

Phylogenetic Relationship of Genus *Microhyla* (Amphibia, Anura) in Sunda Shelf Including
Sumatera, Java, Borneo, and Peninsular Malaysia as Revealed by 16S rRNA mtDNA Gene
Sequences

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ABSTRACT

Sundaland was a single large landmass during Pliocene and Pleistocene period and consisted of Java, Borneo, Sumatra, Malay-Peninsula and others smaller island. We used 26 partial DNA sequences of the mitochondrial DNA genes 16S rRNA from taxa of Microhylidae (23 genus *Microhyla* and 3 outgroups) from GenBank. We aligned sequences using MEGA 5 software. Phylogenetic trees were constructed using maximum parsimony (MP) and maximum likelihood (ML) with 1000 bootstraps. Our results reveal three monophyletic clades which are not supported: clade A (all Sundaland, including *M. malang*, *M. borneensis*, *M. mantheyi*, *M. achatina*, *M. berdmorei*, *M. superciliaris*, and *M. palmipes*); clade B (from Borneo and Malay Peninsular, including *M. annectens*, *M. perparva*, and *M. petrigena*); and clade C (consists of outgroup species, *Metaphrynella pollicaris*, *Chaperina fusca*, and *Kalophrynus heterochirus*). Clade A reveal three subclades with unresolved relationship: AI (*M. malang*, *M. borneensis*, *M. mantheyi*, *M. achatina*, and *M. berdmorei*), AII (*M. superciliaris*), and AIII (*M. palmipes*). Clade B reveal two well-supported subclades: BI (*M. annectens*) and BII (*M. perparva* and *M. petrigena*). Our result show that phylogenetic within genus *Microhyla* was mostly affected species distributions.

Keywords: *Microhyla*, phylogenetic, Sundaland

INTRODUCTION

Sundaland was a single large landmass during Pliocene period. Since the cycles of falling and rising of sea level on the glacial period, now Sundaland was known as Java, Borneo, Sumatra, Malay-Peninsula and others smaller island [1; 2]. Microhylidae is a group of small-sized frog which has 14% percentage of total species in Sundaland. *Microhyla* is a genus of Microhylidae which has the highest total species in Sundaland compared with other genus. Most of *Microhyla* species were spreaded in Borneo, and some were spreaded to 3 other major islands [3; 4; 5; 6; 7].

Separation of genus *Microhyla* into distinct species on the basis of morphological characteristics and mtDNA has long been complicated [8; 9; 10]. Some

studies suggested that speciation of *Microhyla* was mostly affected by geological history due to their wide distribution [8]. Therefore, the aims of this study is to find out that species distributions and phylogenies are strongly related to the geological history of this region as revealed by mitochondrial 16S rRNA mtDNA gene sequences.

MATERIALS AND METHODS

Sample Collection

We examined a total of 26 partial DNA sequences of the mitochondrial DNA genes 16S rRNA from taxa Microhylidae (23 genus *Microhyla* and 3 outgroups) from GeneBank. Genus *Microhyla* included: *M.*

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How to cite:

Firdaus AS et al., (2018) Phylogenetic Relationship of Genus *Microhyla* (Amphibia, Anura) in Sunda Shelf including Sumatera, Java, Borneo, and Peninsular Malaysia as Revealed by 16S rRNA mtDNA Gen Sequences. J.Bioinfo. Biomedic.Research 1(1): 1 – 6.

achatina, *M. annectens*, *M. berdmorei*, *M. borneensis*, *M. malang*, *M. mantheyi*, *M. palmipes*, *M. perparva*, *M. petrigena*, and *M. superciliaris* from Peninsular Malaysia, Sumatra, Borneo, and Java (table 1). We used *Chaperina fusca*, *Kala heterochirus*, and *Metaphrynella pollicaris* as outgroup taxa.

Table 1. Sample of genus *Microhyla* and outgroup species used for mtDNA analysis in this study together with the information on collection locality and GenBank accession numbers.

No	Species	Locality	GenBank (16S rRNA)
1	<i>M. achatina</i>	Indonesia, Central Java, Ungaran	AB598335
2	<i>M. achatina</i>	Indonesia, West Java, Gede Pangrango	AB634657
3	<i>M. annectens</i>	Malaysia, Pahang, Cameron	AB634659
4	<i>M. berdmorei</i>	Malaysia, Selangor, Gombak	AB598338
5	<i>M. berdmorei</i>	Indonesia, Kalimantan, Paramasan	AB634662
6	<i>M. berdmorei</i>	Indonesia, S. Kalimantan, Paramasan	AB634661
7	<i>M. berdmorei</i>	Indonesia, Sumatra, Bengkulu	AB634660
8	<i>M. borneensis</i>	Malaysia: Sarawak, Mt. Serapi	AB598328
9	<i>M. malang</i>	Malaysia: Sabah, Tawau Hills	AB598323
10	<i>M. malang</i>	Malaysia: Sarawak, Kanowit	AB598322
11	<i>M. malang</i>	Malaysia: Mt. Serapi	AB598321
12	<i>M. malang</i>	Indonesia: E.Kalimantan, Balikpapan	AB634677
13	<i>M. mantheyi</i>	Malaysia: Pahang, Temerloh	AB598334
14	<i>M. mantheyi</i>	Malaysia: Selangor, Templer Park	AB598333
15	<i>M. palmipes</i>	Indonesia: Bali, Ubud	AB781464
16	<i>M. palmipes</i>	Indonesia: Sumatra, Bengkulu	AB634671
17	<i>M. palmipes</i>	Indonesia: Bali, Bedugul	AB634670
18	<i>M. perparva</i>	Malaysia: Sarawak, Mulu	AB634673
19	<i>M. perparva</i>	Indonesia: E. Kalimantan, Balikpapan	AB634672
20	<i>M. petrigena</i>	Malaysia: Sarawak, Bukit Kana	AB634675
21	<i>M. petrigena</i>	Malaysia: Sabah, Maliau Basin	AB634674
22	<i>M. superciliaris</i>	Malaysia: Pahang, Temerloh	AB634682
23	<i>M. superciliaris</i>	Malaysia: Negeri Sembilan, Kenaboi	AB634683
24	<i>Chaperina fusca</i>	Malaysia: Sabah, Crocker	AB598342
25	<i>Kalophrynus heterochirus</i>	Malaysia: Sabah, Crocker	AB634697
26	<i>Metaphrynella pollicaris</i>	Malaysia: Pahang, Fraser's Hill	AB634692

Phylogenetic Analysis

Sequences were contained of approximately 800 basepairs each and then aligned using Clustal W an option of MEGA 4 software [11]. Phylogenetic trees were constructed using maximum parsimony (MP) and maximum likelihood (ML) with 1000 bootstraps for both MP and ML tree [8].

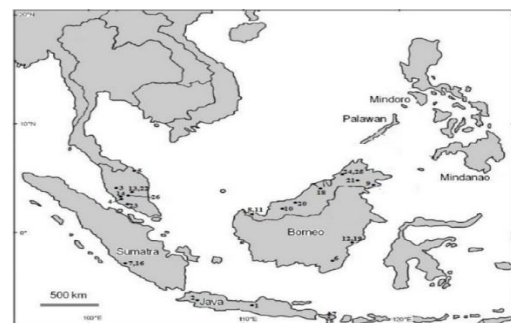


Figure 1. Sample origin of sequenced specimens from Sundaland localities adapted from Matsui et al. (for details see Supplementary Table 1).

RESULTS AND DISCUSSION

This study was using two analysis method, Maximum Likelihood (ML) and Maximum Parsimony (MP)

to construct phylogenetics trees referred to Matsui et al (8). This two methods are based on character-based method phylogenetics.

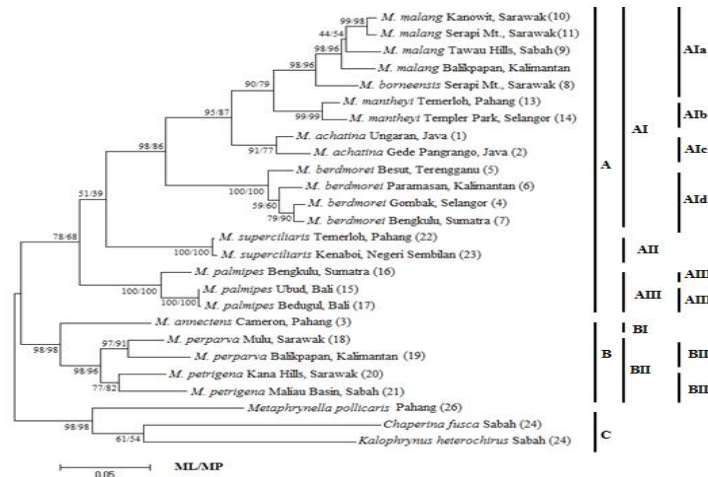


Figure 2. Maximum likelihood and maximum parsimony phylogram of 16S rRNA mitochondrial genes for samples of genus *Microhyla* obtained from several location Sundaland and outgroups. Sample numbers are included in Table 1. Number near branches represent bootstrap support for ML/MP.

The result show two clades of *Microhyla* genus and one clade as outgroup. A and B clades represent all *Microhyla* genus with respect to outgroups (*Metaphrynella pollicaris*, *Chaperina fusca*, and *Kalophrynus heterochirus*) was supported in all trees (ML = 98%, MP = 98%). Monophyly of *Microhyla* was not supported and they were divided into two monophyletic groups: clade A (all Sundaland, including *M. malang*, *M. borneensis*, *M. mantheyi*, *M. achatina*, *M. berdmorei*, *M. superciliaris*, and *M. palmipes*: ML = 78%, MP = 68%) and clade B (from Borneo and Malay Peninsular, including *M. annectens*, *M. perparva*, and *M. petrigena* with supported bootstrap in all trees: ML = 98%, MP = 98%).

In Clade A, there were three subclades (I–III) recovered with unresolved relationships. Subclade AIII form a monophyletic group with subclade AI and AII (ML = 78%, MP = 68%). While subclade AI and AII also formed monophyly (ML = 53%, MP = 39%). In subclade AI, there were four monophyletic groups with supported bootstrap (ML = 98%, MP = 86%): (a) *M. malang* and *M. borneensis* (well supported in all trees, ML = 98%,

MP = 96%); (b) *M. mantheyi* from Pahang and Selangor (all 99%); (c) *M. achatina* from Ungaran and Gede Pangrango (ML = 91%, MP = 77%); and (d) *M. berdmorei* from Terengganu with the other localities (all 100%). In subclade AII, there was group of *M. superciliaris* from Temerloh and Kenaboi (all 100%). In subclade AIII, there were two monophyletic groups between *M. palmipes* Sumatra (a) with Bali (b) (all 100%). *M. palmipes* from Bali form a monophyletic group (all 100%).

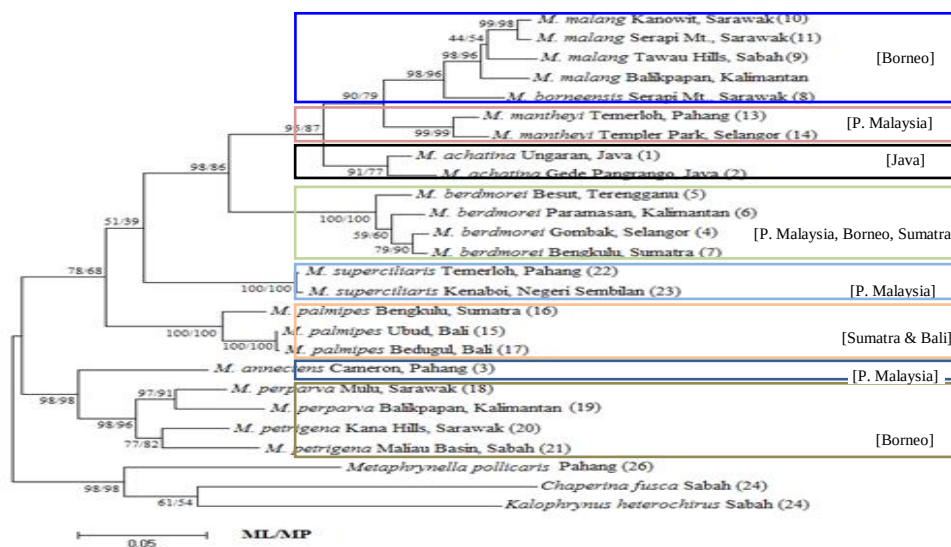
In Clade B, there were two subclades (I and II) recovered which was well supported (ML = 98%, MP = 98%). Subclade BII formed two monophyletic groups of *M. perparva* and *M. petrigena* (ML = 98%, MP = 96%): (a) *M. perparva* from Sarawak and Kalimantan (ML = 97%, MP = 92%); (b) *M. petrigena* from Sarawak and Sabah (ML = 77%, MP = 82%).

Many studies were conducted to resolve relationship among genus *Microhyla*. Relationships among many other species in the genus *Microhyla* remained unknown from the previous studies. Therefore, this

study tried to evaluate relationships of *Microhyla* by using GenBank data. Unfortunately, the available Sundaland region is very limited as exemplified in Fig. 1, which could be constructed using approximately 800 bp of 16S rRNA partial sequences that were comparable among GenBank data [12; 9]. The results show two basal clades among *Microhyla*: clade A (*M. malang*, *M. borneensis*, *M. mantheyi*, *M. achatina*, *M. berdmorei*, *M. superciliaris*, and *M. palmipes*) and clade B (*M. annectens*, *M. perparva*, and *M. petrigena*), as well as out-group taxa of *Metaphrynella pollicaris*, *Chaperina fusca*, and *Kalophrynus heterochirus* were placed among the above the main groups. Clade A were distributed in four main islands of Sundaland, while clade B only in Borneo and Peninsular Malaysia. This grouping indicates an

interesting relationship of *Microhyla* that strengthen the statement, which say species distribution was affected by geographical history.

The result suggests a grouping of *Microhyla* in Sundaland based on its species and distribution. Our results supports one of Dubois suggestion about the relationship of *M. malang* and *M. borneensis*. Dubois [15] classified a group of *M. berdmorei* which consist of *M. mantheyi* and *M. malang* from previous research of Parker [13]. Our result show that *M. malang* were dispersed in Borneo together with *M. borneensis* and both of it show a close relationship. *M. mantheyi* were dispersed in Peninsular Malaysia. Our result also supports Das et al. [6] and Matsui et al. [11] research which suggested that *M. malang* split from *M. borneensis*. Our result show that *M. malang* and *M. borneensis* split from *M. mantheyi*.



Figur 3. Distribution of each species of *Microhyla* in Sundaland, Data from GenBank. Number near branches represents bootstrap support.

M. achatina were dispersed in Java and showed a well supported split between *M. achatina* from Ungaran (Central Java) and Gede Pangrango (West Java). This probably caused by a barrier of mountains between Central Java and West Java as mentioned by Self and ring of fire in Java (series of active mountains).

M. berdmorei as mentioned in group AId were dispersed in Peninsular Malaysia (Besut and Gombak), Borneo (Paramasan), and Sumatra (Bengkulu). *M. berdmorei* showed a wider distribution compared with other species. Our result is against Parker [13] suggestion about group of *M. berdmorei* which consisted of *M. annectens*, *M. berdmorei*, *M. palmipes*, *M. perparva*, *M.*

petrigena, and *M. palmipes*. Our result of *M. berdmorei* show a well supported bootstrap with *M. mantheyi*, *M. borneensis*, and *M. malang*, and support Dubois [15] classification.

M. superciliaris shows unresolved relationship with subclade AI (consists of *M. berdmorei*, *M. achatina*, *M. mantheyi*, *M. borneensis*, and *M. malang*). This species were dispersed in Peninsular Malaysia and shows a well supported tree within Temerloh and Kenaboi. *M. palmipes* also shows unresolved relationship with *M. superciliaris* and subclade AI. *M. palmipes* from Sumatra were split with *M. palmipes* from Bali as shown by well supported bootstraps. The split of *M. palmipes* from Sumatra and Bali probably caused by an emergence of wide sea as a barrier. *M. palmipes* from Ubud also shows a monophyletic group with *M. palmipes* from Bedugul.

The last group consists of *M. annectens*, *M. perparva*, and *M. petrigena* from Peninsular Malaysia and Borneo. *M. annectens* from Pahang, Peninsular Malaysia, shows a distinct group with well supported bootstrap within *M. perparva* and *M. petrigena* from Borneo. *M. perparva* and *M. petrigena* form a monophyletic group of Borneo with a well supported bootstraps. Our result support Matsui et al. [8] which also suggested a monophyletic group within *M. petrigena* and *M. perparva* from Borneo.

CONCLUSION

Our result show that phylogenetic within genus *Microhyla* was mostly affected by species distributions. Validity of our taxonomic arrangement should be tested by both phylogenetically examining additional taxa unavailable in this study, and finding non-genetic (such as morphological and acoustic) evidence to diagnose each group recognize. We also suggest further study about time divergence in genus *Microhyla* in order to know their biogeography and the relationship between phylogenies and its geological history.

ACKNOWLEDGMENT

The authors would like to thank our advisor: Fatchiyah, Widodo and Estri Laras Arumningtyas and also like to thank every who involved in the making of this paper.

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