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Research Article

Phylogenetic Study of Bufonidae (Amphibia: Anura) From West Sumatra (Indonesia) Based on Cytochrome *b* Gene

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ABSTRACT

Bufonidae is a widely distributed amphibian family around the world, including Indonesia (Sumatra) and Asia. Sumatra is an island that has separated because of sea level increase and creates a gene flow barrier for amphibians in Sumatra and Asia. This research aims to analyze Bufonidae phylogenetic, which exists in several locations in West Sumatra and Asia based on gene cytochrome b analysis. Samples were collected from six localities in West Sumatra. DNA extraction and amplification have been done in Genetics and Cell Biology Laboratory, Andalas University, whereas DNA sequencing was analyzed at MacroGen USA DNA Sequencing Laboratory, South Korea. About 14 tissue samples of Bufonidae from West Sumatra have been analyzed. The relationship of Bufonidae phylogenetics in Sumatra and Asia was divided into three main clusters. The first cluster consists of *Duttaphrynus melanostictus, Duttaphrynus himalayanus, Bufo japonicus* and *Ingerophrynus quadriporcatus.* The second cluster consists of *Leptophryne barbonica, Phrynoidis juxtasper* and *Phrynoidis aspera*. The third cluster only consists of *Ansonia* sp. *D. melanostictus* in Sumatra is closer to *D. melanostictus* from India with 0.3-0.5% sequence divergence and it is a group of paraphyletic with *D. melanostictus* from Vietnam, Taiwan, and China. *L. barbonica* from Padang Panjang with Bengkulu possibility different species (sequence divergent 26.1%). *P.aspera* (Solok&Sijunjung) with (Malampah 1-2, Rimbopanti, Palupuh Agam) indicated genetic differences between populations (sequence divergence 1.9%-2.2%). For *Ansonia* sp. and *I.quadriporcatus* need further analysis to determine the relationship phylogenetically.

Keywords: Bufonidae, Cytochrome-b, Phylogenetic, Sequence divergence

Introduction

Bufonidae as a family of Anura order widely spread worldwide, except Australia, Madagascar, and Papua Nugini, has about 33 genus and 400 species [1]. Bufonidae is distributed in altitude from 150-4000 m and the largest distribution of Bufonidae in Indonesia is Kalimantan and Sumatra [2, 3, 4]**.** Sumatra, as a part of Asia, separated a long time ago because of sea level increase on interglacial period, resulting in separation and distribution of flora and fauna. West Sumatra is part of the island of Sumatra which is separated by the Bukit Barisan mountains into western and eastern parts. The mountains are a barrier for some vertebrate animals, including Anura, which do not move from west to east or vice versa. This condition leads to geography and reproduction isolation, also blocked gene flow [5, 6].

Several studies on the effects of the Bukit Barisan mountains on Anura phylogenetics have been carried out, including in the Rhacoporide family based on the cytochrome b gene and in the *Hylarana hosii* species based on microsatellite DNA. The results of this study indicated that there was a geographical separation based on the Bukit Barisan mountains in several *Rhacoporus* species and also in *H. hosii* [7, 8].

Molecular analysis is nowadays able to under-

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stand species phylogenetic relationships. Phylogenetic is a study about the relationship of organisms based on its evolution [9]**.** One of the genes often used for species phylogenetic study is cytochrome *b* gene [10, 11, 12]. This gene founded in the mitochondrial genome and passed maternally [13]. This study aims to analyze the phylogenetic of Bufonidae in West Sumatra based on the cytochrome *b* gene.

Material and Methods

In this research, samples were collected during March-June 2013 in several areas of West Sumatra, such as Solok, Payakumbuh, Sijunjung, Malampah, Palupuah, Agam, Rimbo Panti, Merapi, Siberut, Padang Panjang.

We used QIAGEN kit protocol DNA easy® Blood and Tissue Kit (QIAGEN, Inc., Valencia, California, USA) for DNA isolation. The quality of DNA was tested by 1,2% gel electrophoresis added 3 µl Ethium Bromide (EtBr). Amplification of DNA is programmed for 35 cycles with modified temperature and time (denaturation: 98°C; 10 seconds, annealing: 45°C, 45 seconds, extension: 72°C; 1 minutes, 20 seconds) using PROMEGA PCR Core Kit [14,15]. Primer used were 150 Fow (5'-ACM GGH YTM TTY YTR GCH ATR CAY TA-3') and Rev-1 (5'-TAD GCR AAW AGR AAR TAY CAY TCN GG -3'). DNA amplification identification use 2% gel electro-phoresis added with 5 µl EtBr. The best PCR product was sequenced in *MacroGen USA DNA Sequencing Laboratory* in South Korea. DNA sequence of Bufonidae samples of Asia was accessed from *GenBank* (ncbi.nlm.nih.gov). The parallelization of DNA sequence was done by Clustal X version 1,8 [16]. Alignments were edited by Bioedit program [17]. The analysis of phyloge-netic tree based on DNA sequences of cytochrome b gene mitochondrial DNA used MEGA (Molecu-lar Evolutionary Genetics Analysis) version 5.1 [18]. The tree was constructed and evaluated by bootstrap analyses with 1000 replicates.

The analysis of DNA sequences order for other Bufonidae from Asia were accesed from GenBank with accession numbers: AB159258, AB159256, AB159257, AB159244, AB597923, AB713504, AB597918, AF249082, AF171200, AF171206, AF171205, AF171207, AF171208, AF171209, AF171199, AF171201, AF171202, AF171191, JX564876.

Table 1. Samples Bufonidae from West Sumatra used in this study

Collection localities	Number of samples	Species
Malampah1 Malampah2***** Rimbopanti***** Sijunjung, Batang Palupuh Agam, Solok	6	*Phrynoidis aspera*
Solok	2	*Phrynoidis juxtasper*
Payakumbuh	1	*Ingerophrynus quadriporcatus*
Malampah Marapi***** Siberut*****	3	*Dutta-phrynus mel-anostictus*
Sijunjung	1	*Ansonia sp.*
Padang Panjang*****	1	*Leptophryne barbonica*
Total	14	

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

Results and Discussions

As much as 381 bp part of cytochrome *b* gene mitochondrial DNA was analyzed. Nucleotide bases of cytochrome *b* gene mitochondrial DNA consisted 25.4% A (adenine), 28.1% T (thymine), 33.5% C (cytosine), and 13% G (guanine). This nucleotide bases composition was similar to previously reported amphibian cytochrome *b* gene sequences [11, 16]. From 381 bp obtained, 200 bp (52.49%) were categorized as conserved sites and 181 bp (47.50%) as variable sites.

There were three main clusters in the relationship of the family Bufonidae in Sumatra and Asia based on cytochrome *b* genes (Figure 1). The first cluster consists of *Duttaphrynus melanostictus, Duttaphrynus himalayanus, Bufo japonicus* and *Ingerophrynus quadriporcatus.* The second cluster consists of *Leptophryne barbonica, Phrynoidis juxtasper* and *Phrynoidis aspera.* The first and second cluster consists of three subcluster. The third cluster only consists of *Ansonia* sp.

The members of the first subcluster were formed two species groups *D. melanostictus* with sequence divergence 15.7%-18.5% which was supported with bootstrap value 96/86/86/77 (ML/ NJ/ME/MP). First group were *D. melanostictus* of Vietnam (1-5), *D. melanostictus* of Taiwan, *D. melanostictus* of China (1-3) and *D. himalayanus* of China. Second group were *D. melanostictus* of

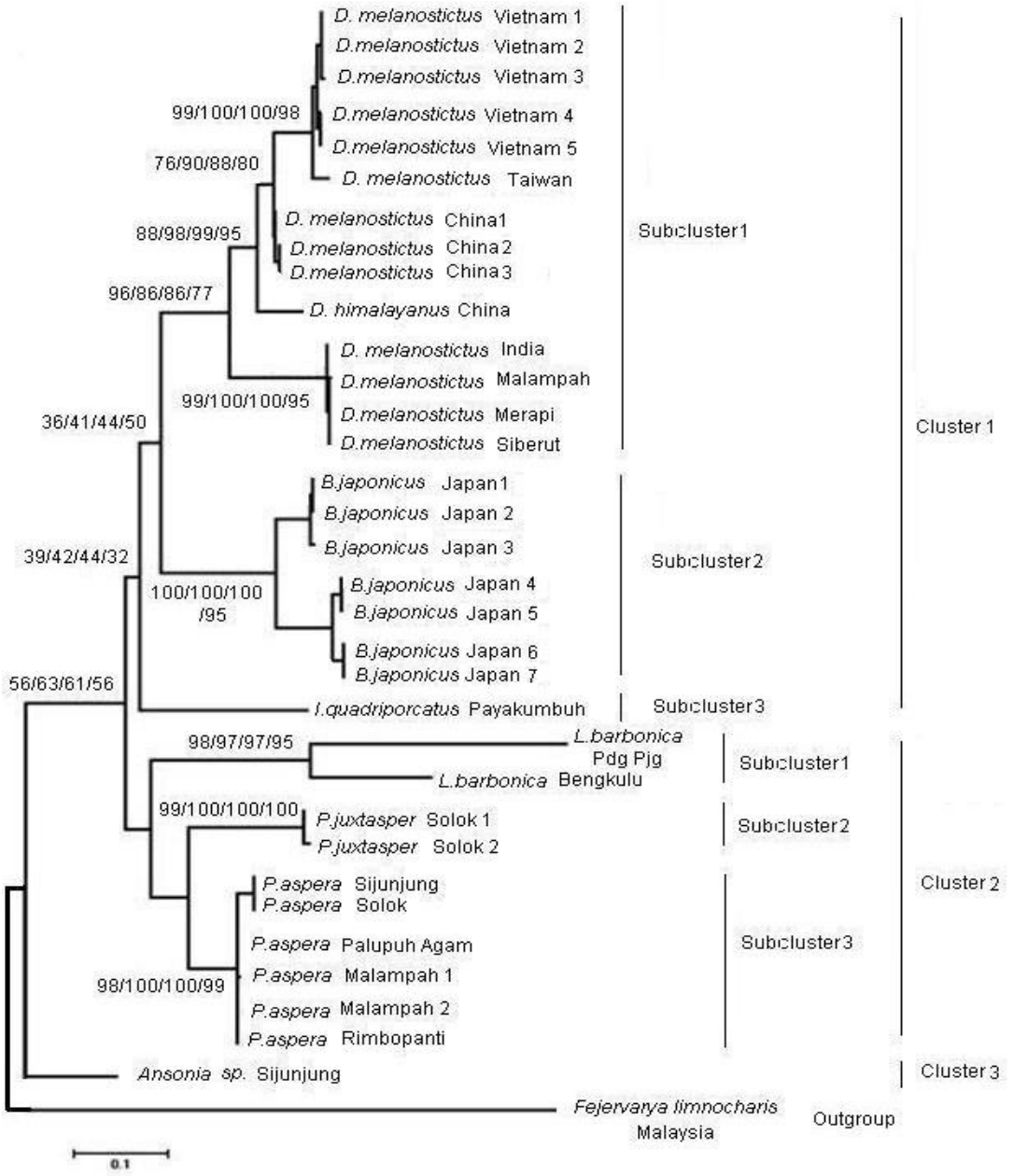

Figure 1. Phylogenetic tree of Bufonidae based on cytochrome *b* gene with bootstrapping 1000 (ML/NJ/ME/MP)

India and West Sumatra (Malampah, Marapi, Siberut). The large sequence divergence between *D. melanostictus* groups indicate that isolation were occured between *D.melanostictus* of West Sumatra with Vietnam, China, and Taiwan and were expected as a different species. The sequence divergent above 11% indicates the form of new species [19]. Second group is *D. melanostictus* of West Sumatra with *D. melanostictus* of India has low sequence divergence (0.3-0.5%) and supported by bootstrap value 99/100/100/95 (ML/NJ/ME/MP). This indicates that *D. melanostictus* of India and West Sumatra came from the same ancestor (monophyletic). *D. melanostictus* is also known as original species from India (type locality) [20].

D. melanostictus is invasive species or introduced species that can spread fast and adapt to degradation and suppress native species [21, 22,

23]. The sequence divergence between D. *melanostictus* of Vietnam with *D. melanostictus* of Taiwan is about 2.5% - 3.0%. *D. melanostictus* (Vietnam and Taiwan) with *D. melanostictus* of China is about 5.1%-5.4%. *D. melanostictus* of China with *D. himalayanus* from China is about 6.5%-7.2 %. The sequence divergence based on gene cytochrome *b* on population is about 7.5% [24].

The second subcluster is species *B. japonicas* which is from Japan and divide into two groups. *B. japonicus* of Japan (1–3) represents Japan west area, while *B. japonicus* Japan (4–7) represents Japan east area. Members of third subcluster were *I. quadriporcatus* (Payakumbuh).

First subscluster from the second cluster is *L. barbonica* from Padang Panjang and Bengkulu has a sequence divergence of about 26.1%, bootstrap value 98/97/97/95. Padang panjang (West Sumatra) is on the east of Bukit Barisan, while Bengkulu in the west. Based on the score of divergent sequence, *L. barbonica* in both locations was expected as different species. Bukit Barisan of Sumatra area is considered geographical isolation for amphibians, especially *L. barbonica*, which exists in the west and east of Bukit Barisan, leading to speculation. A barrier like geographical isolation could block *L. barbonica* gene flow as the result of reproductive isolation.

The second subcluster from the second cluster is *P. juxtasper* Solok (1-2), with sequence divergence 0.5%. The third subcluster is *P. aspera* which is divided into two groups. First group is *P. aspera* from Solok and Sijunjung river which flow to the east Sumatra beach, second group is *P.aspera* from Rimbo Panti, Malampah, Palupuh Agam river which flow to the west Sumatra beach. Both groups of *P. aspera* have sequence divergence 1.9-2.2% with bootstrap 98/100/100/99 (ML/NJ/ME/MP). This value indicated genetic differences between *P. aspera* populations. The sequence divergence based on gene cytochrome *b* on population level is 0.0-7.5% [24]. The sequence divergence based on gene cytochrome *b* on Bufonidae in Europe and East Asia is 5.72% for population divergent, 8.58% for subspecies and 15.39% for species [25].

One of the possibilities that the classification of *P. aspera* because *P. aspera* in West Sumatra was previously a population and had inhabited the island of Sumatra long before the formation of the Bukit Barisan mountains was completed. The formation of the Bukit Barisan mountains can be a geographical barrier that will hinder migration *P. aspera* in the river into the west and east of Sumatra, so there is no genetic mixing.

The third cluster is *Ansonia* sp has 23% – 27.9% sequence divergence from first cluster and 20.9% - 39.7% from the second cluster. These high differences make *Ansonia* sp. being divided and separated from other clusters. This result accords with the phylogenetic analysis result. *Ansonia* sp. Reaches Sumatra via the Bangka Belitung – Karimata from Kalimantan during the Pleistocene.

Conclusion

Phylogenetic of Bufonidae either West Sumatra monophyletic relationship to species *D. melanostictus* (Malampah, Merapi, Siberut). *D. melanostictus* group in Sumatra Barat is closer to *D. melanostictus* from India. It is a parafiletic group with *D. melanostictus* from Vietnam, *D. melanostictus* Taiwan and *D. melanostictus* China.

In contrary to species *L. barbonica* of Padang Panjang (East Bukit Barisan) with Bengkulu (West Bukit Barisan) there is sequence divergence of 26.1% and possibility different species. Species *P. aspera* (Solok and Sijunjung) with (Malampah 1-2, Rimbopanti, Palupuh Agam) indicated genetic differences between populations (sequence divergence 1.9%-2.2%). For *Ansonia* sp. and *I. quadriporcatus* need further analysis to determine the relationship phylogenetic.

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