Phylogenetic analysis of colubrid snakes based on 12S rDNA reveals distinct lineages of *Dendrelaphis pictus* (Gmelin, 1789) populations in Sumatra and Java

FITRA ARYA DWI NUGRAHA[1], FATCHIYAH FATCHIYAH[1], ERIC NELSON SMITH[2], NIA KURNIAWAN[1,♥]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya. Jl. Veteran, Malang 65145, East Java, Indonesia.
Tel.: +62-341-554403, ♥email: wawan@ub.ac.id, wawanunibraw@gmail.com
[2]Department of Biology, University of Texas at Arlington. Arlington, TX 76019, USA

Abstract. *Nugraha FAD, Fatchiyah F, Smith EN, Nia Kurniawan N. 2018. Phylogenetic analysis of colubrid snakes based on 12S rDNA reveals distinct lineages of* Dendrelaphis pictus *(Gmelin, 1789) populations in Sumatra and Java. Biodiversitas 19: 303-310.* The phylogenetic relationship among the major colubrid snakes, particularly those of the subfamily Colubrinae, has been the subject of much debate. Also, there was limited data on the molecular relationships of Sundaland colubrid snakes. This study aimed to examine the relationships among colubrid snakes from Sumatra and Java based on fragments of 12S rDNA gene. We sequenced 17 specimens of colubrid snakes representing 5 genera and 2 subfamilies: Colubrinae and Ahaetullinae. We used maximum likelihood, maximum parsimony and Bayesian inference methods for inferring phylogenetic relationships. The result of our phylogenetic analyses is in line with the previous findings for the separation between Colubrinae and Ahaetullinae. Interestingly, we found two distinct clades of *Dendrelaphis pictus* species with the high genetic divergence between them where *D. pictus* from Sumatra and West Java separated from Central and East Java clade. Our divergence time estimation showed that the differentiation between these clades of *D. pictus* occurred in the late Miocene epoch (8.9 Ma) when Sumatra and Java separated after being inundated in the early Miocene epoch.

Keywords: Colubridae, mtDNA, phylogeny, p-distance, Sundaland

INTRODUCTION

Indonesia is known as one of the top biodiverse countries in the world. It comprises about 17,000 islands of various sizes and geological origins (Mittemeier et al. 1999). Based on the Indonesian Biodiversity Action Plan, 16% of the amphibians and reptiles of the world occur in Indonesia (BAPPENAS 1993). The distribution of herpetofauna within those thousands of islands was affected by the geological activities occurred in the past. These geological activities include volcanic activities, subduction of the Indian ocean crust, and collision of Australia-East Indonesia, which resulted in the rise and widening of Sumatra island at 15 Ma - mid-Miocene. Another region, East Java-West Sulawesi-Sumba, South West Borneo - rifted from the Australia continental block and were added to Sundaland at approximately 145 Ma - Cretaceous (Hall 2013). The Malay Peninsula, Sumatra, Java and Borneo islands were later connected due to decreasing of the sea level to approximately 118 m below the present sea level during ice ages, most recently at 20,500 - 14,000 years ago (Solihuddin 2014).

Previous studies on animal compositions have revealed complexity in their distribution, in particular within the Sundaland region. Snake populations on the Malay Peninsula and Sumatra are more closely related to each other than to those from Java or Borneo. However, snakes on the Malay Peninsula, Sumatra, Java, and Borneo were more closely related than they were to those from Sulawesi (Inger and Voris 2001). Based on a simple spatial proximity similarity model, mammals on Mentawai island and Borneo were more closely related to each other than they were to those from Sumatra, the Malay Peninsula and Java, while reptiles and amphibians on those two islands were closely related to those on Sumatra than to those from the other areas (Wilting et al. 2012). Outside of the Sundaland region, several studies have been conducted on colubrid snakes to resolve their taxonomy issues, evolutionary history, biogeographies, and phylogenies (Guo et al. 2012; Lawson et al. 2005; Pyron et al. 2011b; Vidal et al. 2000; Vidal and Hedges 2002; Zaher et al. 2009). Nevertheless, the phylogeny of Colubridae has not yet been fully resolved, particularly within the paraphyletic Colubrinae (Figueroa et al. 2016; Pyron et al. 2013a). Unfortunately, there have been few genetic samplings of Colubridae from Java and Sumatra (Shaney et al. 2016). However, some species of colubrid snakes in Indonesia intrigued some scientists in the past (Auliya 2002; How et al. 1996; van Rooijen and Vogel 2008a, b; 2010; 2012; Vogel 2008; Vogel and van Rooijen 2007; 2008). Here, we conducted the first study to address the relationship of colubrid snakes within Java and Sumatra islands using 12S rDNA with focusing on Colubrinae and the newly recognized subfamily (Figueroa et al. 2016), Ahaetullinae.

MATERIALS AND METHODS

Specimen collection and ethical clearance

This study analyzed eleven specimens of the genus *Dendrelaphis* from different locations in Sumatra and Java (Figure 1) consisting of *D. pictus* (Gmelin, 1789), *D. caudolineatus* (Gray, 1834), *D. haasi* van Rooijen & Vogel, 2008, and *D. subocularis* (Boulenger, 1888), one specimen of *Boiga dendrophila* (Boie, 1827) from Central Java, three specimens of the genus *Ptyas* from central and east Java, one specimen each of *Lycodon capucinus* (Boie, 1827) and of *Coelognathus radiatus* (Boie, 1827) from Central and East Java, respectively. Additional colubrid and non-colubrid outgroup sequences were obtained from GenBank (Table 1). To focus the assessment of sequence divergence and relationship on colubrid of the Sundaland region, we restricted our GenBank sampling to only the sequences that came from Sundaland. Therefore, many sequences from the same genera were not included in our analysis because they come from outside of Sundaland. To collect our specimens, we obtained ethical clearance with a letter of Ethical Clearance No: 68-KEP-UB.

DNA isolation, PCR and sequencing

Genomic DNA was isolated from muscle tissue using a QIAGEN DNA isolation kit following the manufacturer's instruction. A fragment of the 12S gene was amplified by polymerase chain reaction (PCR) using the primers 12S268 (5'-GTGCCAGCGACCGCGGTTACACG-3') and 12S916 (5'-GTACGCTTACCATGTTACGACTTGCCCTG-3') (Jeong et al. 2013). The PCR amplification was performed in 40 µL reaction volumes with 5 min denaturation at 94º C, followed by 35 cycles of 94º C for 30 s, 56,5º C for 1 min, and 72º C for 1 min, and then by post elongation at 72º C for 10 min. The amplified DNA was purified at Biosains Institute, Brawijaya University and sequenced in both directions using the same primers as for PCR by IndoseqGATC.

Sequence and data analysis

DNA sequences were aligned in MEGA 7.0 by the ClustalW method (Kumar et al. 2016). The alignments were edited by eye, with ambiguous sites trimmed (Figueroa et al. 2016). Genetic distances were calculated as uncorrected pairwise distances (Kumar et al. 2016). Phylogenetic relationships were estimated by maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI). ML analyses were performed with 1000 bootstrap replicates in MEGA 7.0 (Kumar et al. 2016) with Kimura2 parameter model. PAUP 4.0b10 was used to performed MP analyses with 1000 bootstrap replicates (Swofford 2002) and tree bisection recognition (TBR) branch-swapping algorithm on heuristic search option.
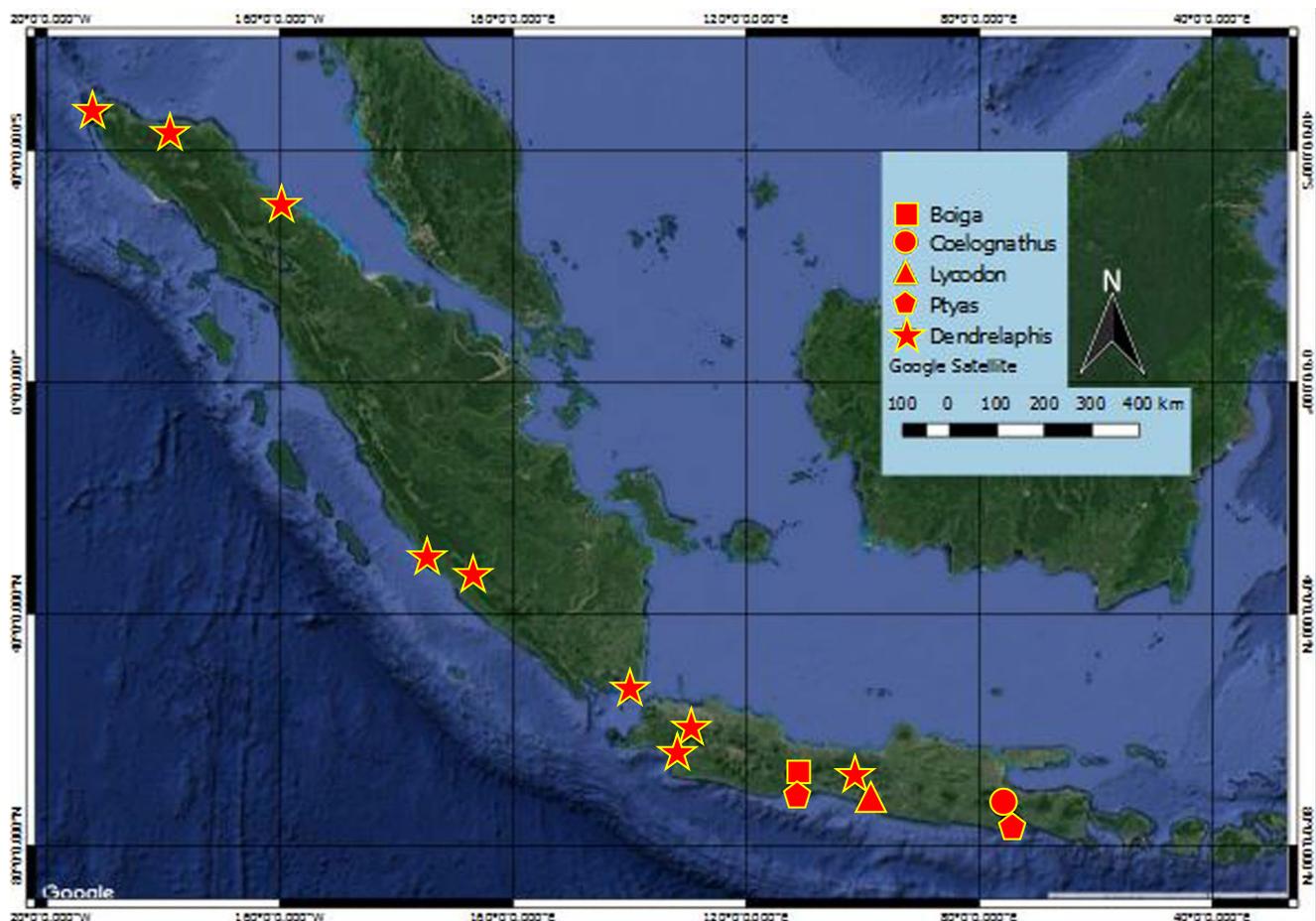
Figure 1. Locations of specimens collected from Sumatra and Java, Indonesia and included in this study

Table 1. List of GenBank accession numbers of sequences used in the phylogenetic analysis.

Specimens	Locality	Country	Genbank accession no.	Source
Boiga dendrophila	Cilacap, Central Java	Indonesia	KY700852	This study
Coelognathus radiatus	Malang, East Java	Indonesia	KY700853	This study
Dendrelaphis caudolineatus	Aceh	Indonesia	KY700854	This study
Dendrelaphis caudolineatus	Bengkulu	Indonesia	KY700855	This study
Dendrelaphis sp.	Sukabumi, West Java	Indonesia	KY700856	This study
Dendrelaphis subocularis	Bogor, West Java	Indonesia	KY700857	This study
Dendrelaphis haasi	Bengkulu	Indonesia	KY700858	This study
Dendrelaphis pictus	Bogor, West Java	Indonesia	KY700859	This study
Dendrelaphis pictus	South Lampung, Lampung	Indonesia	KY700860	This study
Dendrelaphis pictus	Aceh	Indonesia	KY700861	This study
Dendrelaphis pictus	Medan, North Sumatra	Indonesia	KY700862	This study
Dendrelaphis pictus	South Malang, East Java	Indonesia	KY700863	This study
Dendrelaphis pictus	Wonosobo, Central Java	Indonesia	KY700864	This study
Lycodon capucinus	Sadang, Central Java	Indonesia	KY700865	This study
Ptyas korros	Cilacap, Central Java	Indonesia	KY700866	This study
Ptyas korros	Cilacap, Central Java	Indonesia	KY700866	This study
Ptyas mucosa	South Malang, East Java	Indonesia	KY700867	This study
Boiga cynodon	Thailand	Thailand	Z46468	Heise et al. 1995
Boiga forsteni	Sri Lanka	Sri Lanka	KC347314	Pyron et al. 2013b
Lycodon laoensis	Suphanburi	Thailand	Z46455	Heise et al. 1995
Coelognathus radiatus	Thailand	Thailand	AY122676	Utiger at el. 2005
Coelognathus flavolineatus	Java	Indonesia	AY122666	Utiger at el. 2005
Coelognathus erythrurus	Sibutu island	Philippines	AY122776	Utiger at el. 2005
Ptyas mucosa	Kathmundu, Nepal	India	AY122828	Utiger at el. 2005
Dendrelaphis caudolineatus	Thailand	Thailand	AF544782	Vidal and Hedges 2002
Enhydris enhydris	-	-	EF395879	Alfaro et al. 2008
Hypsiscopus plumbea	-	-	EF395884	Alfaro et al. 2008
Naja naja	-	-	AF236683	Unpublished

Transition and transversion were equally weighted and the gaps were treated as missing data. BI was calculated with MrBayes 3.0b4 (Huelsenbeck and Ronquist 2001). The analysis performed under 4 simultaneous metropolis couple Monte Carlo Markov Chains (MCMC) for 1,000,000 generations and sampled a tree in every 1000 generations. The consensus topology was calculated by discarding the tree with the burn-in of 25%.

The divergence time was estimated with BEAST (Drummond and Rambaut 2007) under a Hasegawa-Kishino-Yano (Hasegawa et al. 1985) model of DNA evolution with uncorrelated log-normal relaxed clock rate model (Drummond et al. 2006). The external calibration used the time divergence between Homalopsidae, Colubridae and Elapidae at 49.2 Million years ago (Ma) (CI: 39-61); between Colubridae and Elapidae at 46.3 Ma (CI: 36-58) (Vidal et al. 2009); and between *Enhydris enhydris* and *Enhydris plumbea* at 18.9 Ma (CI: 12.9-24.9) (Alfaro et al. 2008). The internal calibration used the divergence time of Colubridae at 36.6 Ma (CI: 28-46) (Vidal et al. 2009); and between *Ptyas mucosa* and *Ptyas korros* at 21 Ma (Nagy et al. 2004). The analysis used MCMC chain every 1000 generations, for a total of 10,000,000 samples and assessed to the stationary distribution through inspection of the likelihood and parameter sample plots in Tracer v1.6 (Rambaut et al. 2014). All of the phylogenetic trees from the analyses were visualized with Figtree v1.4.2 (Rambaut 2014).

RESULTS AND DISCUSSION

Genetic Distances

The uncorrected pairwise distance or p-distance is described as the difference of nucleotide which being compared (Nei and Kumar 2000). Regardless of the locality, p-distance among *Boiga* ranged from 5.6% to 6.5% (mean±sd; 5.99 ± 0.47). Genetic distances were in the genus of *Coelognathus* (mean±sd;6.40 ± 3.50), which ranged from 4.0% to 9.0%. Sequence divergence within *Ptyas* ranged from 0.62% to 7.4% (mean±sd;5.06 ± 3.68). Within *Dendrelaphis*, p-distance values ranged from 5.9% to 11.8% (mean±sd; 6.36 ± 3.79) (Table 2). The intra-species threshold for 12S/16S was previously set at 3% (Jeong et al. 2013). Based on our result, we propose that 12S threshold for intra-species is 3.7%, inter-species is 11.8% and inter-generic is 16%. Our results showed the higher value of sequence divergence than the previous studies which suggests that the colubrid snakes in Java and Sumatra are genetically more diverse than those of other places.

Interestingly, *D. pictus* of West Java showed a greater distance when it compared to Central or East Java (3.1-3.72%) than to Sumatra (0.31-0.93%). The value of p-distance showed that *D. pictus* from Sumatra and West Java were genetically distinct from those in Central and East Java (Table 3).

Table 2. Uncorrected p-distances (%) for 12S rDNA among different genera of colubrid snakes of Java and Sumatra

Genus	Mean ± SD	Range
Boiga	5.99 ± 0.47	5.6-6.5
Coelognathus	6.40 ± 3.50	4.0-9.0
Ptyas	5.06 ± 3.68	0.62-7.4
Dendrelaphis	6.36 ± 3.79	5.9-11.8

Table 3. Uncorrected p-distances (%) for 12S rDNA of *Dendrelaphis pictus* from different localities of Java and Sumatra

No.	Locality	1	2	3	4	5
1	Aceh, Sumatra					
2	Medan, North Sumatra	0.3				
3	Lampung, Sumatra	0.6	0.3			
4	Bogor, West Java	0.9	0.6	0.3		
5	Wonosobo, Central Java	3.7	3.4	3.7	3.1	
6	Malang, East Java	3.4	3.1	3.4	3.4	0.3

Relationships among Colubridae in Sumatra and Java islands

The ML, BI and MP trees showed identical topology in the case of separation between the subfamilies Colubrinae and Ahaetullinae. In the monophyly of Colubrinae, the group of *Ptyas* found to be paraphyletic, while within Ahaetullinae, *D. pictus* was separated from *D. caudolineatus* (Figure 2). Monophyly of Colubrinae was strongly supported in all trees (Figure 2), while in Ahaetullinae it was strongly supported in the BI and MP trees (BPP=1, MP=70) yet weakly in the ML tree.

In the subfamily of Colubrinae, two *P. korros* from Cilacap formed a clade and was strongly supported in all trees. In the ML and BI trees, this clade was separated from other remaining species consisting of *P. mucosa*, *Coelognthus*, *Lycodon* and *Boiga,* while *P. mucosus* from East Java and *P. mucosa* from India were in a clade which formed a sister to the clade consisting of *Coelognathus*, *Lycodon*, and *Boiga*. The group of *Coelognathus* formed a clade (BPP=0.93) where *C. radiatus* from East Java and *C. radiatus* from Thailand (ML=100, BPP=1, MP=100) separated from *C. erythrurus* and *C. flavolineatus* (ML=84, BPP=1, ML=60). The Colubrinae genus *Coelognathus* clade was separated from the clade consisting of *Lycodon* and *Boiga*, where the clade of *Lycodon* formed a sister to the clade of *Boiga*. The species of *L. laoensis* formed a clade with *L. capucinus* (ML=89, BPP=1, MP=87) and *B. dendrophila* nested in a clade with *B. forsteni* in the ML and BI trees, but with *B. cynodon* in the MP tree.

Within Ahaetullinae, *D. caudolineatus* from Aceh, Bengkulu, and Thailand and *Dendrelaphis* sp. from Sukabumi formed a clade with significant bootstrap values in all trees. The clade consisting of *D. subocularis*, *D. haasi*, and *D. pictus* was divided into two subclades. The first subclade consists of *D. subocularis* from Bogor and *D. haasi* from Bengkulu, which was a sister clade to *D. pictus* from Bogor, Lampung, Medan, and Aceh. The second subclade consists of *D. pictus* from Wonosobo and Malang (ML=93, BPP=1, MP=96).
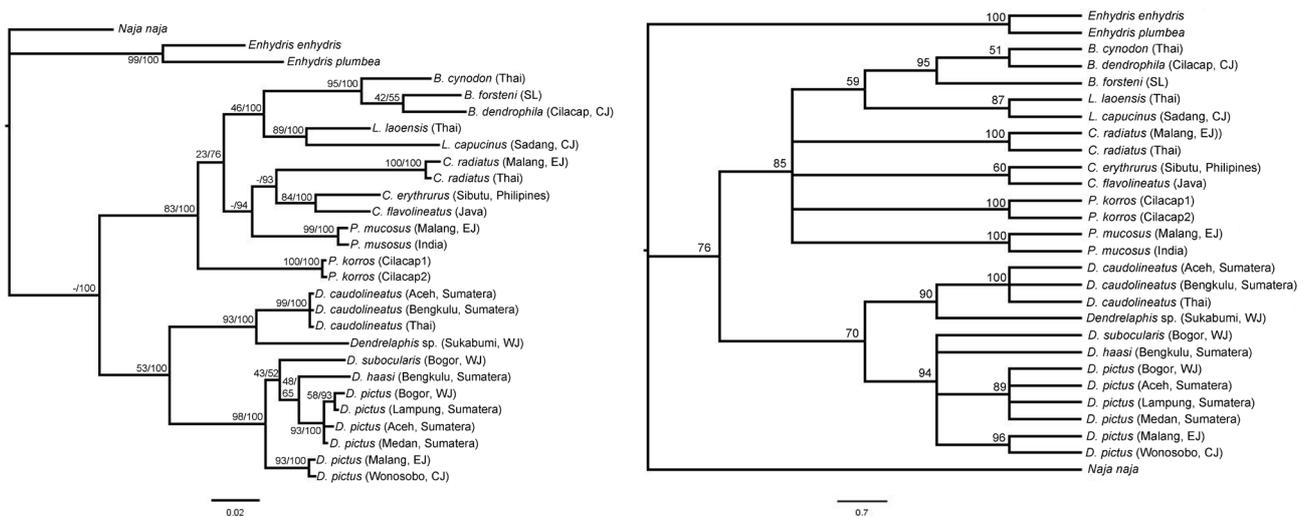

Figure 2. Phylogeny of Colubridae in Sumatra and Java islands based on partial sequence of 12S rDNA. *Left*: ML and Bayesian inference tree. Nodal supports represent ML bootstrap value/Bayesian posterior probability (BPP) (-) means no value. *Right*: MP tree with the bootstrap values on the branches

The present study is consistent with previous studies by Pyron et al. (2011; 2013a, b) and Figueroa et al. (2016) which separated *Dendrelaphis* from the Colubrinae group. Based on their results, Figueroa et al. (2016) proposed a new subfamily Ahaetullinae, which consists of *Ahaetulla, Chrysopelea, Dendrelaphis,* and *Dryiophiops* and covers 56 species. *B. dendrophila* (Cilacap, Central Java) in this study formed a clade with *B. forsteni* (Sri Lanka) and *B. cynodon* (Thailand), which is consistent with the subfamily proposed by Pyron et al. (2013a, b). However, Figueroa et al. (2016) showed that *B. dendrophila* formed a clade with *B. cyanea*, which was a sister clade to *B. forsteni* and *B. cynodon*. Additionally, two specimens of *P. korros* (Cilacap, Central Java) and *P. mucosus* (Malang, East Java) did not form a clade as showed by Utiger et al. (2005) and Pyron et al. (2011; 2013a), yet comprised a sister clade to *Coelognathus* (Utiger et al. 2005).

Dendrelaphis formed a clade with *Oreocalamus* (Kraus and Brown 1998). An individual of *D. caudolineatus*, *Ahaetulla fronticincta*, *Chrysopelea paradisi* formed a clade and became a sister clade to all remaining colubrid snakes (Pyron et al. 2011). Four single-specimens of *Dendrelaphis* formed a clade; *D. caudolineatus* formed a clade with *D. schokari*, *D. tristis* and *D. bifrenalis* (Pyron et al. 2013a), while in Pyron et al. (2013b) *D. bifrenalis* formed a clade with *D. pictus* and *D. calligaster*. The study with more comprehensive specimens and genetic sampling showed that *D. caudolineatus* separated from *D. haasi*, *D. cyanochloris*, and *D. pictus*, which were nested in a paraphyletic clade (Figueroa et al. 2016). In contrast to our expectation, *D. pictus* was found to be paraphyletic, with separation of the clade between Sumatra-west Java and central-east Java. Given that the high p-distance value and separated clade of the *D. pictus* group, we consider the geological history which could influence on the genetic differentiation in this area below; while the taxonomic status of this snake remains an area for further investigation.

The opportunities for gene flow among snakes from Sumatra, Java and Borneo would have been possible until the end of the Oligocene (±25 Ma) and again at the end of Miocene (±10 Ma). The exposed continents of Sundaland during much of Pleistocene were partially covered by savanna or grasslands. It provided the corridor for the migration of the snakes from the north of Sundaland to the south in Java region (Inger and Voris 2001). The prehistoric environmental conditions also had an enormous influence on the divergence of these snakes (Allam and Abo-Eleneen 2012). However, based on the map of Heaney (1991), the Pleistocene savanna or grasslands only covered part of the area between Sumatra-Borneo and Borneo-Eastern Java. It would have led the snakes to come only to East part of Java and then diversified genetically from those in Western Java and Sumatra. Unfortunately, our work did not include *D. pictus* from Borneo, which might be critical to understanding the pattern of distribution.

Historically, the east part of Java was a block rift from continental Australia, as along with SW Borneo and West Sulawesi. The blocks were added to Sundaland at approximately 90 Ma from West Java through Meratus mountains in the north (Hall 2014). According to this, snakes would have existed in these blocks since the snakes arose at approximately 125 Ma and they have become a distinct population from those in West Java. However, this scenario is not supported by the oldest known fossil of colubrid snake found in Wai Lek, Thailand, which was considered to date from the late Eocene (Rage et al. 1992). Moreover, we still consider this scenario as the likely speciation process between Sumatra-west Java and Central-East Java population due to limited fossil information, particularly for *D. pictus*.

The divergence time of Colubrid snakes

Our analysis on divergence time estimated that Colubrid snakes started to diverge in the late Eocene approximately 37.6 Ma. It was the period when most of western of Sundaland formed a connected landmass with many large freshwater lakes; while southern Sundaland comprised volcanic arcs that formed new islands. This age was considered as the collision time between India and Asia (Hall 2013), which might have facilitated many migrations of terrestrial fauna. From late Eocene, colubrid snakes began to spread from Asia to Europe by Oligocene, to North America through Beringia and to the west of America (Holman 1984). Moreover, colubrid rat-snakes began to spread from the eastern Palearctic to the western Palearctic and Nearctic at about 20 Ma (Chen et al. 2017). The result was also in line with the oldest Colubridae fossil in Wai Lek pit, which was dated from the late Eocene (Rage et al. 1992). The subfamily Colubrinae began to diversify in early Miocene at about 25 Ma, when rainforest extended to the north over much of Sumatra and when shallow seas inundated Java while Ahaetullinae diversified later in the Miocene, at about 24 Ma. *D. caudolineatus* began to diverge in the late Miocene at about 11.3 Ma, while genetic differentiation among *D. pictus* populations from Sumatra-West Java and Central-East Java occurred later at about 9.0 Ma when many small volcanic islands emerged in Sumatra and Java (Hall 2013).

Although the diversification of colubrids was estimated to have started in the Eocene epoch, our relaxed normal clock showed that most of the inter-specific divergence occurred in the Miocene epoch, when the majority of Java and Sumatra was inundated by a shallow sea. Glaciation in the Pleistocene epoch caused lowering of the sea level, which formed a continuous landmass of Sundaland. Savannah and open woodlands that grew up in this region may have facilitated a huge spread of colubrid snakes. To address the distinct lineage of *D. pictus* between Sumatra-West Java and Central-East Java, we discussed three possibilities that caused the distinction.
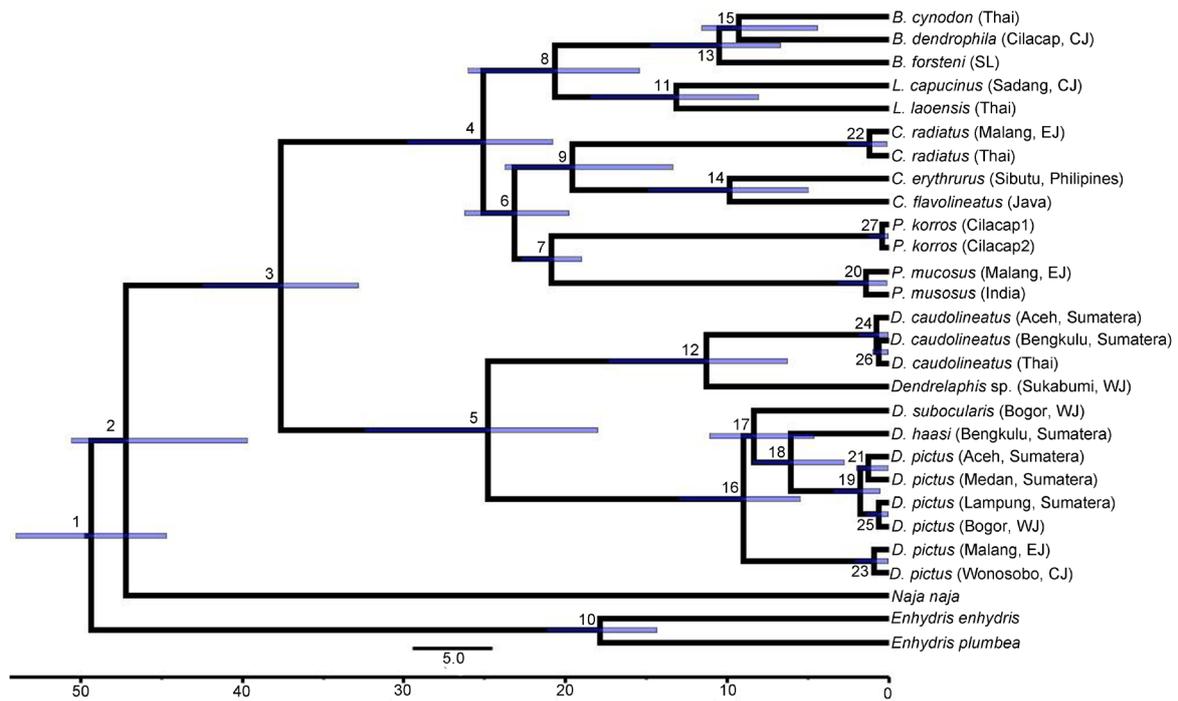
Figure 3. The divergence chronogram of colubrid snakes of Sumatra and Java based on 12S rDNA. The tree was constructed based on a relaxed normal clock and Bayesian inference with 95% credible interval

Table 4. The time of divergence of Colubridae with 95% credible interval

Clade	Divergence time (Ma)	95% CI (Ma)
1	49.3	45.488-54.977
2	47.2	40.466-51.489
3	37.6	33.419-43.257
4	25.1	21-30.204
5	24.8	18.279-32.931
6	23.1	20.093-26.669
7	20.9	19.326-23.076
8	20.7	15.669-26.46
9	19.6	13.558-24.105
10	17.9	14.581-21.5
11	13.1	8.163-18.722
12	11.3	10.455-17.787
13	10.5	6.831-15.094
14	9.9	5.094-15.281
15	9.2	4.53-11.854
16	9.0	5.634-13.334
17	8.4	4.742-11.314
18	6.0	2.817-8.615
19	1.8	0.564-3.545
20	1.4	0.094-3.075
21	1.2	0.07-2.019
22	1.2	0.141-2.653
23	0.9	0.094-2.09
24	0.8	0.07-1.784
25	0.6	0.07-1.432
26	0.6	0.094-0.986
27	0.4	0.094-1.286

First, if Rage (1992) is correct about the Asiatic origin of Colubridae, the migration through a land bridge for *D. pictus* must come from South of Java which is Borneo or Sumatra and began to diversify. The possibility to migrate occurred only in the Pleistocene epoch because there was no a land bridge which connected these islands since Eocene through Miocene. However, it was not corroborated by the divergence time data. The Pleistocene began at 1.8 Ma, while the separation of *D. pictus* populations in Sumatra-west Java from those in Central-East Java evidently occurred at 9 Ma. Second, West Sulawesi and East Java were continental blocks that rifted from Australia and were added to Asia in the Cretaceous period (Hall 2014). If *D. pictus* from Central-East Java came from these continental blocks, Colubridae should have appeared in the Jurassic period or at least in the Cretaceous, but the oldest fossil Colubridae was considered only from the late Eocene which is very far from Jurassic. Hence, the two possibilities described above are contradicted by other data and cannot explain the high genetic differentiation between *D. pictus* populations of Sumatra-West Java and Central-East Java.

The last possibility uses the migration theory proposed by Inger and Voris (2001), material rafting. When *D. pictus* reached the west part of Java, the volcano activities caused migration of the snakes down from their habitat, they randomly attached to material that floats on the water, and the sweepstakes migration followed the wind or cyclonic

storm to the central part and east part of Java. The scaly epidermis may have facilitated and protected them from crossing the saline water. Once *D. pictus* reached central or east part of Java, the gene flow was obstructed between the regions of Sumatra-West Java with Central-East Java. However, the possibility of inhibition of gene flow only lasted until the Ice Age in the Pleistocene, because in this epoch the distribution of snakes becomes larger than the previous wave of distribution in the final epoch of the Eocene, Oligocene, Miocene or Pliocene. This happened due to the lowering sea level and the growth and development of savanna in the mainland of Sundaland. In addition, the river system linking the island of Borneo to Central and East Java, as well as West Java with the central and eastern part also provided a very suitable corridor for the migration of *D. pictus* to and from those areas. As a result, the gene flow that had been interrupted in the previous epoch (Eocene-Pliocene), which was not strong enough to make *D. pictus* in the West Java significantly different from Central and East Java, became reopened in the Pleistocene epoch. Hence, the gene flow could still occur again among populations of *D. pictus* of western Java with those in central and eastern Java.

This study showed that geological history and ecological variation of Sumatra and Java facilitated the distribution and the diversification of colubrid snakes in those islands. Moreover, our study has found evidence that East and Central Java has a unique diversity compared to West Java and Sumatra as it found in *L. hasseltii* (Hamidy and Matsui 2017). However, the present study should be confirmed with further studies using more genes and more comprehensive methods, since we only used one mitochondrial gene in this study. More specimens from adjacent islands such Bali, Madura, Borneo, and Sulawesi are also crucial to reveal the origin of East Java diversity, especially that of *D. pictus*.

ACKNOWLEDGEMENTS

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