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Original research article

Genetic Divergence and Phylogenetic Relationships Among Indonesian Species of Monitor Lizards of the Genus *Varanus* Based on Cytochrome Oxidase I Sequences

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ABSTRACT

The application of cytochrome oxidase subunit I for genetic divergence and phylogenetic analyses in monitor lizards seems to be limited, despite the practicality and relevance to use the short sequence of this region known as the DNA barcode. Some Indonesian species of monitor lizards are morphologically similar, some of which are legally exported as pet animal commodities and some others being protected by Indonesian national law. Thus, a practical molecular tool that can be useful to help clarify their species identity is essential, especially for closely related species. This study used the DNA barcode to test the application of this mitochondrial DNA region as a molecular tool to identify some species of Indonesian monitor lizards for the first time. Results showed that the Barcodes can facilitate molecular species identification based on their relative amount of genetic divergence and phylogenetic relationships. Closely related species can be distinguished based on the short sequences, as well as a likelihood of species misidentification among samples in this study. Further study should be performed in the future using more species, especially those belong to groups of species complex from the eastern Indonesia and species protected by the Indonesian national law.

1. Introduction

Indonesia's export of pet reptiles includes species of monitor lizards, some of which are not easy to identify by means of morphological characters such as colour pattern, especially by nonexperts. The international trades of monitor lizards are legal and regulated by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) through an annual quota system. In 2015, e.g. the export quotas for six Indonesian species of *Varanus* for pets ranged from 270 to 5400 individuals. The six species of monitor lizards for pet export were three species with distribution areas in the eastern Indonesia and three species in the western part of the archipelago, including the Asian water monitor, *Varanus salvator* (CITES 2015). This species is among the most exploited monitor lizard species in the world for its skin and as pets (Pernetta 2009). A case of misidentification for *V. salvator* may occur for the protected, endemic, and closely related species, i.e. *Varanus togianus* (Böhme 2003; Koch *et al.* 2013). Furthermore, falsification of species identity for shipping information can also occur for protected and nonprotected species of monitor lizards without a CITES quota. In the case of such circumstances, a rapid, accurate, and cost-effective molecular method such as the DNA barcoding might be useful to clarify species identity (Hebert *et al.* 2003).

Previous studies on the systematics and biogeography of the genus *Varanus* have applied different mitochondrial DNA markers, such as 12s rRNA (Fuller *et al.* 1998), ND4 (Fitch *et al.* 2006; Doughty *et al.* 2014), and 16S rRNA (Ziegler *et al.* 2007). In addition, Ast (2001) used several markers, i.e. the full length of ND1 and ND2, as well as partial 16S rRNA and cytochrome oxidase subunit I (COI). Others have used a combination of mitochondrial and nuclear genes (e.g. Welton *et al.* 2010; Vidal *et al.* 2012). However, the application of the COI marker to analyse phylogenetic relationships among species within this group seems to be scarce. Besides, an effort to barcode these species using the short sequence of this mitochondrial gene for a practical molecular identification seems to be lacking, despite the importance of this method and availability of DNA barcoding primers (Francis *et al.* 2010; Nagy *et al.* 2012).

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This study attempts to generate molecular data, which is aimed at providing a starting basis for further applications in the identification of economically important species in Indonesia, such as those within the genus *Varanus*. In addition, DNA barcoding method as a practical molecular tool for identifying protected species of Indonesian monitor lizards is being tested in this study.

2. Materials and methods

Twelve DNA materials used in this study are tissue samples from several sources, including trade and zoo animals, as well as zoological museum tissue collections from previous field works in Indonesia. All tissues are deposits in the tissue sample collection of Herpetology Section of MZB in Cibinong, Indonesia. Morphological examination was performed on specimens associated with the tissues following Ziegler *et al.* (2007) and Koch *et al.* (2007). Otherwise, some of the tissues were identified only by its label. Whole genome extraction is modified from phenol-chloroform method described in Sulandari and Zein (2003).

Primers for polymerase chain reactions (PCRs) are based on Nagy *et al.* (2012) and Folmer *et al.* (1994), which target sequence sizes of 664 base pairs and 710 base pairs, respectively. Nagy *et al.*'s primer pair, i.e. RepCOI-F (5′-TNT TMT CAA CNA ACC ACA AAG A-3′) and RepCOI-R (5′-ACT TCT GGR TGK CCA AAR AAT CA-3′) is shorter in its oligo-sequence than Folmer *et al.*'s, i.e. LCO1490 Forward (5′-GGT CAA CAA ATC ATA AAG ATA TTG-3′) and HCO2198 Reverse (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′). Both primers are aimed at amplifying a fragment of the COI gene of the mitochondrial DNA, which is termed as the "DNA barcode" (Hebert *et al.* 2003). Each of these primer pairs was applied randomly to amplify any tissue samples in this study.

PCR mixes were modified from Sulandari and Zein (2003) and adjusted to its final concentrations of primers and bovine serum albumin for a reaction volume of 30 μL. PCR conditions were set following conditions given for each primer pair by their designers with manual adjustments of annealing temperatures that ranged from 48.0°C to 55.0°C. All PCR products were sent to international commercial sequencing facilities and manually edited based on their traces using the software BioEdit version 7.2.5 (Hall 1999). Sequence identity was checked with previously analysed sequence data at GenBank using BLAST (Basic Local Alignment Search Tool) for highly similar sequences. Seven COI sequences of *Varanus* were downloaded from GenBank nucleotide database, including three sequences of complete COI gene of about 1600 bp for *Varanus komodoensis*, *Varanus niloticus*, and *Varanus salvator*. All seven sequences were incorporated in the data set.

Multiple sequence alignment, amino acid translation, genetic divergence, and phylogenetic analyses were conducted in MEGA version 5.2 (Tamura *et al.* 2011). The neighbor-joining method was applied to search for phylogenetic tree in MEGA with 1000 bootstrap replications. Visualization of resulting tree and tree editing for publication was carried out using the freeware Dendroscope (Huson and Scornavacca 2012).

3. Results

Following a random application of primer pairs for tissue samples in this study, it seems that both DNA barcoding primers worked well to amplify the target mitochondrial DNA fragments. Original contigs resulting from pairwise alignments of forward and reverse sequences showed various lengths with a range of 677–687 base pairs (bp) for samples amplified using Nagy *et al.*'s primer pair and a range of 688–711 bp for samples amplified using Folmer *et al.*'s primer pair. The BLAST results indicated up to 98% of sequence similarity and 99% query cover for some samples such as *V. salvator*, for which complete COI sequence accessible at GenBank.

A total of 19 sequences were aligned and translated into their amino acids as original data set. Homologous sequences of 629 bp were obtained using Clustal W and the default setting in MEGA. No premature stop codon was found in any of the sequences after translation. The absence of premature stop codons suggests that all sequences in my data set are not nuclear mitochondrial pseudogenes (numts), which have been found in the genomes of vertebrate and invertebrate groups (Antunes and Ramos 2005; Buhay 2009; Moulton *et al.* 2010; Lobo *et al.* 2013). Thus, all sequences in the final data set are the target mitochondrial fragments of the DNA barcodes. All the 12 sequences resulted in this study have been submitted to GenBank, i.e HR033 (KY354294), V012 (KY354295), V011 (KY354296), V014 (KY354297), V013 (KY354298), HR098 (KY354299), HR008 (KY354300), HR003 (KY354301), HR037 (KY354302), HR004 (KY354303), HR031 (KY354304), and HR006 (KY354305).

Table. Pairwise genetic distance based on uncorrected *p*-distance

Sequence name	ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
EU621818_bengalensis	1																			
KF766939_bengalensis	2	0.126																		
AB080275_komodoensis	3	0.183	0.176																	
AB185327_niloticus	4	0.189	0.188	0.167																
HQ219069_niloticus	5	0.186	0.188	0.175	0.022															
EU621817_salvator	6	0.129	0.148	0.189	0.169	0.169														
EU747731_salvator	7	0.127	0.146	0.188	0.170	0.170	0.002													
V011_cf. indicus_Aru	8	0.167	0.169	0.167	0.167	0.170	0.143	0.141												
V012_cf. finschi_KeiKecil	9	0.167	0.167	0.165	0.165	0.169	0.141	0.140	0.003											
V013_doreanus_Batanta	10	0.180	0.159	0.183	0.189	0.186	0.169	0.170	0.127	0.130										
V014_cf. rainerguentheri_Kofiau	11	0.176	0.170	0.173	0.173	0.176	0.154	0.153	0.030	0.033	0.127									
HR003_salvator_TanahJampea	12	0.126	0.153	0.184	0.172	0.170	0.021	0.019	0.143	0.141	0.165	0.156								
HR004_togianus_Togean	13	0.135	0.149	0.186	0.175	0.180	0.035	0.033	0.143	0.141	0.178	0.156	0.030							
HR006_salvator_Kalaotoa	14	0.124	0.151	0.188	0.172	0.167	0.022	0.021	0.145	0.143	0.164	0.157	0.005	0.029						
HR008_prasinus_RagunanZoo	15	0.169	0.172	0.183	0.181	0.186	0.162	0.161	0.143	0.143	0.161	0.154	0.162	0.157	0.161					
HR031_yuwonoi_Trade	16	0.165	0.162	0.170	0.184	0.188	0.164	0.162	0.103	0.107	0.105	0.114	0.617	0.173	0.169	0.135				
HR033_beccarii_Trade	17	0.172	0.165	0.191	0.202	0.210	0.181	0.180	0.154	0.154	0.165	0.154	0.180	0.170	0.178	0.076	0.153			
HR037_salvator_TuloCSulawesi	18	0.132	0.159	0.183	0.173	0.175	0.038	0.037	0.145	0.143	0.170	0.157	0.033	0.029	0.032	0.151	0.156	0.164		
HR098_nebulosus_Singapore	19	0.000	0.126	0.183	0.189	0.186	0.129	0.127	0.167	0.167	0.180	0.176	0.126	0.135	0.124	0.169	0.165	0.172	0.132	

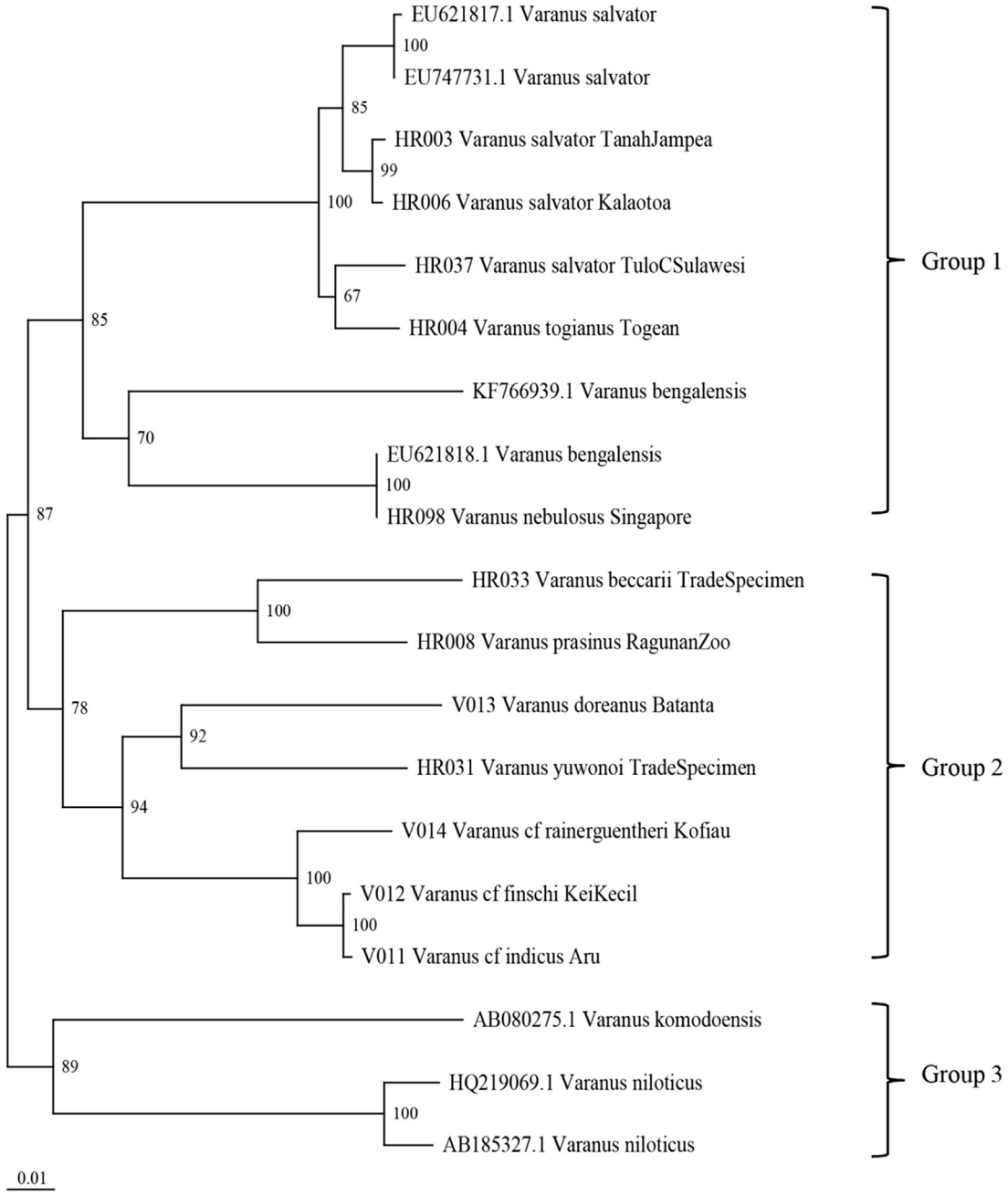

Figure. Neighbor-joining tree based on uncorrected *p*-distance and 1000 bootstrap replications.

Genetic divergence and phylogenetic analyses involved all 19 nucleotide sequences, of which all three codon positions included and all positions containing gaps and missing data eliminated. Estimates of evolutionary divergence (*p*-distance) between sequences are shown in Table, and results of phylogenetic analysis are shown in Figure. Sequences generated in this study are coded with their laboratory tag and those from GenBank are coded with their accession number.

4. Discussion

Both primers used in this study seem to show similar success for amplifying DNA barcode sequences of monitor lizard species in this study, albeit the resulting shorter sequences. Sequence size in this study is 29 bp shorter than the standard 658-bp DNA barcode (Hebert *et al.* 2003). The shorter aligned sequences are due to the inclusion of two relatively short sequences of *Varanus bengalensis* EU621818.1 and *V. salvator* EU621817.1, i.e. 644 bp in the multiple sequence alignment process.

Phylogenetic analysis shows that there are three major groups based on these shorter DNA barcode sequences (Figure). In this analysis, *V. komodoensis* and *V. niloticus* are used as the root of the tree because tissue sample of *Lanthanotus borneensis*, which is the next extant relative of family Varanidae is not available and the species is nationally protected in Indonesia. Overall, this grouping shown by the neighbor-joining method seems to agree with Ast's (2001) well-sampled phylogenetic tree, which includes three mitochondrial genes and is reconstructed based on more than

2000 bp nucleotide sites. Thus, the DNA barcodes can facilitate identification based on the phylogenetic relationships with previously known species and the relative amount of genetic divergence among them. Some limitations of this identification method include availability of tissue samples of rare and/or endemic species as well as published Barcode sequences associated with museum specimens. However, the DNA barcode can be used reliably as a molecular tool to identify species of monitor lizards in Indonesia in general.

Three Indonesian species of monitor lizards included in group 1 are *V. salvator* or the Asian water monitor, *V. togianus* or Togian Islands monitor, and *Varanus nebulosus* or the clouded monitor. All Indonesian samples of *V. salvator* in this study were collected during fieldwork on Sulawesi and two other islands in the south of Sulawesi *V. togianus* seem to be closely related to all individuals of *V. salvator* in this study, with 2.9%—3.5% genetic divergence between the two species. These results seem to indicate that there might be a species complex among *V. salvator* distributed on Sulawesi and the small islands nearby, e.g. because the genetic divergences between Central Sulawesi sample and the two samples from the small islands south of Sulawesi are 3.3% and 3.2%, respectively, for Tanah Jampea and Kalaotoa. This amount of genetic differentiation falls within the range of genetic divergence between *V. salvator* and *V. togianus*, and may be enough to distinguish the small island individuals from those distributed on Sulawesi genetically. Therefore, an examination of whole specimens to compare morphological characteristics of these individuals would be helpful to determine the prospect of species complex for *V. salvator* on Sulawesi and the nearby islands. Contrary to this view, the range of genetic divergence among all samples of *V. salvator* in this study is between 1.9% and 3.8% (Table), with the highest divergences found accounted for between the sample from Central Sulawesi and the two sequences from GenBank without locality information.

It is interesting to note that the sequence of *V. nebulosus* from Singapore are identical to that of *V. bengalensis* EU621818. The identity of these sequences is accounted for by the absence of sequence divergence (Table) and seems to indicate that a misidentification of one of these specimens occurred. However, genetic distance between *V. nebulosus* and *V. bengalensis* KF766939 is about 13%. This suggests that EU621818 may be misidentified as *V. bengalensis*. A misidentification may have happened because *V. nebulosus* has been raised to full species rank based on morphological characters from being a previous subspecies of the Bengal monitor, *V. bengalensis*. The distribution areas of both species seem to overlap only in Thailand, with *V. bengalensis* being distributed in India and some parts of the mainland Southeast Asia and *V. nebulosus* being distributed south of those, including islands east of Sumatra in Indonesia and probably also Sumatra and Java (Böhme and Ziegler 1997; Böhme 2003; Arida and Setyawatiningsih, 2015). Nonetheless, locality information for EU621818 is not available from published literatures. It becomes necessary now for a further scrutiny on the phylogenetic relationships among *V. bengalensis* group based on molecular data to shed light the evolutionary relationships between the two closely related species.

All samples included in group 2 of the neighbor-joining tree are members of the subgenus *Euprepiosaurus* that are distributed in the Malukus, New Guinea, and islands in the Pacific. Two species complexes are known in this group, i.e. *Varanus prasinus* and *Varanus indicus* species complexes (Ziegler *et al.* 2007). Each of the two subtrees within this group correctly identifies the two species complexes. In other words, species included in each of the subtrees belong to its corresponding species complex.

In the subtree of *V. prasinus*, the sample from Ragunan Zoo in Jakarta is grouped with a sample of *Varanus beccarii* obtained from the pet trade. Indeed, the black *V. beccarii* is a relative of the green *V. prasinus* that is limited in its distribution or endemic to the Aru Islands, Maluku (Ziegler *et al.* 2007). Despite being grouped in the same clade (Figure), the two species show a relatively high genetic divergence of 7.6% (Table). Interestingly, the amount of genetic differentiation shown between the two species based on the COI sequences in this study is about three times higher than the amount of differentiation between samples of the two species based on 16s rRNA sequences, i.e. 2.15%—2.74% (Ziegler *et al.* 2007). This result might suggest that the DNA barcodes or the COI sequences in general are more polymorphic than some fragments in the mitochondrial genome. Nevertheless, *V. beccarii* seems to be distantly related to *V. prasinus* following inclusion of more species within the group for phylogenetic analysis (e.g. Ast 2001; Ziegler et al. 2007).

Sister to the subtree of *V. prasinus* is another subtree that includes *Varanus* cf. *indicus* collected from Aru Island. Some of the species within the *V. indicus* species complex are morphologically similar to one another. Therefore, some of the samples are temporarily assigned with “confer” (cf.) in this study, pending further detailed morphological examination. It is interesting to note that the two samples from the Maluku islands of Aru and Kei Kecil are almost identical in their sequences, i.e. with a genetic divergence of 0.3% (Table). These two samples come from two specimens with different colour patterns resembling to *Varanus finschi* and *V. indicus*, respectively. Nevertheless, the low genetic divergence indicates otherwise. A very close phylogenetic relationship between the two samples are shown by the low genetic divergence and the presence of colourmorphs maybe a case within *V. indicus* in general. Further studies on the external morphological characters of these two samples can be made possible in the near future because whole specimens of these samples are available in the herpetological collection at MZB. In addition, studies on both species complexes from eastern Indonesia is expected by adding more DNA samples from species described previously and possibly new samples from future fieldworks in the Malukus and Papuan region.

In group 3, *V. komodoensis* is outside the clade for *V. niloticus* from Africa, which suggests that the Komodo dragon is closer phylogenetically to African species than to all the Indo-Asian species in this study. This phylogenetic grouping is incongruent with Ast's (2001) tree, which shows that *V. komodoensis* belongs to the Indo-Australian clade and sister to Indo-Asian species. In fact, the grouping of the Komodo dragon with the African water monitor, *V. niloticus* is likely to be an effect of limited species sampling in this study that includes only 12 of 78 currently described species of monitor lizards worldwide (Uetz 2010). Additionally, it is likely that rooting the tree with *Lanthanotus borneensis* would recover a topology that is congruent with previous phylogeny estimate of *Varanus*.

Besides the Komodo dragon, three other species in this study are among the eight protected species of monitor lizards in Indonesia, which includes the Borneo-endemic earless monitor, *Lanthanotus borneensis*. The Indonesian national law protecting these species is the Peraturan Pemerintah No. 7 Tahun 1999 that lists 294 species of animals and plants. The availability of first COI sequence data for these protected species, i.e. the emerald monitor, *V. prasinus*; the clouded monitor, *V. nebulosus*; and the Togean water monitor, *V. togianus* serves as a starting basis of a potentially rapid species identification in the future. Thus, misidentification or falsification of protected species identity with a purpose of illegal wildlife trade

may be detected with little time and financial cost. In addition, these sequences can be used as a reference for future studies on their respective taxonomic groups. The mangrove monitor, *V. indicus* is also listed as a protected species in Indonesia; however, the species identity for this sample in this study still remains to be confirmed. More sampling for this group is essential to clarify phylogenetic relationships among its members and to determine their species identity.

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Conflict of interest

None.

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