

Phylogenetics and Biogeography of Cobra (Squamata: *Naja*) in Java, Sumatra, and Other Asian Region

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Abstract

The separation of Sumatra and Java occurred at the end of the Miocene (10 mya) to the end of Pliocene (1.8 mya). The existence of ecological variations and geographic barriers inhibits gene flow through the isolation of adaptation, geography, reproduction, inbreeding, and leading to population segregation. Cobra (genus *Naja*) distribution became greatly influenced by the geologic condition and sea level. This study was conducted by phylogenetic analysis towards the 16S rRNA gene. Survey was done with Visual Encounter Survey (VES) method on 6 locality. There were 3 location in Sumatra Island and the others in Java Island. Sample from other Asian region was obtained from Genbank, which are 11 individuals from China, Thailand, and Nepal. DNA extraction was done according to the QIAmp[®] DNA Mini Kit standard protocol. The forward and reverse 16S sequences are combined with the Sequencher[™] version 4.1.4 program, then in BLAST (Blast Local Alignment Search Tool) at www.ncbi.nlm.nih.gov. Phylogenetic analyzes of clade A (MP = 60, ML = 54, BI = 88) indicate the presence of division into two monophyletic subclade (AI and AII). Subclade AI consists of groups of Cobra from Sunda (Thailand, Sumatra and Java). Subclade AII is a group of species *N. kaouthia* originating from Chumpon Province, Southern Thailand with (MP = 96, ML = 95, BI = 100). Clade B is divided into two subclasses (BI and BII). The result is supported by bootstrap value MP = 93, ML = 99, BI = 100. *N. atra* of Fujian Province is a sister lineage of the same species from Jiangxi Province (MP = 86, ML = 86, BI = 100).

Keywords: Asian, 16s rRNA, Java, Cobra, Sumatra.

INTRODUCTION

The evolution of South Asia's diversity was occurred in Paleocene epoch (60 mya-million years ago), when the Malay Peninsula, Java, Sumatra, and Borneo still merged as a big continent, called as Sundaland. Then, the formation of the volcanic rings during Eocene (40 mya) and sea level fluctuations at the beginning of Miocene (23.03 mya) initiated the separation between the Malay Peninsula and Borneo. On the late Miocene, sea level fluctuations occurred again and half of Java Island were flooded by water. The separation of Sumatra and Java as well as Borneo rotational changes occurred at the end of the Miocene (10 mya) to the end of Pliocene (1.8 mya) [1]. The movement of the earth's plate during the land evolution was separate several regions of Asia. In addition, Sumatra Island is estimated to occurred in last 25 million years ago (late Miocene) and divided into west and east regions by Bukit Barisan mountains. The existence of ecological

variations and geographic barriers inhibits gene flow through the isolation of adaptation, geography, reproduction, inbreeding, and leading to population segregation [2]. Cobra (genus *Naja*) distribution became greatly influenced by the geologic condition and sea level [3]. This study was conducted by phylogenetic analysis towards the 16S rRNA gene. The purpose of this study is to estimate the relationship of *Naja* genus in the island of Java, Sumatra, and other Asian region based on the 16S rRNA mitochondrial gene. Zoo-geography analysis is also examined for more advance understanding between the correlation and evolution of Sundaland.

MATERIALS AND METHODS

Sampling and Preservation

Survey was done with Visual Encounter Survey (VES) method on 6 locality. There were 3 location in Sumatra Island and the others in Java Island (Table 1). Sample from other Asian region was obtained from Genbank, which are 11 individuals from China, Thailand, and Nepal (Table 2). VES method identification was done based on morphological characteristics, then preserved by alcohol 70%. The preserved sample tissue was further extracted and cured in EDTA 4%.

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Table 1. Cobra (Genus: *Naja*) Samples Used on This Research

Species	Locality	Island	Accession Number
<i>Naja sputatrix</i>	Malang, East Java	Java	MG012885
<i>Naja sputatrix</i>	Bondowoso, East Java	Java	MG012888
<i>Naja sputatrix</i>	Cilacap, Central Java	Java	MG012889
<i>Naja sumatrana</i>	Mandailing Natal, North Sumatra	Sumatra	MG012884
<i>Naja sumatrana</i>	Medan, North Sumatra	Sumatra	MG012886
<i>Naja sumatrana</i>	Bengkulu, Sumatra	Sumatra	MG012887

Table 2. Sequences Data of the Genus *Megophrys* and Outgroup Species from GenBank

Species	Accession Number	Locality
<i>Naja sumatrana</i>	JN687929.1	Thailand
<i>Naja kaouthia</i>	GQ359757.1	Thailand
<i>Naja kaouthia</i>	EU624269.1	Thailand
<i>Naja atra</i>	EU729432.1	Fujian, China
<i>Naja atra</i>	HM439991.1	Jiangxi, China
<i>Naja atra</i>	EF413645.2	Guangdong, China
<i>Naja atra</i>	EF413642.2	Guangdong, China
<i>Naja atra</i>	EU729431.1	Yunnan, China
<i>Naja naja</i>	GQ359756.1	Nepal, India
<i>Naja naja</i>	EU624270.1	Nepal, India
<i>Ophiophagus hannah</i>	JN687931.1	Thailand

DNA Extraction

DNA extraction was done according to the QIAmp® DNA Mini Kit standard protocol. A total of 25 mg of tissue was cut in tiny pieces and placed in 1.5 ml microtube. The tissue was given 180 µL of ATL buffer and 20 µL of K-proteinase. Samples were incubated in waterbath-shaker, and was done in 56°C for 1-3 hours, then added 200 µL of AL buffer. Sample incubation at a temperature of 56°C was done for 10 minutes, then added 200 µL of 90-100% ethanol. Each procedure is terminated with vortex. Samples were pipetted and inserted into 2 mL collection tube and placed in Dneasy Mini Spin. Centrifugation was done at 8000 rpm (one minute), supernatant is then discarded. Spin Column was placed in new collection tube and was given 500 µL of AW1 buffer. Sample then centrifuged on 8000 rpm (one minute), supernatant is then discarded. Spin column then placed in new collection tube (2 mL) and was added 500 µL of AW2 buffer. Centrifugation was done at 14.000 rpm (3 minute), supernatant is then discarded. Spin column then placed in 1.5 or 2 mL microtube. DNA was thawed with 200 µL AE buffer in the middle of spin column. Incubation was done at one minute (15-25°C), then centrifugated at 8000 rpm (1 minute).

Qualitative Analysis

Qualitative analysis was done by agarose gel (1%) electrophoresis [4]. Gel was made using 0.3 g agarose and 30 mL Tris-Boric-EDTA (TBE) with pH 8. Gel was made inside Erlenmeyer flask until boiled a little. Warm agarose was given 1 µL EtBr

and then homogenized. Homogenate was poured slowly into gel cast, and allowed to harden. The hardened gel was inserted into electrophoresis chamber and soaked in TBE (pH 8). DNA sample is mixed with loading dye, then inserted into wells as well as the DNA marker ladder 1 kb (1:1). Electrophoresis then conducted at half voltage for 45-60 minute. The results were documented with Gel Doc.

DNA Amplification

The components were placed in microtube: 3.6 µL of dH₂O; 5 µL of PCR mix (Go Taq Green); 0.2 µL of 16 SAR primer (F) 5'CGCCTGTTTATCAAAAACAT-3' and 16 SBR (R) 5'-CCGGTCTGAACTCAGATCACGT3' [5]; 0.5 µL of DNA sample. PCR was set at: Hot start at 95°C (3 minute), denaturation at 95°C (30 second), annealing at 52°C (60 second), extension at 72°C (60 second), and post-extension at 72°C (7 minute). PCR cycle was repeated 34 times.

Phylogenetic Analysis

The forward and reverse 16S sequences are combined with the Sequencher™ version 4.1.4 program, then in BLAST (Blast Local Alignment Search Tool) at www.ncbi.nlm.nih.gov. As outgroup, *Ophiophagus hannah* (Family Elapidae) is used with DNA sequence data that is accessed from the site www.ncbi.nlm.nih.gov. Both sample sequences and outgroup species were aligned with ClustalW analysis on the MEGA6.0 program.

The reconstruction of phylogenetic tree was done by Maximum Parsimony (MP) analysis, with bootstrap 1000 in PAUP 4.0b10a program. The Jmodeltest 3.06 program is used for the best model of likelihood analysis. Analysis of MP, Maximum Likelihood, and Bayesian Inference were performed on Mr.Bayes program ver 3.1.2 MCMC 10 million generation. Haplotype Network Analysis is done with DNAsp program and Bandelt Network.

RESULT AND DISCUSSION

Qualitative Analysis

The results of electrophoresis of all *N. sputatrix* DNA samples and *N. sumatrana* showed the presence of DNA with an amplicon size of

500-600 bp (Fig. 1). An amplicon length range of 550-590 bp [6] is the result obtained from the amplification of 16S rRNA gene with 16 SBR primary and 16 SAR as reverse and forward respectively. Visualization of DNA bands describes the purity of DNA resulting from isolation process, hence the visualized DNA can be used as a further molecular analysis material [6].

Genetic Distance of *Naja*

The determination of new species on *N. haje* is indicated by the percentage of p-Distance value of the 16S rRNA gene $\geq 5\%$ [7]. The genetic characteristics of a species are influenced by differences in geographical conditions, while geographical barriers can alter the genetic distance of a population [8]. The p-Distance results showed a low value (1-2%) between the species of *N. sputatrix* (Java) and *N. sumatrana* (Sumatra). The obtained value is supported with a similarity rate of 96-97% in the BLAST analysis. The p-Distance value between *N. sumatrana* from Sumatra and Thailand is low (p-Distance 1%), which is supported 97-98% similarity level on BLAST result. The p-Distance value between

N. atra (China), *N. sputatrix* (Java), and *N. sumatrana* (Sumatra) is high (p-Distance 4-16%). The p-Distance value between *N. kaouthia* and *N. sputatrix* (Java) and *N. sumatrana* (Sumatra) is low (p-Distance 2-3%), while p-Distance value between *N. kaouthia* with *N. atra* and *N. naja* classified as high (p-Distance 9-12%). The p-Distance analysis concluded that *N. sputatrix*, *N. sumatrana*, and *N. kaouthia* were spread in the Sundaland and had close phylogenetic relationships. Based on the p-Distance value of $\geq 5\%$ for the determination of new species, the three *Naja* species may be possible still within the same species (Table 3).

Phylogenetic Analysis

The phylogenetic analysis of *Naja* genus based on 16S rRNA (MP=98, ML=80, BI=100) shows the formation of two clades of *Naja* (A and B), and one *outgroup*, which is *Ophiophagus hannah* (C) (Fig. 2). The results of this study led to the idea to review the results of research by Philip [9] which states that *Ophiophagus hannah* is a sister lineage of the genus *Naja*.

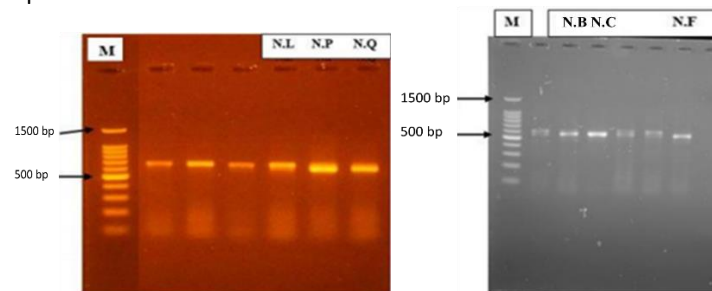


Figure 1. Electrophoresis result of *Naja* DNA sample by 16SAR and 16SBR primers. **Description:** M=marker; N.L.=*N. sumatrana* (Bengkulu); N.P.=*N. sputatrix* (Bondowoso); N.O.=*N. sputatrix* (Cilacap); N.B.=*N. sputatrix* (Malang); N.C.=*N. sumatrana* (Mandailing Natal); N.F.=*N. sumatrana* (Medan).

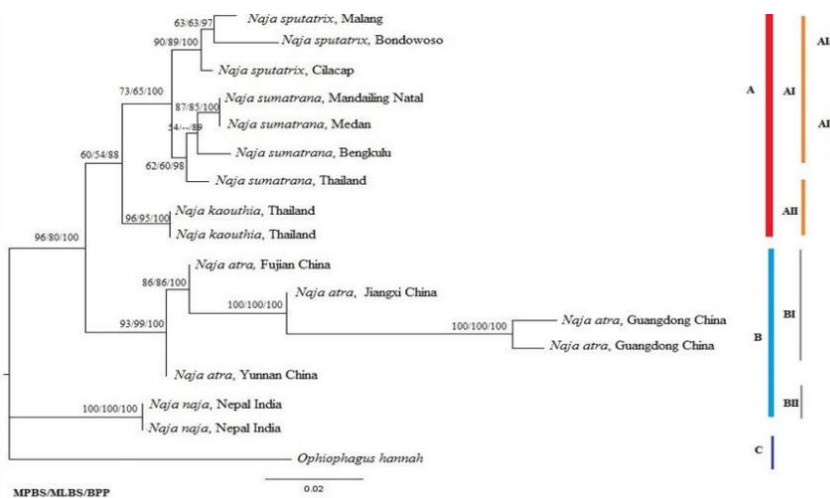


Figure 2. The Reconstructed Phylogenetic Tree of the *Naja* Genus Based on the 16S rRNA Gene from Java, Sumatra and Some Areas of Asia. **Description:** MPBS = Maximum Parsimony, MLBS = Maximum Likelihood, BPP = Bayesian Inference, sign-indicates no bootstrap values on phylogenetic tree branches.

Table 3. Value of Uncorrected p-Distance of Genus *Naja* (Laurenti, 1768) Based on mtDNA 16S rRNA

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
<i>N. sputatrix</i> ¹																
<i>N. sputatrix</i> ²	0.01															
<i>N. sputatrix</i> ³	0.00	0.01														
<i>N. sumatrana</i> ⁴	0.02	0.02	0.02													
<i>N. sumatrana</i> ⁵	0.02	0.02	0.02	0.00												
<i>N. sumatrana</i> ⁶	0.02	0.03	0.02	0.01	0.01											
<i>N. sumatrana</i> ⁷	0.02	0.02	0.02	0.01	0.01	0.02										
<i>N. atra</i> Fujian ⁸	0.03	0.04	0.04	0.04	0.04	0.04	0.03									
<i>N. atra</i> Jiangxi ⁸	0.06	0.06	0.06	0.06	0.06	0.06	0.05	0.02								
<i>N. atra</i> Guangdong ⁸	0.14	0.14	0.14	0.14	0.14	0.14	0.13	0.10	0.07							
<i>N. atra</i> Yunnan ⁸	0.03	0.04	0.03	0.03	0.03	0.04	0.03	0.00	0.02	0.10						
<i>N. atra</i> Guangdong ⁸	0.12	0.13	0.13	0.12	0.12	0.13	0.12	0.08	0.06	0.02	0.09					
<i>N. kaouthia</i> ⁷	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.04	0.12	0.02	0.10				
<i>N. kaouthia</i> ⁷	0.02	0.03	0.03	0.03	0.03	0.03	0.03	0.02	0.04	0.12	0.02	0.10	0.00			
<i>N. naja</i> ⁹	0.06	0.07	0.06	0.06	0.06	0.06	0.05	0.06	0.08	0.15	0.05	0.14	0.06	0.06		
<i>N. naja</i> ⁹	0.06	0.07	0.06	0.06	0.06	0.06	0.05	0.06	0.08	0.15	0.05	0.14	0.06	0.06	0.00	
<i>Ophiophagus hannah</i>	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.08	0.10	0.18	0.08	0.17	0.08	0.08	0.09	0.09

Notes: 1=Malang, 2= Cilacap, 3= Bondowoso, 4= Mandailing Natal, 5= Medan, 6= Bengkulu, 7= Thailand, 8= China, 9= Nepal.

Clade A

Phylogenetic analyzes of clade A (MP = 60, ML = 54, BI = 88) indicate the presence of division into two monophyletic subclade (AI and AII). Subclade AI consists of groups of *Naja* from Sunda (Thailand, Sumatra and Java). Phylogenetic analysis of subclade AI (MP = 73, ML = 65, BI = 100) divided the subclade into two monophyletic groups (Ala and Alb). The grouping of Ala group (MP = 90, ML = 89, BI = 100) indicates the division of *N. sputatrix* originating from East Java (Malang, Bondowoso) and Central Java (Cilacap). The construction of the phylogenetic tree shows that *N. sputatrix* from Cilacap is a sister lineage of *N. sputatrix* from Malang and Bondowoso. *Naja sputatrix* from Malang and Bondowoso were identified as sister species or close relatives (MP = 63, ML = 63, BI = 97).

According to BLAST results, the Ala group (*N. sputatrix* from Java) is similar to *Naja sumatrana* and *Naja kouthia* with 96-97% similarity level. Wuster and Thrope [10] show that *N. kouthia* and *N. sumatrana* in Asia are one group with *N. sputatrix* and represent a lineage with appropriately similar morphology or distribution and ecological characteristics. The results showed that *N. sputatrix* from Malang and Bondowoso (Fig. 3) had closer relationship than *N. sputatrix* from Cilacap, in accordance with similar morphological characters (brown scales on *N. sputatrix* from Malang and Bondowoso, while black on *N. sputatrix* from Cilacap).

Das [3] revealed that *N. sputatrix* has 1189 mm (SVL) and 155 mm (TL). Meristic characters are two nasal scales, no loreal scales, one supraocular scales, one preocular scoop, three postocular scales, supralabial scales, nine infralabial scales, 19 dorsal central scales, smooth surfaces, 180 ventral scales, and anal scales are divided. Generally, there is no pattern behind the

hood, but if there is, it usually resembles the shape "V". The presence of small scale accessories [11] on the head scales is not directly related to the process of adaptation, but in contrast, color is possible to be one of the adaptation factor. Populations in Central Java to West Java have dominant blackish color and occupy a thick humid rainforest, while the population from East Java has a silver to brownish scales, occupying habitat with dry soil conditions.

The Alb group (*N. sumatrana*) originating from Sumatra has a similar likelihood of 97-98% with the same species from Thailand, while it has a similarity of 95-96% with *N. kaouthia* (AII) of Thailand, in accordance with BLAST results. The result of BLAST is in accordance with the result of phylogenetic tree reconstruction using bootstrap value: MP = 62, ML = 60, BI = 98, which indicate that *N. sumatrana* species from Indonesia is the sister lineage of *N. sumatrana* from Thailand. The groupings performed on Alb (MP = 54, ML = -, BI = 89) indicate that *N. sumatrana* from Bengkulu is identified as sister lineage with the same species from Medan and Mandailing Natal. Meanwhile, *N. sumatrana* from Medan and Mandailing Natal is a sister species (MP = 87, ML = 85, BI = 100).

The phylogenetic reconstruction of the Alb group is in accordance with the research of Wuster and Thrope [10], i.e. the comparison of Sumatran *N. sumatrana* with similar species from South Thailand and the Philippines based on morphological characters. Some of the morphological characters observed in the juvenile phase of *N. sumatrana* are bright and striking colors on the back, dark base that surrounds the body, and a dot mark on the back of the head. The adult of *N. sumatrana* has a whole black body color, and only brightly colored in its head scales (Fig. 4). Climatic conditions

affect the distribution process of this species in Southern Thailand resulting in competition between *N. sumatrana* and *N. kaouthia*. *N. sumatrana* has a body length of 1.5 m with a strong body, large head differs from the neck, round muzzle, and round hood (adult) or oval (juvenile). This species has a single preocular scales, postocular scales of two to three, seven supralabial, four infralabial, 179-206 ventral scales, 40-57 subcaudal scales. The back color is related to geographical origin and size [3].

Subclade All is a group of species *N. kaouthia* originating from Chumpon Province, Southern Thailand with (MP = 96, ML = 95, BI = 100). The results of the phylogenetic reconstruction trees in accordance with the results of research Wuster [12] which showed that three evolutionary lines separated from the lineage of the genus *Naja*. The main sub-lineage representing the *Naja* genus of Asia represented by *N. naja* where the reconstructed phylogenetic tree by Bayesian analysis shows that *N. kaouthia* is a sister lineage of *N. atra* supported by a valid matching value (bootstrap 95%).

Clade B

Clade B is divided into two subclasses (BI and BII). Sequences with valid matching values from the BI subclass *N. atra* are from China (Fujian, Guangdong, Jiangxi, Yunnan province) are taken from GenBank. The result is supported by bootstrap value MP = 93, ML = 99, BI = 100. *N. atra* of Fujian Province is a sister lineage of the same species from Jiangxi Province (MP = 86, ML = 86, BI = 100). *N. atra* of Guangdong Province is a sister lineage of the same species of Jiangxi Province with a strong matching value (MP = 100, ML = 100, BI = 100).

Subclade BII is *N. naja* from Nepal (India), and has a valid topology (MP = 100, ML = 100, BI = 100). *N. naja* according to the results of the reconstruction of phylogenetic trees is the species with the closest kinship with outgroup species *Ophiophagus hannah*. The results of the reconstruction are in accordance with research from Wuster [12] and Wallach [13], where *N. naja* are separated from clade of *N. kaouthia*, *N. sputatrix*, and *N. Sumatrana*.



Figure 3. *N. sputatrix* Based On the Color Difference of Scales Among Localities. **Description:** A1. Bondowoso with whole body, A2. Ventral scales, A3. Head Scale; B1. Malang, with whole body, B2. Ventral scale, B3. Head scales.



Figure 4. *N. sumatrana* Muller, 1890. (a) Bengkulu; (b) Medan

Naja Biogeography

The divergence time of Elapidae occurs at the beginning of the Neogenic Period (approximately 16 mya) to the end of the Cretaceous subperiod [14]. The distribution of this family is related to the evolution of Sundaland. There are two main factors affecting the distribution of cobra in the Malay Peninsula and Indonesia, i.e. ecological conditions and sea surface changes [3].

The separation of Elapidae occurred in about 16 mya [16]. *N. sputatrix* is recorded only in Indonesia (Java, Bali, Lombok, Sumbawa, Flores, and possibly the surrounding islands). The abundance of *N. sputatrix* in Java is varies depend on locality [17]. Ecological conditions is found to be correlated with patterns of diversity among *N. sputatrix*, and occur in various climatic zones in Java and the Sunda Islands. The phylogenetic trees reconstruction and the value of p-Distance show that *N. sputatrix* in Malang (East Java) with Bondowoso (East Java) is closely related.

Separation of Java begins at late Miocene, which accompanied by sea water fluctuations that cause half of Java to be submerged by water (Fig. 5). Separation of Java and Sumatra occurs during the end of Miocene (10 mya) to the end of Pliocene (1.8 mya) [1]. The process of land

separation causes a geographical isolation on *N. sputatrix*. Geographical isolation ultimately limits the spread of this species due to its inability to adapt to the Sumatra, Kalimantan, and other Asian environments. This insights explain why *N. sputatrix* is only found in Java. Furthermore, at the beginning of the Pleistocene (250,000 years ago) there was a decrease of sea water surface, allowing the distribution of *N. sumatrana* in southern Thailand. This species is able to adapt to climatic and ecological conditions in Thailand and is able to spread from Sumatra and Kalimantan [18].

N. kaouthia that used in this study was came from Southern Thailand. *N. kaouthia* experienced separation with the divergence time of 1-0.8 mya [16]. The species is distributed almost in various regions: Myanmar, Thai Peninsula, South Laos, Cambodia, South Vietnam, northern Peninsular Malaysia, India, Nepal, Bangladesh, South China, Sichuan Province, and Yunnan [3]. *N. kaouthia* throughout the mentioned region is the result of the evolution of the Sundaland. The Distribution was began in the early Eocene to the late Oligocene (25 million years ago), when Burma, Malay Peninsula, Java and Sumatra were still in the mainland [2].

N. naja is separated from Elapidae family with divergence time of 1-0.8 mya [16]. The relation of this species with another *Naja* in Sundalandis quite distant. This evidence can be explained by the separation process of Indian and African land that occurred at the end of the Mesozoic era (120 mya) to the beginning of the Cenozoic era (65 mya). The expansion of the ocean floor that occurred during the Tertiary period led to the movement of the Indian mainland towards the Asian Continent (Fig. 5). The movement of mainland India resulted in the occurrence of collisions and continues on the formation of the Himalayas and the Tibet plateau [19]. The *N. naja* species subsequently experienced an allopathic speciation due to the separation of population by the ecological barrier (Himalayan Mountains).

N. atra has a divergence time of about 3 mya. Nanling and Luoxiao mountains do not become a

barrier in the distribution of *N. atra* in mainland China, where in this study is represented by the provinces used as sampling data (Fujian, Yunan, Jiangxi and Guangdong). The existence of *N. atra* in Taiwan, an island separated from mainland China was seen as any divergence split progress. However, *N. atra* in Taiwan differs from the species of mainland China. *N. atra* in Taiwan has a distinct genetic character and shows the separation of the phylogenetic tree lineage between populations in mainland China and Taiwan. Formation of Taiwan Island occurred during the Miocene, then it was separated from mainland from the southeast coast of mainland China by Taiwan Strait. Climate change during the Pleistocene caused most of the Taiwan Strait to become a landmass, thus providing an opportunity for *N. atra* to migrate from mainland China [20].

Eon	Era	Period	Epoch	Millions of Years Ago	Major Geologic and Biological Events
PHANEROZOIC	CENOZOIC	QUATERNARY	Recent	0.01	Modern humans evolve; major extinction event is now underway.
			Pleistocene	1.3	
		TERTIARY	Pliocene	6.3	Tropics, subtropics extend poleward. Climate cools; dry woodlands and grasslands emerge. Adaptive radiations of mammals, insects, birds.
			Miocene	22.0	
			Oligocene	33.7	
			Eocene	65.5	
			Paleocene	66	
	MESOZOIC	CRETACEOUS	Late	99	Major extinction event, perhaps precipitated by asteroid impact. Mass extinction of all dinosaurs and many marine organisms. Climate very warm. Dinosaurs continue to dominate. Important modern insect groups appear (bees, butterflies, termites, ants, and herbivorous insects including aphids and grasshoppers). Flowering plants originate and become dominant land plants.
			Early	145	
		JURASSIC	213	Age of dinosaurs. Lush vegetation; abundant gymnosperms and ferns. Birds appear. Pangea breaks up into North America, Eurasia, and Gondwana.	
		TRIASSIC	249	Major extinction event. Recovery from the major extinction at end of Permian. Many new groups appear, including turtles, dinosaurs, pterosaurs and mammals.	
		PALEOZOIC	PERMIAN	286	Major extinction event. Supercontinent Pangea and world ocean form. Adaptive radiation of conifers. Cycads and ginkgos appear. Relatively dry climate leads to drought-adapted gymnosperms and insects such as beetles and flies.
			CARBONIFEROUS	360	High atmospheric oxygen level fosters giant arthropods. Spore-releasing plants dominate. Age of great lycophyte trees; vast coal forests form. Ears evolve in amphibians; penises evolve in early reptiles (vaginae evolve later, in mammals only). Major extinction event.
	DEVONIAN		410	Land tetrapods appear. Explosion of plant diversity leads to tree forms, forests, and many new plant groups including lycophytes, ferns with complex leaves, seed plants.	
	SILURIAN		440	Radiations of marine invertebrates. First appearances of land fungi, vascular plants, bony fish, and perhaps terrestrial animals (millipedes, spiders). Major extinction event.	
	ORDOVICIAN		505	Major period for first appearances. The first land plants, fish, and reef-forming corals appear. Gondwana moves toward the South Pole and becomes frigid.	
	CAMBRIAN	544	Earth thaws. Explosion of animal diversity. Most major groups of animals appear (in the oceans). Trilobites and shelled organisms evolve.		
PROTEROZOIC				2,500	Oxygen accumulates in atmosphere. Origin of aerobic metabolism. Origin of eukaryotic cells, then protists, fungi, plants, animals. Evidence that Earth mostly freezes over in a series of global ice ages between 750 and 600 mya.
ARCHAEAN AND EARLIER					3,800–2,500 mya. Origin of prokaryotes. 4,600–3,800 mya. Origin of Earth's crust, first atmosphere, first seas. Chemical, molecular evolution leads to origin of life (from proto-cells to anaerobic prokaryotic cells).

Figure 5. Geological Time Scale and Some Important Events [15]

CONCLUSION

N. sputatrix from Java is similar to *Naja sumatrana* and *Naja kouthia* with 96-97% similarity level. The results showed that *N. sputatrix* from Malang and Bondowoso had closer relationship than *N. sputatrix* from Cilacap, in accordance with similar morphological characters. *N. sumatrana* from Sumatra has a similar likelihood of 97-98% with the same

species from Thailand. *N. atra* of Fujian Province is a sister lineage of the same species from Jiangxi Province (MP = 86, ML = 86, BI = 100). *N. atra* of Guangdong Province is a sister lineage of the same species of Jiangxi Province with a strong matching value. *N. naja* is the species with the closest relationship with outgroup species *Ophiophagus hannah*.

ACKNOWLEDGEMENTS

Authors acknowledges the support of PEER-USAID for herpetofauna studies in Java and Sumatra (2016). We are very grateful to NK research members: Bagus Priambodo, M. Alif Fauzi, Ari Ardiantoro, Anggun S. Firdaus, for valuable work on herpetofauna research. We are also thankful to Erintha E. Wardani for her help in making many corrections and improvements to the experimental procedure. We are also grateful for the revisions, suggestions and criticisms for Prof. Nashi Widodo and Dr. Bagyo Yanuwadi for the preparation of the earliest manuscripts.

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