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Phylogenetic of Rhacophoridae (Amphibia: Anura) from Sumatra based on Cytochrome-b gene.

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ABSTRACT

We examined the phylogenetic relationship of Rhacophorid species on Sumatra based on cytochrome-*b* (*cyt-b*) sequences in relation to the geological history of Asia. The alignment showed that phylogenetic reconstruction tree of Rhacophorids in Sumatra has 613 base pairs (bp) and 312 bp for Rhacophorid Asia. The phylogenetic tree showed that all of the Rhacophorids species in Sumatra were divided in three clades, then phylogenetic Rhacophorids in Asia were divided in two clades. This result showed that phylogenetic relationship of Rhacophorid on Sumatra and in Asia was monophyletic. In this study, several species from West Sumatra were reanalyzed, includes *Polypedates lecomystax* (Sawahlunto), *Rhacophorus* sp (Mentawai), *Nyctixalus pictus* (Palupuh) and *Rhacophorus* sp 1-5 and those species were include in Phylogenetic tree in Asia.

Keywords: Rhacophoridae, Phylogenetics, DNA mitochondrial, Cytochrome-*b*.

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INTRODUCTION

Rhacophoridae is one family belong to tree frog group which is distributed in Asia, Madagascar and Africa [1-4]. Generally, this family were found throughout Indomalayan, it called Asian tree-frogs or Asian tree-frogs family [5]. Rhacophoridae can be found at 60-3050 meters above sea level [5,6]. Rhacophoridae in Indonesia, including in Sumatra consist of five genera: *Nyctixalus*, *Philautus*, *Polypedates*, *Rhacophorus* and *Theiaderma* [2,5,6]. In West Sumatra Rhacophoridae diversity has been known based on survey and fieldwork. There was lack of knowledge about genetic study of this family bringing the phylogenetic tree into unknown for West Sumatra population. Most of phylogenetic analyses using molecular marker as assessed to explain the relationship among species or taxa. Cytochrome-*b* is one of gene in mitochondrial region which is commonly used in phylogenetic analysis. This gene believed has rapid evolution, high mutation rate and were maternally derived. The aim of this study was to determine and analyze the relationship of Rhacophoridae in Sumatra related to the establishment of the geological history of Asia.

MATERIALS AND METHODS

Taxon sampling and data collection

Samples were collected from eight localities in West Sumatra (Figure 1). DNA extraction and amplification has been done in Genetics and Cell Biology Laboratory, Andalas University, whereas DNA sequencing were analyzed at MacroGen USA DNA Sequencing Laboratory, South Korea. About 25 tissue samples of Rhacophoridae from West Sumatra (Table 1) has been analysed and in total 22 additional sequences data from GenBank with genera *Buergeria* as the outgroup (Accession number are shown in Table 2) were used for this study.

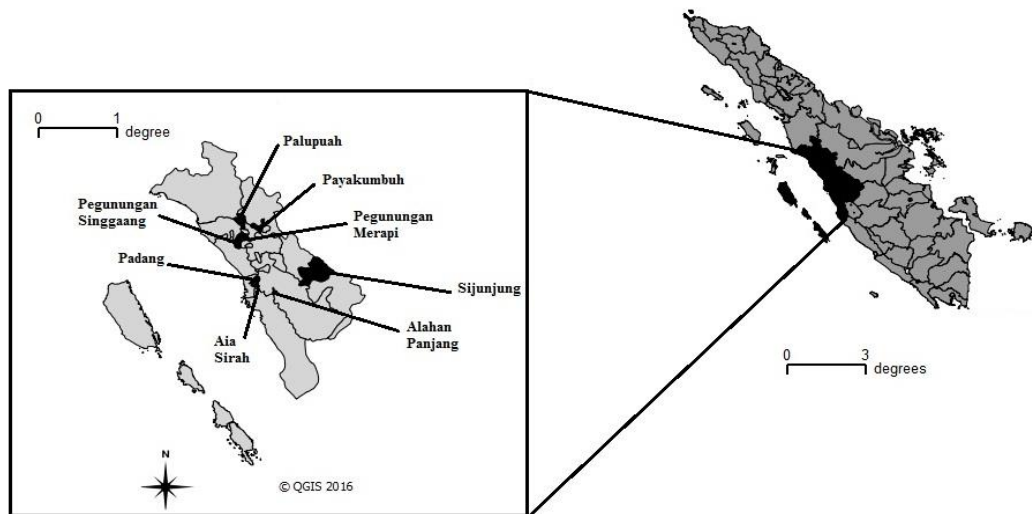


Fig 1:

Research location of Rahcophoridae in Sumatera.

Table 1: Samples Rhacophorids from West Sumatra used in this studies.

| Collection localities | Number of samples | Species |
|-----------------------|-------------------|---|
| Aia Sirah | 2 | <i>Philautus</i> sp <i>Rhacophorus</i> sp1 |
| Alahan Panjang | 1 | <i>Polypedates leucomystax</i> |
| Marapi | 5 | <i>Rhacophorus</i> sp4 <i>Rhacophorus</i> sp5 <i>Philautus</i> sp1* <i>Philautus</i> sp2* <i>Philautus</i> sp3* |
| Mentawai* | 3 | <i>Nyctixalus pictus</i> |

| | | |
|--------------|-----------|--|
| | | <i>Rhacophorus</i> sp <i>Philautus</i> sp4 |
| Malampah* | 2 | <i>Rhacophorus</i> sp2 <i>Rhacophorus</i> sp3 |
| Padang | 3 | <i>P. leucomystax</i> <i>P. pseudotilpohus</i> <i>Rhacophorus pardalis</i> |
| Palupuah | 1 | <i>Nyctixalus pictus</i> |
| Pasaman* | 2 | <i>Polypedates</i> sp1 <i>Polypedates</i> sp2 |
| Payakumbuh | 1 | <i>P. leucomystax</i> |
| Sawahlunto* | 1 | <i>P. leucomystax</i> |
| Sijunjung | 3 | <i>P. leucomystax</i> <i>P. macrotis</i> <i>P. pseudotilpohus</i> |
| Singgalang | 1 | <i>Philautus</i> sp5 |
| Total | 25 | |

*The Tissue samples from Genetic and Cell Biology Laboratory, Biology Departement, Andalas University

Table 2: Accession number used this studies.

| No. | No Akses | Spesies | Lokasi |
|-----|----------|----------------------------------|-------------------|
| 1. | AB530008 | <i>Buergeria buergeri</i> | Japan (OUTGROUP) |
| 2. | KC151123 | <i>Buergeria japonica</i> | Japan (OUTGROUP) |
| 3. | JF802921 | <i>Buergeria robusta</i> | Taiwan (OUTGROUP) |
| 4. | GQ204549 | <i>Nyctixalus pictus</i> | Bengkulu |
| 5. | GQ204522 | <i>Philautus aurantium</i> | Malaysia |
| 6. | AF249095 | <i>Philautus charius</i> | India |
| 7. | GQ204521 | <i>Philautus disgregus</i> | Malaysia |
| 8. | GQ204523 | <i>Philautus ingeri</i> | Borneo |
| 9. | EU924619 | <i>Philautus rhododiscus</i> | China |
| 10. | AY708186 | <i>Philautus</i> sp6 | Sri Lanka |
| 11. | AY708195 | <i>Philautus</i> sp7 | Sri Lanka |
| 12. | AY708173 | <i>Philautus</i> sp8 | India |
| 13. | AY708171 | <i>Philautus</i> sp9 | India |
| 14. | AY708174 | <i>Philautus</i> sp10 | India |
| 15. | AB451715 | <i>Polypedates leucomystax</i> | Filipina |
| 16. | JX393399 | <i>Polypedates leucomystax</i> | Laos |
| 17. | JX393400 | <i>Polypedates leucomystax</i> | Vietnam |
| 18. | GQ204510 | <i>Polypedates maculatus</i> | Sri Lanka |
| 19. | GQ204512 | <i>Polypedates macrotis</i> | Malaysia |
| 20. | EU924601 | <i>Polypedates megacephalus</i> | China |
| 21. | KF933135 | <i>Rhacophorus bimaculatus</i> | Filipina |
| 22. | GQ204529 | <i>Rhacophorus chenfui</i> | China |
| 23. | GQ204527 | <i>Rhacophorus nigropalmatus</i> | Malaysia |
| 24. | GQ204532 | <i>Rhacophorus dulitensis</i> | Malaysia |
| 25. | GQ204528 | <i>Rhacophorus pardalis</i> | Malaysia |

DNA extraction, amplification and sequencing

The DNA were extracted from liver tissue according to standard protocol of QIAGEN DNAeasy® Blood and Tissue Kit. Amplification using Gotaq® Green Master Mix (PROMEGA) Kit with 5 µl DNA isolate; 12.5 µl Gotaq Green, 3.5 µl Nuclease free water, 2 µl primer forward and reverse. A pair primer of *cyt-b* and cycling condition which has been modified according Kurniawan [7]. DNA identification by 2% agarose electrophoresis. Purified PCR product and sequencing conducted at MacroGen USA DNA Sequencing Laboratory in South Korea.

Phylogenetic analyses

The *cyt-b* DNA sequences from Rhacophorids were aligned using Clustal X version 1.8 [8]. The sequences edited using Bioedit [9]. Outgroup species for phylogenetic analyses of West Sumatra population has chosen from three species of *Buergeria* genera and one species for Asian tree frog. Phylogenetic analyses were performed by the Maximum Likelihood (ML), Neighbor Joining (NJ), Minimum Evolution (ME) and Maximum Parsimony (MP) methods by MEGA version 5.1 [10]. The tree were constructed and evaluated by bootstrap analyses with 1000 replicates.

RESULTS AND DISCUSSIONS

Phylogenetic Rhacophorids from Sumatra

Alignment of *cyt-b* genes for 28 Rhacophorids taxa (including 3 species outgroup, *Buergeria buergeria*, *B. japonica* and *B. robusta*) had comprise at 613 bp contained 291 (47.47%) parsimony informative, 342 (55.79%) variable site, 268 (43.71%) conserve site. Frequencies of nucleotide base were Thymine (T)= 30.2, Cytosine (C)= 28.4%, Adenine (A)= 26.4% and Guanine (G)= 15%.

The tree were reconstructed by MEGA 5.1 showing relationship of Rhacophorids in West Sumatra are monophyletic (Figure 2). Some researchers have been said that relationship of Rhacophoridae is monophyletic based on synapomorphic character from Ranoidea [1,3,11,12,13]. Those characters are intercalary elements of fingers and toes digits between ultimate and penultimate phalange.

The ML analyses did well resolve the dichotome the phylogenetic tree. Figure 2 shown that relationship of Rhacophorid from West Sumatra divided to three clades. Genus *Polypedates* and *Rhacophorus* on first clade, *Philautus* on second clade and *Philautus* and *Nyctixalus* in last clade. This cluster separation based to bootstrap values and outgroup. Most of the Rhacophorids species complete separate, it is supported by bootstrap values above 80%. Bootstrap value for branches of >90% are often considered very strong support, with moderate strength accorded to values between 60% and 90%, and <50-60% generally indicating poor support [14].

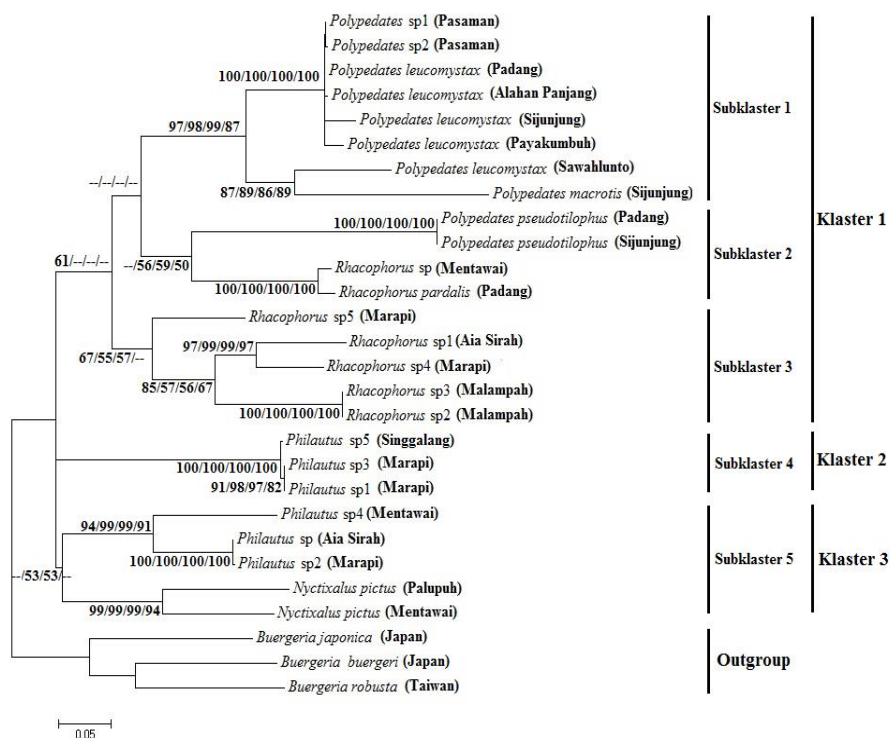


Fig 2: Maximum likelihood tree of Sumatran Rhacophorids based on 613 bp segment of mitochondrial *cyt b* gene, including outgroup. Bootstrap support values are listed in order for the ML/NJ/ME/MP analyses.

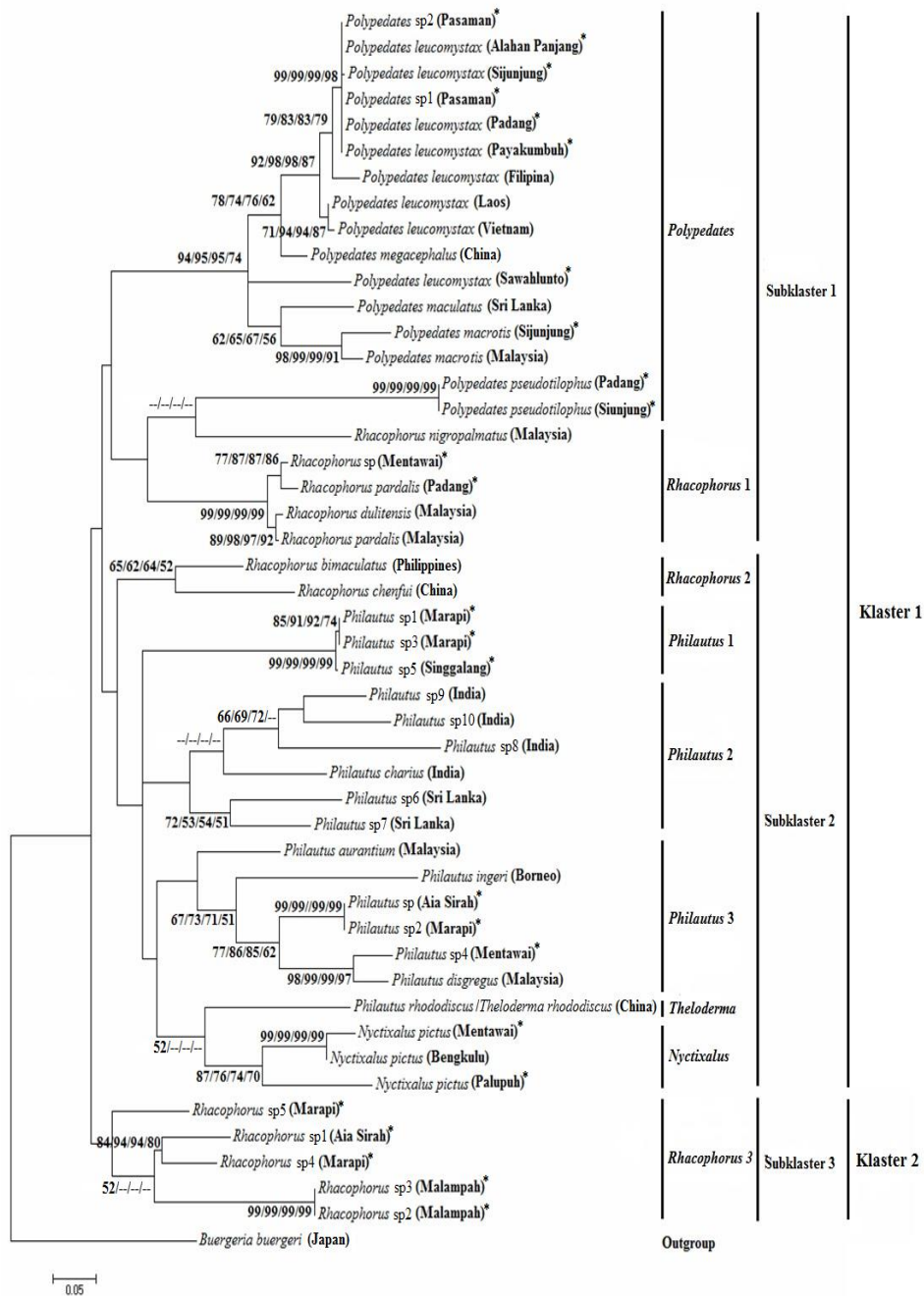


Fig 3: Maximum likelihood tree of Asian Rhacophorids based on 312 bp segment of mitochondrial cyt b gene, including outgroup. Bootstrap support values are listed in order for the ML/NJ/ME/MP analyses.

The following relationship among Rhacophorids in Sumatra are: all spesies *P. leucomystax* divided to one cluster with sequence divergence values 0.2%-5%, except *P. leucomystax* (Sawahlunto) which separated with another population. It has sequence divergence 21.4%-26.3%, indicating that *P. leucomystax* from Sumatra have genetic variation in cyt-b intrapopulation, and Sawahlunto population were not *P. leucomystax* based on that sequence divergence. Species *P. pseudotilophus* have not difference in two population, because they have sequence divergence 0% and bootstrap value 100%.

Separation of *Polypedates* and *Rhacophorus* were weak support, because that genus still debated and *Polypedataes* was part of *Rhacophorus* [15]. There are several morphological characters that distinguish genus *Polypedates* and *Rhacophorus*: 1) a portion of the scalp is fused to the skull in genus *Polypedates*, 2) the

ringfold on the lower side of digital disk typical for *Rhacophorus* is missing in *Polypedates*, and 3) while *Rhacophorus* posses webbings between the finger, it absent in *Polypedates* [5].

Genus *Rhacophorus* divided to five species: *Rhacophorus pardalis*, *Rhacophorus* sp1, *Rhacophorus* sp2-3, *Rhacophorus* sp4 and *Rhacophorus* sp5. Base on Figure 3, *Rhacophorus* sp (Mentawai) was include to *R. pardalis* (Padang). It is indicated that those species are same with strong support and sequence divergence 0.3%. For *Rhacophorus* sp1-5 have sequence divergence 23.6%-24% with moderate value for separating them.

Genus *Philautus* divided to two clades with 0%-19.8%. First clade include *Philautus* sp1, *Philautus* sp3 and *Philautus* sp5. Those species have sequence divergence 0%-0.5%, so we conclude they are same species. Second clade include *Philautus* sp, *Philautus* sp2 and *Philautus* sp4 have sequence divergence 0.2%-19.8%, indicating it may have two species. Species *N. pictus* (Palupuh and Mentawai) have sequence divergence 23.8%. It is indicated that two species may different species support to bootstrap value 99%.

Sequence divergence for each species showed the presence of a genetic variation in the population (0% - 5%), different species (over 11%), indicating the existence of subspecies (above 8.58%) to a different genus (19% - 31%). Based on the phylogenetic analyses, these species may need revition, consisting of *Polypedates* sp1 (Pasaman), *Polypedates* sp2 (Pasaman), *P. leucomystax* (Sawahlunto), *Rhacophorus* sp (Mentawai), *N. pictus* (Palupuh).

Phylogenetic Rhacophorids from Asia

Alignment of cyt-b for 48 Rhacophorids taxa (including a outgroup species, *Buergeria buergeria*) had comprise at 312 bp contained 166 (53.20%) parsimony informative, 179 (53.37%) variable site, 131 (41.99%) conserve site. Frequencies of nucleotide base were (T)= 30.6%, (C)= 29.9%, (A)= 26.7% and (G)= 12.7%.

Phylogenetic tree reconstruction of Rhacophorids in Asia shown in Figure 3 are monophyletic. This tree have two clades consisting of four genera (*Polypedates*, *Rhacophorus*, *Philautus*, *Nyctixalus*) in the first clade and the genus *Rhacophorus* from West Sumatera in the second clade. Based on alignment result of cyt-b, genus *Polypedates* has sequence divergence 0%-42.9%, *Rhacophorus* has 1.3-35.7%, *Philautus* has 0-42.7%, *Nyctixalus* has 4.1-27% and *Rhacophorus* from West Sumatera has 0-29.5%.

The phylogenetic tree showed the same species in one clade by close distribution. *Polypedates* occurs from Southeast Asia to Sri Lanka, which consists of five species; *P. leucomystax*, *P. megachepalus*, *P. maculatus*, *P. macrotis* and *P. Pseudotilophus*. *Rhacophorus* occurs from Southeast Asia to China consisting species *R. pardalis*, *R. bimaculatus* and *R. chenfui*. Genus *Philautus* were divided into three groups; *Philautus* Mountains of West Sumatera, *Philautus* in South Asia and *Philautus* in Southeast Asia.

Phylogenetic analysis showed that *P. leucomystax* in Asia consisted of eight populations, including West Sumatera (Alahan Panjang, Padang, Pasaman, Payakumbuh and Sijunjung), Philippines, Laos and Vietnam. These species are in the same cluster with bootstrap 92% and sequence divergence 0%-7.6%. sequence divergence species *P. leucomystax* (Sawahlunto) has 20.5%-23.1% to another populations, so we conclude that the *P. leucomystax* (Sawahlunto) is a different species. *P. leucomystax* (Philiphine) has close relationship with West Sumatera population and bootstrap 79% with sequence divergence 5.2%-5.6%. *P. leucomystax* (Laos and Vietnam) with West Sumatera has 7.2%-7.6%. Between Laos and Vietnam, this spesies has 1%.

Based on the geography, Philippines, Sumatra, Laos and Vietnam are in the same region, namely South-East Asia. Based on geological history, West Sumatera, which is part of Sumatra Island is part of the Sunda Shelf. Laos and Vietnam are part of Indochina and the Philippines is an archipelago formed of several mainland. So the kinship between *P. leucomystax* Philippines with the West Sumatera is probably caused by the formation of the geological history of the Philippines [16]. There are other possibilities between Borneo and Philippines have a landbridge when the sea level rises due to a cold climate [17].

Based on Figure 3, genus *Polypedates* concisting of five species include *P. leucomystax*, *P. megachepalus*, *P. maculatus*, *P. macrotis* and *P. pseudotilphus*. *P. leucomystax* species are species that widespread in Southeast Asia. This spesies has the ability to adapt quickly to the environment and able to

maintain their genetic composition. A result of the rapid process of adaptation, some species can be categorized as a species complex. Complex species are some species that have similar morphology, but there are some morphological characters that distinguish it. *P. leucomystax* complex is a group of species that are difficult to classify. There are some species that are said to be *P. leucomystax* complex test based on mitochondrial 12S rRNA and 16S rRNA tRNA^{val} namely *P. macrotis*, *P. braueri*, *Polypedates cf. mutus*, and *P. megachepalus* [15].

The dichotomy of *P. macrotis* (Sijunjung and Malaysia) was reconstructed with strong support value 98%. This population have sequence divergence 9.5%. Sequence divergence values for each species indicated to subspecies, if it has sequence divergence 8.58% [18]. Species *P. pseudotilophus* (Padang and Sijunjung) were same species with strong support 100% and 0% for sequence divergence.

Genus *Rhacophorus* based on phylogenetic tree consisting four species; *Rhacophorus* sp, *R. pardalis*, *R. dulitensis* and *R. nigropalmatus*. Species *Rhacophorus* sp (Mentawai) and *R. dulitensis* (Malaysia) as *R. pardalis* because of the clustering supported by sequence divergence values 1.3%-6.8% and 99% bootstrap.

Based on their distribution, genus *Philautus* (Figure 3) showed that relationship phylogenetic divided to three clades. First to *Philautus* from West Sumatera, *Philautus* from South Asia and *Philautus* from South East Asia. *Philautus* West Sumatera has sequence divergence 0%-0.7%, it indicated as same species with strong support bootstrap 99%. *Philautus* South Asia 1.2%-37.7% and *Philautus* South East Asia 0%-35.5% with moderate support bootstrap 77%. This clade divided to five species *Philautus*; *P. aurantium*, *P. ingeri*, *Philautus* sp-sp2, *Philautus* sp4 and *P. disgregus*. Species *Philautus* sp (Aia Sirah) and *Philautus* sp2 (Marapi) were same species with sequence divergence 0% and strong support bootstrap value 99%. *Philautus* sp4 is different species from another species in clade.

Genus *Nyctixalus* just have one species; *N. pictus* from Mentawai, Bengkulu and Palupuh. Mentawai and Bengkulu population have sequence divergence 4.1% than Palupuh population with 22.3%-27%. Based on that value, Palupuh population were different species. The last clade genus *Rhacophorus* from West Sumatera were separation to four species; *Rhacophorus* sp5, *Rhacophorus* sp1, *Rhacophorus* sp4 and *Rhacophorus* sp2-3, based on sequence divergence and bootstrap value.

The all result of phylogenetic *Rhacophoridae* Sumatra and Asia showed relationship among *Rhacophoridae* by distribution area. We conclude that there are some species that need to be revised and reanalyzed based on the phylogenetic tree, such as *P. leucomystax* (Sawahlunto) which may be a different species from other populations of *P. leucomystax*, *Rhacophorus* sp (Mentawai) and *R. dulitensis* (Malaysia) which concluded the species *R. pardalis*, *N. pictus* (Palupuh) and *Rhacophorus* sp1-5 of West Sumatra. Some species who separated by marine such as *P. leucomystax* (West Sumatra and Philippines), *Rhacophorus pardalis* (Padang, Mentawai and Malaysia), and *N. pictus* (Mentawai, Palupuh and Bengkulu). It is caused by geological movements like tectonic plates, sea level changes, climate and ocean circulation direction. It can be concluded that the amphibian is an organism that is appropriate in explaining the history of biogeography. This is because the amphibians are often considered incapable of crossing the ocean, intolerant of sea barrier, it can not survive in sea water and has a level of sensitivity to osmotic sea water is very high [19-21].

CONCLUSIONS

Phylogenetic of *Rhacophoridae* either Sumatra or Asia showed monophyletic relationship. Based on that relationship, there are some species to need reanalyzed such as *P. leucomystax* (Sawahlunto), *Rhacophorus* sp (Mentawai), *R. dulitensis* (Malaysia), *Rhacophorus* sp1-5 (West Sumatra).

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