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Molecular barcoding of venomous snakes and species-specific multiplex PCR assay to identify snake groups for which antivenom is available in Thailand

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ABSTRACT. DNA barcodes of mitochondrial *COI* and *Cytb* genes were constructed from 54 specimens of 16 species for species identification. Intra- and interspecific sequence divergence of the *COI* gene (10 times) was greater than that of the *Cytb* gene (4 times), which suggests that the

former gene may be a better marker than the latter for species delimitation in snakes. The *COI* barcode cut-off scores differed by more than 3% between most species, and the minimum interspecific divergence was greater than the maximum intraspecific divergence. Clustering analysis indicated that most species fell into monophyletic clades. These results suggest that these species could be reliably differentiated using *COI* DNA barcodes. Moreover, a novel species-specific multiplex PCR assay was developed to distinguish between *Naja* spp, *Ophiophagus hannah*, *Trimeresurus* spp, Hydrophiinae, *Daboia siamensis*, *Bungarus fasciatus*, and *Calloselasma rhodostoma*. Antivenom for these species is produced and kept by the Thai Red Cross for clinical use. Our novel PCR assay could easily be applied to venom and saliva samples and could be used effectively for the rapid and accurate identification of species during forensic work, conservation study, and medical research.

Key words: Snake; Barcode; Mitochondrial DNA; Multiplex PCR; Venom; Saliva

INTRODUCTION

Snakes (Serpentes) are carnivorous squamate reptiles that exhibit phenotypically diverse radiation (Secor and Diamond, 1998; Castoe et al., 2008, 2009). Extant snakes consist of over 3,000 known species worldwide (Uetz, 2014). The draft genome assemblies of the Burmese python (*Python bivittatus*) and the king cobra (*Ophiophagus hannah*) provide new perspectives on the comparative genomics of Reptilia and Amniota, which in turn facilitate extensive comparisons between genomic structures at the molecular level (Castoe et al., 2013; Vonk et al., 2013). Snakes form the most divergent group in Squamata and their speciation occurred over a relatively short period. These characteristics make snakes difficult to place among iguanians and anguimorphs, to which they are closely related (Srikulnath et al., 2010; Pyron et al., 2013). It is also difficult for evolutionary biologists to classify all snakes with conserved morphology within the sublevels of Serpentes. However, snake biodiversity is decreasing globally owing to hunting and trading for health food, medicinal products, and pets. This issue requires serious attention in the context of conservation biology, and has led to an effort to produce a collection of entire diversity of snake alongside a modern, accurate taxonomy. The Convention on International Trade in Endangered Species of Wild Fauna and Flora regulates the trade of certain snakes. It is essential to develop reliable methods to forensically identify snake products in order to enforce trading laws. Conventional morphology-based taxonomic procedures for snakes are well established (Cox et al., 2012), but are time-consuming due to the limited availability of snake specimens and differences between life stages and sexes, which can lead to misidentification. This suggests that modern techniques, such as molecular approaches, are needed in addition to traditional taxonomic methods in order to identify species.

DNA sequences of a standardized region from an unknown species can be compared to sequences available in databases in order to identify them as belonging to a particular species. This technique is known as DNA barcoding. The reference sequence library is constructed from a known species and becomes the barcode. The degree of nucleotide divergence between individuals can

facilitate identification of species. The gene most commonly used as a marker for the barcode is the mitochondrial *COI* gene because it has been studied in many vertebrates and exhibits interspecific nucleotide divergence that is greater than its intraspecific nucleotide divergence (Chaves et al., 2008). Other mitochondrial genes, such as *Cytb* and 16S rRNA (Xia et al., 2012; Nicolas et al., 2012), have also been employed as barcodes with varying levels of success. Snake DNA barcodes based on *COI*, *Cytb*, and 12S rRNA genes are well established in India, China, and the USA (Wong et al., 2004; Pook and McEwing, 2005; Dubey et al., 2011; Gaur et al., 2012). However, these successes were based on studies of only those species that are abundant in a given country, and there is no single standard gene for the reliable identification of snakes at the species level. Global expansion of the snake DNA barcode library is therefore necessary to aid work in conservation biology, medicine, and forensic science. In Thailand, venomous snakebites remain a serious daily occurrence. Even though several specific antivenoms are available, bites from venomous snakes that cause morbidity and mortality often occur. There are seven effective antivenoms available from the Thai Red Cross for clinical use. These include antivenoms to *Naja kaouthia*, *O. hannah*, *Trimeresurus albolabris*, *Daboia siamensis*, *Bungarus fasciatus*, *Bungarus candidus*, and *Calloselasma rhodostoma*. Clinically, the most serious problem for antivenom therapy is snake identification. The classification of snakebites is first performed by investigation of the clinical signs and symptoms or examination of the carcass of the snake and the local ecology. However, this is time-consuming and must be performed by a specialist. Therefore, several approaches have been developed to the examination of snakebites, such as radioimmunoassay, agglutination assay, enzyme-linked immunosorbent assay, and DNA fingerprinting (Selvanayagam and Gopalakrishnakone, 1999; Suntrarachun et al., 2001). However, there remain problems with false positives, low sensitivity, and availability that is limited to local-species-specific markers (Tibballs, 1992; Mead and Jelinek, 1996).

In this study, we generated *COI* and *Cytb* barcodes for 53 venomous snakes and one non-venomous snake (*Python bivittatus bivittatus*) found in Thailand, and identified snakes using the degree of nucleotide divergence between barcodes and clustering analysis with a phylogenetic tree. Species-specific multiplex PCR markers were then developed from barcodes to rapidly delimit eight groups of venomous snakes. Effective antivenom is available from the Thai Red Cross for most of these groups.

MATERIAL AND METHODS

Specimen collection and DNA extraction

Fifty-four live snakes were collected from several locations in Thailand, and kept at Queen Saovabha Memorial Institute (The Thai Red Cross Society; Table 1).

Morphologic identification of the species was performed as previously described (Cox et al., 2012). Blood samples were collected from each snake through the ventral tail vein using a 25-gauge needle treated with 10 mM ethylenediaminetetraacetic acid (EDTA) and attached to a 1-mL disposable syringe. All experimental procedures using animals conformed to the guidelines established by the Animal Care Committee, National Research Council, Thailand. Whole-genomic DNA was extracted from the blood of snakes in accordance with a standard salting-out protocol, and used as a template for polymerase chain reaction (PCR). Briefly, the blood cells were digested at 55°C for 1 h using 1.6 µg/µL proteinase K in extraction buffer [50 mM Tris-HCl, pH 8.0, 20 mM EDTA, and 1% (w/v) sodium dodecyl sulfate]. The mixture was then extracted using a salt solution

Table 1. Classification and accession numbers of species used in sequence analyses.

Species name	Common name	Family	Sex	Locality	Code	*COI* accession No.	*Cytb* accession No.
Ophiophagus hannah	King Cobra	Elapidae	Female	Captive-born (Southern parents origin)	OHA1	AB920180	AB920234
Ophiophagus hannah	King Cobra	Elapidae	Female	Captive-born (Southern parents origin)	OHA2	AB920181	AB920235
Ophiophagus hannah	King Cobra	Elapidae	Female	Captive-born (Southern parents origin)	OHA3	AB920182	AB920236
Naja kaouthia	Monocled Cobra	Elapidae	Male	-	NKA1	AB920183	AB920237
Naja kaouthia	Monocled Cobra	Elapidae	Female	Bangkok	NKA2	AB920184	AB920238
Naja kaouthia	Monocled Cobra	Elapidae	Male	Bangkok	NKA3	AB920185	AB920239
Naja sumatrana	Sumatran Cobra	Elapidae	Female	-	NSU1	AB920186	AB920240
Naja siamensis	Indo-Chinese spitting cobra	Elapidae	Male	-	NSI2	AB920187	AB920241
Naja siamensis	Indo-Chinese spitting cobra	Elapidae	Male	Rayong	NSI3	AB920188	AB920242
Bungarus fasciatus	Banded Krait	Elapidae	Female	-	BFA1	AB920189	AB920243
Bungarus fasciatus	Banded Krait	Elapidae	Female	-	BFA2	AB920190	AB920244
Bungarus fasciatus	Banded Krait	Elapidae	Female	-	BFA3	AB920191	AB920245
Bungarus candidus	Malayan Krait	Elapidae	Male	Nakhon Ratchasima	BCA1	AB920192	AB920246
Bungarus candidus	Malayan Krait	Elapidae	Female	-	BCA2	AB920193	AB920247
Bungarus candidus	Malayan Krait	Elapidae	Female	-	BCA3	AB920194	AB920248
Hydrophis brookii	Brooke's Sea Snake	Elapidae	Male	Songkhla	HBR1	AB920212	AB920266
Hydrophis brookii	Brooke's Sea Snake	Elapidae	Female	Songkhla	HBR5	AB920213	AB920267
Hydrophis brookii	Brooke's Sea Snake	Elapidae	Female	Songkhla	HBR6	AB920214	AB920268
Hydrophis obscurus	Russel's Sea Snake	Elapidae	Male	Ranong	HOB2	AB920215	AB920269
Hydrophis obscurus	Russel 's Sea Snake	Elapidae	Male	Ranong	HOB3	AB920216	AB920270
Hydrophis obscurus	Russel 's Sea Snake	Elapidae	Male	Ranong	HOB4	AB920217	AB920271
Hydrophis obscurus	Russel 's Sea Snake	Elapidae	Female	Ranong	HOB5	AB920218	AB920272
Hydrophis obscurus	Russel 's Sea Snake	Elapidae	Female	Ranong	HOB8	AB920219	AB920273
Hydrophis obscurus	Russel 's Sea Snake	Elapidae	Female	Ranong	HOB15	AB920220	AB920274
Enhydrina schistosa	Beaked Sea Snake	Elapidae	Female	Songkhla	ESC4	AB920221	AB920275
Enhydrina schistosa	Beaked Sea Snake	Elapidae	Female	Songkhla	ESC5	AB920222	AB920276
Enhydrina schistosa	Beaked Sea Snake	Elapidae	Female	Songkhla	ESC6	AB920223	AB920277
Enhydrina schistosa	Beaked Sea Snake	Elapidae	Male	Songkhla	ESC7	AB920224	AB920278
Enhydrina schistosa	Beaked Sea Snake	Elapidae	Female	Songkhla	ESC9	AB920225	AB920279
Enhydrina schistosa	Beaked Sea Snake	Elapidae	Male	Songkhla	ESC12	AB920226	AB920280
Enhydrina schistosa	Beaked Sea Snake	Elapidae	Female	Songkhla	ESC13	AB920227	AB920281
Daboia siamensis	Siamese Russell's Viper	Viperidae	Male	Bangkok	DSI1	AB920195	AB920249
Daboia siamensis	Siamese Russell's Viper	Viperidae	Female	Bangkok	DSI2	AB920196	AB920250
Trimeresurus albolabris	White-lipped Pit Viper	Viperidae	Female	Bangkok	TAL3	AB920197	AB920251
Trimeresurus albolabris	White-lipped Pit Viper	Viperidae	Female	Chanthaburi	TAL4	AB920198	AB920252
Trimeresurus macrops	Big-eyed Pit Viper	Viperidae	Female	Nong Bua Lamphu	TMA2	AB920199	AB920253
Trimeresurus macrops	Big-eyed Pit Viper	Viperidae	Female	Bangkok	TMA7	AB920200	AB920254
Trimeresurus macrops	Big-eyed Pit Viper	Viperidae	Female	Sara Buri	TMA14	AB920201	AB920255
Trimeresurus macrops	Big-eyed Pit Viper	Viperidae	Female	Ratchaburi	TMA19	AB920202	AB920256
Trimeresurus macrops	Big-eyed Pit Viper	Viperidae	Female	Ratchaburi	TMA20	AB920203	AB920257
Trimeresurus macrops	Big-eyed Pit Viper	Viperidae	Female	Ratchaburi	TMA21	AB920204	AB920258
Trimeresurus macrops	Big-eyed Pit Viper	Viperidae	Female	Ratchaburi	TMA24	AB920205	AB920259
Trimeresurus macrops	Big-eyed Pit Viper	Viperidae	Female	Nakhon Ratchasima	TMA31	AB920206	AB920260
Trimeresurus macrops	Big-eyed Pit Viper	Viperidae	Male	Trat	TMA35	AB920207	AB920261
Trimeresurus macrops	Big-eyed Pit Viper	Viperidae	Female	Chanthaburi	TMA36	AB920208	AB920262
Trimeresurus macrops	Big-eyed Pit Viper	Viperidae	Female	-	TMA37	AB920209	AB920263
Trimeresurus macrops	Big-eyed Pit Viper	Viperidae	-	Loei	TMA39	AB920210	AB920264
Calloselasma rhodostoma	Malayan Pit Viper	Viperidae	Female	Chon Buri	CRH1	AB920211	AB920265
Boiga cynodon	Dog-toothed Cat Snake	Colubridae	Female	-	BCY4	AB920228	AB920282
Boiga cynodon	Dog-toothed Cat Snake	Colubridae	Male	-	BCY5	AB920229	AB920283
Rhabdophis subminiatus	Red-necked Keelback	Colubridae	Male	Nakhon Ratchasima	RSU1	AB920230	AB920284
Rhabdophis subminiatus	Red-necked Keelback	Colubridae	Female	Nakhon Ratchasima	RSU2	AB920231	AB920285
Rhabdophis subminiatus	Red-necked Keelback	Colubridae	Female	-	RSU3	AB920232	AB920286
Python bivittatus bivittatus	Burmese python	Pythonidae	-	Bangkok	PBI1	AB920233	AB920287

(0.05 volumes of 5 M NaCl) and the DNA was precipitated using two volumes of isopropanol.

After washing in 70% ethanol, genomic DNA was air-dried, resuspended in 10 mM Tris-HCl, pH 8.0, and kept at -80°C. DNA quality and concentration were determined using 1% agarose gel electrophoresis and spectrophotometric analysis.

PCR amplification and sequencing

Partial mitochondrial *COI* and *Cytb* DNA fragments were amplified using PCR primers (Table 2). A standard PCR was carried in 20 µL 1X ThermalPol reaction buffer containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 5 pM specific primers, 0.25 U *Taq* polymerase (Vivantis Technologies, Selangor Darul Ehsan, Malaysia), and 25 ng genomic DNA. The temperature conditions used for PCR involved an initial denaturation step at 94°C for 2 min; 35 cycles each involving incubation at 94°C for 30 s, 52°C for 30 s, and 72°C for 35 s; and a final extension step at 72°C for 10 min. PCR products were cloned using the pGEM-T Easy Vector System I (Promega, Madison, WI, USA). Nucleotide sequences of the DNA fragments were determined by 1st Base DNA sequencing service (Seri Kembangan, Malaysia). The complete *Cytb* gene amplification is about 1100 bp in length and can only be obtained through two sequencing reactions. Because of the high cost of sequencing, we sequenced only the first part of this gene, obtained in one sequencing reaction. The first part of the gene was selected because it has been used more often in phylogenetic studies than the second part of the gene (Nicolas et al., 2012).

Table 2. Primers used for the amplification of the mitochondrial *COI* and *Cytb* genes.

Primer name	Targeted gene	Forward primer (5'-3')	Specific snake	Reference	Amplified size (bp)
COI forward	*COI*	TCAGCCATACTCCTGTGTTCA	All snakes	Makowsky et al. (2010)	700
COI reverse	*COI*	TAGACTTCTGGGTGGCCAAAGAATCA	All snakes	Makowsky et al. (2010)	700
H16064	*Cytb*	CTTTGGTTTACAAGAACAATGCTTTA	All snakes	Palumbi (1996)	1100
Gludg	*Cytb*	TGACTTGAARAACCAYCGTTG	All snakes	Burbrink (2000)	1100
HYD	*COI*	AGGGCCCTGAGTGAACAATA	Snake in Hydrophiinae	In this study	411
TC	*COI*	GAAAGCCATGTCTGGGGTT	*Trimeresurus* spp	In this study	272
DB	*COI*	TAATAGCATAGTAATTGCTGCTGCAAG	*Daboia siamensis*	In this study	621
DRS	*COI*	CGGATCAAACAAATAGAGGGAAGTTAA	*Daboia siamensis* and *Bungarus candidus*	In this study	579
BF	*COI*	AGGACTGGTAGGGCTAGTAAA	*Bungarus fasciatus*	In this study	590
OH	*COI*	TAAATGCGTGGGCAGTTACTAAA	*Ophiophagus hannah*	In this study	181
NK	*COI*	AGAGAAGTAGGAGGGATGGAGG	*Naja* spp	In this study	322
CR	*COI*	CGAGTGAACTAGGTTTCCGG	*Calloselasma rhodostoma*	In this study	414

Sequence divergence and phylogenetic tree

The nucleotide sequences we obtained were used to search for homology with mitochondrial genes in the National Center for Biotechnology Information database using the BLASTn programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and were deposited in the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/index-e.html; Table 1). The partial mitochondrial *COI* and *Cytb* sequences of the 54 individual snakes and six GenBank accessions were aligned using the Molecular Evolutionary Genetics Analysis 4 software with its default parameters (Center for Evolutionary Functional Genomics, The Biodesign Institute, Tempe, AZ, USA; Kumar et al., 2004). All unalignable sites and gap-containing sites were carefully removed from these data sets. Analysis of sequence divergence was conducted using two data sets (*COI* and *Cytb* nucleotide

sequences) with uncorrected pairwise distances (*p*-distances). The base composition for each nucleotide data set was measured using PAUP* v. 4.0b10 (Swofford, 2002). A chi-square (χ^2) test of base heterogeneity was also carried out for each data set using the same program. Phylogenetic trees were constructed using Bayesian inference (BI) with MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001). The Markov chain Monte Carlo process was set to run four chains simultaneously for one million generations. After the log-likelihood value plateaued, a sampling procedure was performed every 100 generations to obtain 10,000 trees and subsequently to provide a majority-rule consensus tree with average branch lengths. All sample points prior to reaching convergence were discarded, and the Bayesian posterior nodal relationship in the sampled tree population was obtained in percentage terms.

Species-specific multiplex PCR markers

Species-specific primers were designed based on all mitochondrial *COI* nucleotide sequences that differed between clusters of snake species. Primers were examined using two different approaches. First, single-primer-pair PCR was used to examine different snake clades [*Naja* spp (1), *O. hannah* (2), *Trimeresurus* spp (3), Hydrophiinae (4), *D. siamensis* (5), *B. fasciatus* (6), *B. candidus* (7), and *C. rhodostoma* (8)] using the appropriate annealing temperatures (Table 2). A standard PCR was performed as described in the PCR amplification and sequencing section, and the PCR products were examined by electrophoresis on 1% agarose gel. Second, single-tube multiplex PCR was performed to simultaneously detect different snake clades, and multiplex PCR reactions were performed as described above.

To test the sensitivity of species-specific single PCR markers and multiplex PCR markers, serial dilutions of the DNA template ranging from 0.0001 to 5 ng/20 µL were subjected to PCR. The specificity of the single and multiplex PCR systems was determined by adding a mixture of DNA from all snake species to each PCR, and amplification was performed as described above. Snake venom and saliva were also collected from all snake species, except for *Boiga cynodon*, *Rhabdophis subminiatus*, *Python bivittatus bivittatus*, and Hydrophiinae snakes. Whole-genomic DNA was isolated as described in the Specimen collection and DNA extraction section. Multiplex PCRs were performed with 10-15 ng DNA template, and the PCR products were examined by electrophoresis on 1% agarose gels.

RESULTS

Barcode construction

DNA barcodes of mitochondrial *COI* and *Cytb* sequences were constructed from 54 specimens representing 16 species from 11 genera. Most species were represented by two or more individuals. High-quality sequences of approximately 528 bp for the *COI* gene and 396 bp for the *Cytb* gene were obtained from all samples used in this study. Average nucleotide frequencies for the entire data set were A = 26.49%, T = 30.51%, C = 26.60%, and G = 16.39% for the *COI* data set; and A = 31.60%, T = 30.22%, C = 29.57%, and G = 8.6% for the *Cytb* data set. There were no statistically significant proportional differences among snake sequences (data not shown), which indicates that there was no heterogeneity of base frequencies in either data set. No stop codons were found in these sequences, which indicates that nuclear pseudogenes were included in the analysis.

All barcodes represent functional mitochondrial *COI* and *Cytb* sequences. Moreover, different *COI* gene haplotypes were found in *Naja siamensis*, *B. fasciatus*, *B. candidus*, *Trimeresurus albolabris*, *Trimeresurus macrops*, *Hydrophis brookii*, *Hydrophis obscurus*, and *Enhydrina schistosa* (Table 3). Different *Cytb* gene haplotypes were found in *O. hannah*, *Naja kaouthia*, *B. fasciatus*, *B. candidus*, *T. albolabris*, *T. macrops*, *H. obscurus*, *E. schistosa*, and *R. subminiatus* (Table 4).

Nucleotide sequence divergence

Intraspecific sequence divergence of mitochondrial *COI* gene data set for three species (*Naja sumatrana*, *C. rhodostoma*, and *P. bivittatus bivittatus*) could not be analyzed because these species were each represented by a single specimen. The average nucleotide divergence within a species was 0.59% (Table 3, **Table S1**). The intraspecific sequence divergence for *O. hannah*, *N. kaouthia*, *D. siamensis*, *B. cynodon*, and *R. subminiatus* was 0%, and the maximum intraspecific sequence divergence was 3.6% in *T. macrops*. The interspecific nucleotide divergence ranged from 3.1% (between *N. sumatrana* and *N. siamensis*) to 21.9% (between *N. kaouthia* and *D. siamensis*). For the mitochondrial *Cytb* gene data set, the mean intraspecific divergence was 2.22%. Nucleotide divergence within species was 0% in *H. brookii*, *D. siamensis*, and *B. cynodon*. The maximum intraspecific divergence was 14.4% in *B. candidus* (Table 4, **Table S2**). The interspecific *Cytb* nucleotide divergence ranged from 5.1% (between *H. brookii* and *H. obscurus*) to 32.7% (between *B. candidus* and *P. molurus*).

Phylogenetic analysis

BI cladograms were reconstructed based on the *COI* and *Cytb* data sets. They exhibited similar topologies and strongly supported the monophyletic clade of sequences of the same species from both GenBank and this study (Figure 1a, b). This indicates that *COI* and *Cytb* can be used to identify species correctly. However, almost no *T. macrops* or *T. albolabris* individuals were included within a species-specific monophyletic group based on either the *COI* or the *Cytb* data set.

Species-specific PCR assays

Interspecific nucleotide sequence differences were considered in order to design eight species-specific reverse primers. These primers were paired with a forward primer (COI forward), and amplified in eight snake groups: *Naja* spp (1); *O. hannah* (2); *Trimeresurus* spp (3); snakes in Hydrophiinae (4); *D. siamensis* (5); *B. fasciatus* (6); *B. candidus* (7); and *C. rhodostoma* (8) (Table 2). Whereas the primers NK, OH, TC, HYD, DB, BF, and CR allowed specific amplifications for groups (1)-(6) and (8), respectively, the primer DRS could be used to amplify two target species, *D. siamensis* (5) and *B. candidus* (7). For more efficient analysis, a multiplex PCR assay was developed to differentiate among all eight snake groups using two sequential panels. Each multiplex PCR panel allowed the rapid distinction between individuals assigned to each group. A first multiplex PCR (panel A) was performed using three species-specific primers: TC, HYD, and DRS, in combination with COI forward (used as a common forward primer), which produced the following diagnostic bands: i) one band of 277 bp for *Trimeresurus* spp (3), ii) one band of 418 bp for Hydrophiinae snakes (4), and iii) one band of 621 bp for both *D. siamensis* (5) and *B. candidus* (7) (Figure 2a, Table 5). However, a cross-reaction was detected for *O. hannah* with two DNA bands

Table 3. Nucleotide sequence divergence (*p*-distance) in the mitochondrial *COI* gene across 528 bp for 16 snake species. Diagonal values are *p*-distance for intraspecific comparisons.

Species	*O. hannah*	*N. kaouthia*	*N. sumatrana*	*N. siamensis*	*B. fasciatus*	*B. candidus*	*H. brookii*	*H. obscurus*	*E. schistosa*	*D. siamensis*	*T. albolabris*	*T. macrops*	*C. rhodostoma*	*B. cynodon*	*R. subminiatus*	*P. bivittatus bivittatus*
O. hannah	0.000 (1)															
N. kaouthia	0.156	0.000 (1)														
N. sumatrana	0.150	0.067	[a]													
N. siamensis	0.153	0.054	0.031	0.002 (2)												
B. fasciatus	0.146	0.172	0.165	0.161	0.009 (2)											
B. candidus	0.165	0.159	0.151	0.145	0.127	0.006 (3)										
H. brookii	0.191	0.198	0.193	0.186	0.169	0.167	0.001 (2)									
H. obscurus	0.189	0.189	0.183	0.169	0.166	0.160	0.052	0.001 (2)								
E. schistosa	0.190	0.179	0.174	0.173	0.170	0.168	0.064	0.064	0.007 (5)							
D. siamensis	0.188	0.219	0.207	0.202	0.199	0.194	0.217	0.208	0.203	0.000 (1)						
T. albolabris	0.151	0.182	0.183	0.184	0.183	0.177	0.214	0.210	0.212	0.181	0.015 (2)					
T. macrops	0.211	0.205	0.195	0.200	0.196	0.196	0.211	0.202	0.203	0.192	0.131	0.036 (6)				
C. rhodostoma	0.165	0.177	0.171	0.178	0.186	0.181	0.207	0.206	0.201	0.192	0.164	0.175	[a]			
B. cynodon	0.183	0.194	0.181	0.191	0.172	0.179	0.200	0.208	0.196	0.211	0.196	0.205	0.181	0.000 (1)		
R. subminiatus	0.183	0.203	0.183	0.185	0.175	0.172	0.203	0.189	0.196	0.207	0.207	0.211	0.186	0.181	0.000 (1)	
P. bivittatus bivittatus	0.186	0.190	0.188	0.185	0.189	0.186	0.217	0.209	0.209	0.196	0.190	0.201	0.198	0.196	0.207	[a]

The numbers in parentheses indicate the number of haplotypes per species. [a]Samples were collected from only one individual.

Table 4. Nucleotide sequence divergence (*p*-distance) in the mitochondrial *Cytb* gene across 396 bp for 16 snake species. Diagonal values are *p*-distance for intraspecific comparisons.

Species	*O. hannah*	*N. kaouthia*	*N. sumatrana*	*N. siamensis*	*B. fasciatus*	*B. candidus*	*H. brookii*	*H. obscurus*	*E. schistosa*	*D. siamensis*	*T. albolabris*	*T. macrops*	*C. rhodostoma*	*B. cynodon*	*R. subminiatus*	*P. bivittatus bivittatus*
O. hannah	0.004 (2)															
N. kaouthia	0.194	0.011 (2)														
N. sumatrana	0.189	0.097	[a]													
N. siamensis	0.208	0.112	0.057	0.040 (2)												
B. fasciatus	0.194	0.244	0.215	0.238	0.009 (3)											
B. candidus	0.258	0.295	0.260	0.266	0.208	0.144 (3)										
H. brookii	0.215	0.235	0.214	0.223	0.200	0.260	0.000 (1)									
H. obscurus	0.205	0.222	0.211	0.226	0.193	0.256	0.051	0.003 (4)								
E. schistosa	0.228	0.230	0.222	0.242	0.210	0.284	0.081	0.075	0.001 (2)							
D. siamensis	0.252	0.270	0.274	0.286	0.265	0.288	0.266	0.267	0.263	0.000 (1)						
T. albolabris	0.226	0.288	0.298	0.307	0.269	0.289	0.288	0.264	0.267	0.240	0.011 (2)					
T. macrops	0.234	0.277	0.282	0.292	0.262	0.279	0.301	0.286	0.295	0.218	0.139	0.061 (8)				
C. rhodostoma	0.217	0.263	0.266	0.285	0.233	0.284	0.274	0.267	0.282	0.216	0.214	0.196	[a]			
B. cynodon	0.223	0.260	0.261	0.284	0.246	0.314	0.251	0.244	0.243	0.256	0.251	0.258	0.237	0.000 (1)		
R. subminiatus	0.233	0.286	0.279	0.285	0.256	0.305	0.265	0.251	0.255	0.271	0.281	0.295	0.257	0.233	0.005 (3)	
P. bivittatus bivittatus	0.257	0.293	0.282	0.298	0.285	0.327	0.274	0.263	0.264	0.293	0.272	0.274	0.259	0.253	0.288	[a]

The numbers in parentheses indicate the number of haplotypes per species. [a]Samples were collected from only one individual.

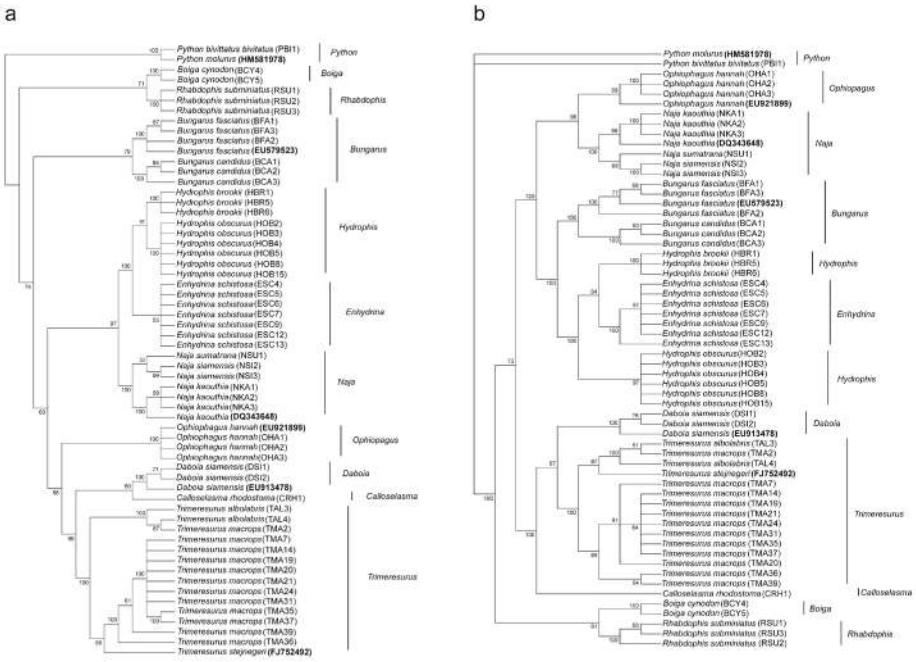

Figure 1. A Bayesian cladogram clarifying the phylogenetic relationships among 54 individual snakes and six GenBank accessions (bold) constructed using *COI* (**a**) and *Cytb* (**b**) mitochondrial gene sequences. The 50% majority-rule consensus of post-burn-in sample trees from the Bayesian inference is shown.

at 500 and 600 bp, indicating the presence of a non-specific band derived from the combination of multiple primers in the PCR. A second multiplex PCR (panel B) was comprised of five primers: DB, BF, OH, NK, and CR, in combination with COI forward. Species-specific PCR products of 323, 176, 541, 599, and 400 bp were observed for *Naja* spp (1), *O. hannah* (2), *D. siamensis* (5), *B. fasciatus* (6), and *C. rhodostoma* (8), respectively, but no PCR product was found for *B. candidus* (Figure 2b, Table 5). However, cross-species amplification was detected in *T. macrops*, with two DNA bands at 400 and 500 bp, indicating the presence of a non-specific band that resulted from the combination of multiple primers in the PCR. Specificity was also analyzed in single and multiplex PCR assays by pooling the DNA of all snake species. The results indicated that DNA fragments from all species were detected as indicated above (data not shown). The sensitivity of each species-specific DNA marker and each multiplex PCR marker was examined by determining the detection limit of the specific primers for the amplification of DNA templates consisting of 5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, and 0.0001 ng/20 µL. The results showed that the minimum detection limit was 0.0005 ng for COI forward-BF primers (**Table S3**). In contrast, the detection limit for multiplex PCR products of panel A and panel B was 0.5 ng.

To extend the applicability of our species-specific multiplex PCR markers, venom and saliva samples from 10 of the examined species (*O. hannah*, *N. kaouthia*, *N. sumatrana*, *N. siamensis*, *B. fasciatus*, *B. candidus*, *D. siamensis*, *T. albolabris*, *T. macrops*, and *C. rhodostoma*) were subjected to multiplex PCR. Two panels of multiplex PCR successfully differentiated all snake venom and saliva with the same species-specific PCR products (Figure 3a, b, c, d).

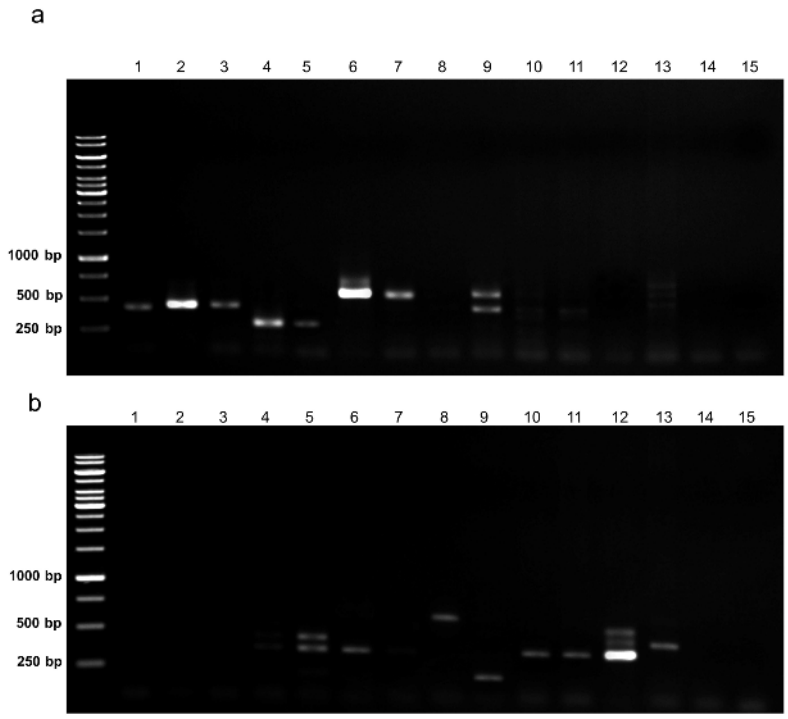

Figure 2. Agarose gel electrophoresis of PCR products using multiplex PCR assays with primer panels **a** and **b**. All snake genomic DNA templates were from blood. Mouse (*Mus musculus*) genomic DNA isolated from blood and human (*Homo sapiens*) genomic DNA isolated from cell line 9948 (Promega) was used for outgroups. *Lane 1 = Hydrophis brookii*; *lane 2 = Enhydrina schistosa*; *lane 3 = Hydrophis obscurus*; *lane 4 = Trimeresurus albolabris*; *lane 5 = Trimeresurus macrops*; *lane 6 = Daboia siamensis*; *lane 7 = Bungarus candidus*; *lane 8 = Bungarus fasciatus*; *lane 9 = Ophiophagus hannah*; *lane 10 = Naja siamensis*; *lane 11 = Naja sumatrana*; *lane 12 = Naja kaouthia*; *lane 13 = Calloselasma rhodostoma*; *lane 14 = Mus musculus*; *lane 15 = Homo sapiens*.

Table 5. Summary of species-specific multiplex PCR assay.

Family	Species name	Common name	Multiplex PCR set				Antivenom
			Panel A	DNA banded size (bp)	Panel B	DNA banded size (bp)	
Elapidae	*Ophiophagus hannah*	King Cobra	-[a]	-	+	176	*Ophiophagus hannah*
Elapidae	*Naja kaouthia*	Monocled Cobra	-	-	+	323	*Naja kaouthia*
Elapidae	*Naja sumatrana*	Sumatran Cobra	-	-	+	323	*Naja kaouthia*
Elapidae	*Naja siamensis*	Indo-Chinese spitting Cobra	-	-	+	323	*Naja kaouthia*
Elapidae	*Bungarus fasciatus*	Banded Krait	-	-	+	599	*Bungarus fasciatus*
Elapidae	*Bungarus candidus*	Malayan Krait	+	621	-	-	*Bungarus candidus*
Elapidae	*Hydrophis brookii*	Brooke's Sea Snake	+	418	-	-	-[b]
Elapidae	*Hydrophis obscurus*	Russel's Sea Snake	+	418	-	-	-[b]
Elapidae	*Enhydrina schistosa*	Beaked Sea Snake	+	418	-	-	-[b]
Viperidae	*Daboia siamensis*	Siamese Russell's Viper	+	621	+	541	*Daboia siamensis*
Viperidae	*Trimeresurus albolabris*	White-lipped Pit Viper	+	277	-[a]	-	*Trimeresurus albolabris*
Viperidae	*Trimeresurus macrops*	Big-eyed Pit Viper	+	277	-[a]	-	*Trimeresurus albolabris*
Viperidae	*Calloselasma rhodostoma*	Malayan Pit Viper	-	-	+	400	*Calloselasma rhodostoma*

[a]Non-specific DNA bands were found in the PCR. [b]There is no specific antivenom available in Thailand. ''+'' = Possible to identify based on the appearance of a specific DNA band. ''-'' = Impossible to identify.

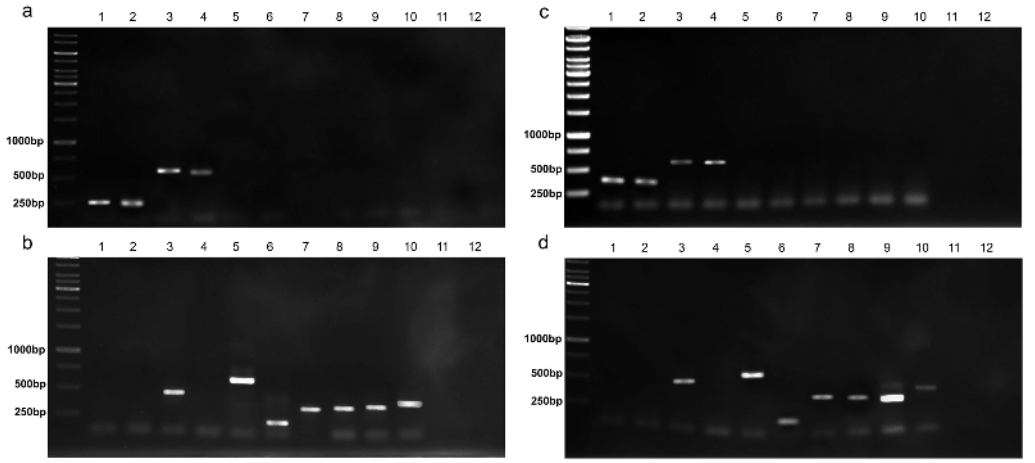

Figure 3. Agarose gel electrophoresis of PCR products using multiplex PCR assays with primer panels A (**a**, **c**) and B (**b**, **d**). Snake genomic DNA templates were from venom (a-b) and saliva (c-d). Mouse (*Mus musculus*) genomic DNA isolated from blood and human (*Homo sapiens*) genomic DNA isolated from cell line 9948 (Promega) was used for outgroups. *Lane 1 = Trimeresurus albolabris*; *lane 2 = Trimeresurus macrops*; *lane 3 = Daboia siamensis*; *lane 4 = Bungarus candidus*; *lane 5 = Bungarus fasciatus*; *lane 6 = Ophiophagus hannah*; *lane 7 = Naja siamensis*; *lane 8 = Naja sumatrana*; *lane 9 = Naja kaouthia*; *lane 10 = Calloselasma rhodostoma*; *lane 11 = Mus musculus*; *lane 12 = Homo sapiens*.

DISCUSSION

Sequence divergence, barcoding gap, and clustering analysis

There was some sequence variation in most of the snake species in the present study, but the mtDNA-based identification enabled differentiation between intraspecific and interspecific variation. The level of intraspecific sequence divergence of the *COI* gene was low (0.59%). This is similar to the intraspecific sequence divergence of 0.30% reported for *N. siamensis* (Wüster and Thorpe, 1994). In vertebrates, the sequence divergence of intraspecific variation is about 2%, whereas the sequence divergence of interspecific variation ranges from 4% to 32% (mean = 9.6%; Avise, 2000; Hebert et al., 2003a). The level of sequence divergence of the *Cytb* gene was about 2.22% for intraspecific and 10.47% for interspecific distance in our snake data set. This result agrees with that obtained for African cobras, for which the range is reported to be 0-11% (Wüster et al., 2007). However, these percentages are high compared with the interspecific distances between mammals and reptiles in general, which are approximately 3% (Johns and Avise, 1998). Among turtles, the intraspecific distances are mostly lower than 1%, with an interspecific divergence rate of more than 8% (Shen et al., 2013). This implies that, in the snake lineage, the mutation rate of the *COI* gene has been slower than that of the *Cytb* gene over the course of evolution.

In terms of the barcoded cut-off scores for snake identification, these might vary depending on the specimen collection, the gene studied, and its length (Dawnay et al., 2007). In this study, we applied three analytical methods: 1) 98% identity (Dove et al., 2008), 2) 10X rule threshold values (Hebert et al., 2004), and 3) 3% threshold values (Hebert et al., 2003b). First, the DNA sequences of the unidentified organism must be at least 98% similar to the most common haplotype of the described taxon. However, using this criterion, more than three snake species were misidentified

at the intraspecific level regardless of whether the *COI* or *Cytb* genes were considered. To employ 10X rule threshold values, the interspecific distance should be 10 times the intraspecific distance in order to differentiate between species. Our standard *COI* barcode sequence divergence between species within a genus (average: 5.10%) was about 10 times the average divergence within species (0.59%; Table 3). In contrast, the average interspecific sequence divergence of the *Cytb* gene within each genus (average: 10.47%) was about four times the intraspecific distance (2.22%; Table 4). This result suggests that the interval divergence at the species and genus levels was greater for the *COI* gene than for the *Cytb* gene. This indicates that the *COI* gene may be a more suitable marker than the *Cytb* gene for species identification in snakes. However, the barcoded cut-off scores of the *COI* barcode were insufficient to differentiate species of *Naja* spp and Hydrophiinae spp, which suggests that these species recently diverged from one another (Wüster et al., 2007; Pyron et al., 2013). Hebert et al. (2003b) asserted that, in order to differentiate among species, interspecific divergence should be at least 3%. This threshold agrees with our *COI* barcode, which can be used to differentiate all snake species in this study, except for *T. macrops*, which has a high degree of intraspecific nucleotide divergence (3.6%).

To determine the barcoding gap, which can be used to monitor the difference between intraspecific and interspecific divergence, graphic representations were produced by plotting the minimum interspecific divergence on the y axis and the maximum intraspecific divergence on the x axis for both *COI* and *Cytb* barcodes (Rasmussen et al., 2009). There was a distinct barcoding gap between intra- and interspecific divergence, even though the species from *Naja* and Hydrophiinae were not differentiated using 10X rule threshold values (Figure 4a, b). However, *T. macrops* could not be differentiated using the *COI* barcoding gap, and *T. macrops* and *B. candidus* could not be differentiated using the *Cytb* barcoding gap. One green viper specimen (*T. macrops*: TMA2) was collected from Nong Bua Lamphu (17°10□0□□N, 102°23□0□□E) in the northeast of Thailand, whereas most green viper specimens were collected from the central regions. This suggests that the barcoding gap decreased when species were sampled across a broad geographic area, owing to intraspecific divergence. While BI clustering analyses of both *COI* and *Cytb* indicated that most species of the same genus and family clustered together, one *T. macrops* individual (TMA2) was positioned in the clade of *T. albolabris* (Figure 1a and b). The snakes in the genus *Trimeresurus* (Asian green pit vipers) are one of the most diverse radiations of pit vipers; they inhabit southern Asia and the Indo-Malay Archipelago. The species complex is remarkable for its extreme morphological similarity, occupies a wide range of ecological habitats, and exhibits diverse life histories and behaviors (Malhotra et al., 2011). Our results suggest that the *Trimeresurus* species complex has frequently been misidentified. Interspecific divergence of the *COI* gene between TMA2 and two *T. albolabris* individuals was 1%, whereas divergence between TMA2 and other *T. macrops* specimens was 13%-15% for the *COI* gene (**Table S1**). These results collectively suggest that TMA2 is a variant of *T. albolabris* or is a cryptic species that is closely related to *T. albolabris*. However, morphologic studies and more specimens are needed to examine the barcode of *Trimeresurus* spp.

DNA marker to differentiate among snakes for which antivenom is available in Thailand

Species-distinguishing sites were investigated in the *COI* barcode for snake identification. Eight DNA markers were designed based on distinctive sites of *COI* sequences. These markers were successfully used to distinguish *Naja* spp (1), *O. hannah* (2), *Trimeresurus* spp (3), Hydrophiinae

snakes (4), *D. siamensis* (5), *B. fasciatus* (6), *B. candidus* (7), and *C. rhodostoma* (8). Multiplex PCR involves running a single PCR that produces many species-specific PCR products simultaneously. Such multiplex PCR assays have been applied to identify a variety of vertebrate species (Unajak et al., 2011). Our primer sets are the first DNA markers to correctly identify the eight snake groups examined. The antivenoms of seven of these species are produced and kept by the Thai Red Cross for clinical use. Selection of an effective antivenom for the treatment of people bitten by venomous snakes requires an accurate taxonomic identification. Saliva samples at the bite wound can be used for molecular identification of a snake. A venom-antivenom snake laboratory has also shown the possibility of using some pooled venoms derived from multiple snake species (Pook and McEwing, 2005). Our two-panel multiplex PCR assay clearly identified seven snakes (*Naja* spp, *O. hannah*, *Trimeresurus* spp, *D. siamensis*, *B. fasciatus*, *B. candidus*, and *C. rhodostoma*) from both venom and saliva, and the detection limit of the DNA sample was 0.5 ng. This suggests that our markers could be used to check venom purity and identity during production. Moreover, the multiplex PCR markers we developed may be adequate for the rapid and accurate identification of species during forensic work, conservation study, and medical research.

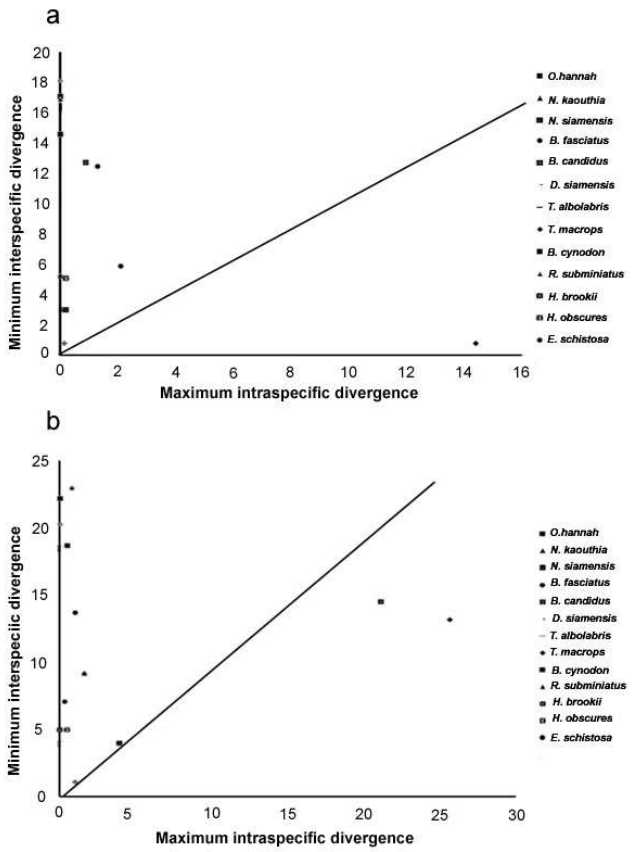

Figure 4. Dot plot analysis of sequence divergence with the *COI* barcode (**a**) and the *Cytb* barcode (**b**). A straight line represents a 1:1 ratio between minimum interspecific sequence divergence and maximum intraspecific sequence divergence. Sequence divergences of species represented by more than one sample are shown.

An effective DNA barcode requires that the DNA target sequences are easily obtained from all species and provide species-level identification. These criteria were fulfilled by our *COI* and *Cytb* barcodes. However, the identification of snake barcode has begun to collect a comprehensive library of *COI* sequences. Blaxter (2004) suggested that the use a large number of samples from a wide geographical area could affect intraspecific genetic distances and lead to more effective species identification. Multiplex PCR markers were then developed for rapid identification without prior sequencing. This is a promising development in the search for an efficient means of examining snakebites, which could ensure the application of appropriate antivenom therapy. However, improvements in the sensitivity of markers, the limitation of locally restricted markers, and protocols for DNA isolation from a remnant swap are still required in order to produce an optimal and practical procedure for the delimitation of snake species.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

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