at 5 %. This can be due to the considerable distance between Sumatra Island and Thailand and Myanmar, but it is still part of Peninsular Malaysia, so it is doubtful to be recommended as a cryptic species or even a new species [10].

The genetic distance between M. heymonsi species of Sumatra and the Vietnamese and Chinese groups was 0.63 or 6.3 % and 0.60 or 6.0 %, respectively. This genetic distance has exceeded 3 % by [23] or 5 % by [28]. It is thought to be caused by the distant geographical distance between Sumatra and Vietnam and China [29], sea barriers, and the Annamites highland and Mekong rivers barriers, so those factors form a process of reproductive isolation, mutation of genes, and evolution. Vietnam and China are also areas of Indochina that have different regional reconstructions from the past with the Sunda region [30] so from the value of the genetic distance, it can be said that M. heymonsi species from Sumatra with M. heymonsi species from China and Vietnam are likely to be cryptic species and can be further investigated through morphological and bioacoustic identification to clarify their taxonomic status.

5. Conclusion
The Sumatran Microhyla heymonsi form a separate clade in the phylogenetic tree. This was supported by significant bootstrap values in all methods. There is a genetic distance below 3 % as the species delimitation of new species between Sumatran, Singaporean, and Malaysian Microhyla heymonsi indicating that the population of the area belong to the same species. However, the genetic distance above 3 % between the species Microhyla heymonsi from Sumatra, Myanmar, Vietnam, and China indicates that the population of those areas can be proposed as different species after being investigated further through other support methods.

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