J.Natn.Sci.Foundation Sri Lanka 2024 52 (2): 199 - 214
DOI: http://dx.doi.org/10.4038/jnsfsr.v52i2.11820

RESEARCH ARTICLE

Phylogeny

Russell's viper (*Daboia russelii*) in the Jaffna peninsula, Sri Lanka bears signatures of incipient genetic divergence from the South Indian population

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Submitted: 22 August 2023; Revised: 04 December 2023; Accepted: 14 December 2023

Abstract: The Russell's viper (*Daboia russelii*) is a medically important viper found in South Asia, including Sri Lanka. This study focused on the phylogeny of Russell's vipers in the geographically segregated coastal peninsula of Jaffna, Sri Lanka. The study aimed to find out whether the specimens collected in Jaffna are different from previously reported forms since such an investigation has not been carried out so far specifically in this area, and to find out whether geographical segregation has had an impact on it. We obtained mtDNA sequences of samples representing six geographical locations in the Jaffna peninsula for the mitochondrial protein-coding genes *Cytb*(576bp) and *ND2*(270bp). Our molecular analyses recovered two distinct clades: *D. russelii* and *D. siamensis*. The clade of *D. russelii* comprises two sister lineages, Pakistan and India/Sri Lanka. The uncorrected pairwise *Cytb* genetic distance between the species range from 5.0 to 14.5 percent. The current study confirms a sister group relationship between the Indian/Sri Lankan lineage and the Pakistani lineage of the Russell's viper (*D. russelii*). Additionally, it discloses the presence of an incipient genetic divergence between Russell's viper populations in Jaffna and South India.

Keywords: *Daboia russelii*, Jaffna, mitochondrial gene, mtDNA, phylogeny, Viperidae, Viperinae.

INTRODUCTION

Sri Lanka is a tropical island located south of India. It has an explicit variation of topography that favours isolation of populations and directing them towards speciation (Gunatilleke *et al*., 2017). Home to one of the highest snake bite rates in the world, the island houses about 89 species of inland terrestrial snakes (Pyron *et al*., 2013b), out of which five species are of medical importance; including the Russell's viper, *Daboia russelii* (Shaw & Nodder, 1797). This reptile is found in South and Southeast Asian countries and classified under the family Viperidae as a true viper (Subfamily: Viperinae) and they lack loreal pits and possess solenoglyphous fangs (Wall, 1921; Smith, 1943). The venom glands that produce a cocktail of venom, coupled with its excellent camouflage, robust body, and defensive behaviour accounts for a higher number of mortalities due to its bite, earning its medically important status (Wüster, 1998). On the other hand, due to the ecological niche that it holds as a carnivore of third or higher trophic levels (Wall, 1921), the snake also contributes to the ecological balance in the environment; especially in the human-made ecosystems of paddy fields where it is generally found, maintaining the populations of fast-growing pests like mice under control (Beaupre & Douglas, 2009; Warrell, 2010; Glaudas, 2021a, 2021b; Martin, 2021).

The current taxonomy of *Daboia russelii* is a result of the integration of morphological studies of several authors including Smith (1917;1943), Deraniyagala (1955) and Wüster *et al*., (1992), molecular genetic studies including that of Thorpe *et al*., (2007) and

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information on envenomation symptoms (Warrell, 1989). The latest update on its classification includes two species, namely, *Daboia russelii* (West of the Bay of Bengal) to which the Sri Lankan haplotype belongs, and *Daboia siamensis* (East of the Bay of Bengal) (Wüster *et al*., 1992; Thorpe *et al*., 2007). Because a synonymous nomen *Daboia pulchella* (Gray, 1842) originating from Sri Lanka is present, Thorpe *et al*., (2007) rightly included Sri Lankan population sequences too. The sequence was sourced from Gampola, a wet zone place in Sri Lanka known for its biotic distinction from the Indian mainland (Gunawardene *et al*., 2007). However, the molecular taxonomic analysis process that led to the above classification has not incorporated a sufficient number of samples from Sri Lanka, presumably based on the small geographical area and the fact that the Russell's viper population in Sri Lanka is similar or closely related to the South Indian haplotype (Thorpe *et al*., 2007). Studies on symptoms of envenomation have also revealed varying patterns, showing distinct symptoms such as neurotoxicity in Sri Lanka, in addition to the most common haemolytic and anticoagulant symptoms shown by both Sri Lankan and Southern Indian populations (Warrell, 1989; Tan *et al*., 2015). Furthermore, experiments carried out on the protein composition of venom from varying geographical locations have also produced concordant results (Pla *et al*., 2019).

Records on snake bite envenomation shows that the Russell's viper is abundant in the Wet Zone, Intermediate Zone and mainly in the Dry Zone of Sri Lanka, which includes Jaffna (Kasturiratne *et al*., 2005; Abyerami & Sivashanthini, 2008). The revised taxonomical work of Russell's viper, as reported by Thorpe *et al*. (2007), included only a sample from the wet zone of Sri Lanka and did not include samples from other climatic areas. Therefore, we aimed to find out whether the specimens collected in Jaffna are different from the *Daboia russelii* population in Tamil Nadu, India. A previous study has also reported that Jaffna experiences the highest incidence of venomous snake bites in agricultural fields, specifically paddy fields, primarily attributed to the Russell's vipers (Ravichandren & Thirunavukarasu, 2016), which further enhanced our interest on this study. During the current study, we examined the morphological characteristics following the literature of Wall (1921), Smith (1917; 1943), Deraniyagala (1955), characteristics of Western subspecies (Wüster, 1998), and notes on the Russell's vipers in Jaffna peninsula (Abyerami & Sivashanthini, 2008) to identify the Jaffna samples in fine scale resolution.

MATERIALS AND METHODS

Daboia russelii specimens were obtained from the Jaffna Teaching Hospital (JTH), which were collected from different locations in Jaffna (9.668^{0}N, 80.029^{0}E) and brought along with the bite victims. A total of eight specimens were collected within the time period from 11 September 2019 to 25 October 2021. After assigning the reference numbers and collecting the preliminary information, we examined the detailed morphological characters to identify the authentic specimens. The ethical clearance for animal handling and obtaining tissue samples, given by the Animal Ethics Review Committee of the University of Jaffna, Sri Lanka, holds the reference number AERC/2021/05.

Laboratory protocols

The liver and spleen tissues of the specimen were obtained by making an incision 2-3 head-lengths behind the head under sterile conditions. Exactly 0.02 g of the frozen tissue samples were used to extract the whole genome of each tissue sample, using the DNeasy® Blood and Tissue Kit. After quantifying its purity, successful extracts (of six individual specimens) were used to amplify partial mitochondrial gene fragments of Cytochrome-b (*Cytb*) and *NADH* dehydrogenase subunit-2 (*ND2*) using the specific primers (Table 1).

Table 1: Primers used in PCR amplification

Gene	Primer	Sequence (5'-3')	Specific snake	Reference	Amplified size (bp)
Cytb	Forward	TCAAACATCTCAACCTGATGAAA	*Daboia russelii*	(Thorpe *et al*., 2007)	758
	Reverse	GGCAAATAGGAAGTATCATTCTG	*Daboia russelii*	(Thorpe *et al*., 2007)	758
ND2	Forward	CCTTGAAGCACTTCTGGGAATCAGA	All snakes	(Hackett, 1996)	363
	Reverse	TATCGGGCCCATACCCCGAAAAT	All snakes	(Hackett, 1996)	363

A standard PCR was carried out for each gene using the master mix, which consisted of 5.0 µL of 5x Buffer, 3.0 µL of $MgCl_2$ (25 µM), and 0.2 µL of *Taq* DNA polymerase. For *Cytb* replicates, the reaction mix included 3.0 µL of DNTP (2 µM), 2.0 µL of forward primer (10 µM), 2.0 µL of reverse primer (10 µM), and 2.0 µL of Template DNA; while for *ND2* replicates, the reaction mix included 4.0 µL of DNTP (2 µM), 2.5 µL of forward primer (10 µM), 2.5 µL of reverse primer (10 µM), and 3.0 µL of Template DNA. Finally, each replicate was topped up to 25µL with nuclease free water, and a total of four replicates were maintained for PCR amplification of each gene from each sample. The PCR conditions for *Cytb* gene consisted of an initial denaturation at 95^0C for 3 min, 35 cycles of denaturation at 95^0C for 30 s, annealing at 50^0C for 45 s, extension at 72^0C for 1 min, and a final extension at 72^0C for 5 min. The PCR conditions for *ND2* gene consisted of initial denaturation at 95^0C for 5 min, 35 cycles of denaturation at 95^0C for 45s, annealing at 50^0C for 45s, extension at 72^0C for 1 min, and a final extension at 72^0C for 3 min. The PCR amplified product was detected through agarose gel electrophoresis (1.5%) at 80V for 45minutes (Figure 1). The purity and the size of the product were confirmed with DNA quantifier. The four replicates of each PCR product were pooled together to obtain a higher yield. Finally, all the samples were purified using a DNA purification kit.

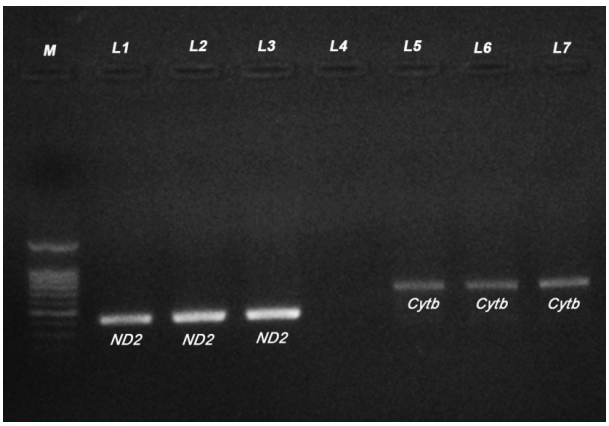

Figure 1: Agarose gel electrophoresis (1.5%) analysis of amplified PCR products from *Cytb* and *ND2* mitochondrial genes. The lane M displays the molecular weight marker 100 bp step ladder (Promega) is positioned in the left corner. Lanes L1-L3 depict the *ND2* marker; Lane L4 serves as the negative control (Nuclease-free water); Lanes L5-L6 display the *Cytb* marker.

Phylogenetic analysis

A dataset with a total of 34 taxa including out-group taxa (576 bp of *Cytb* and 270 bp of *ND2*) was used to carry out the phylogenetic analysis. A list of the 34-taxa dataset included in current study, with their isolate number, geographical location, GeneBank accession number, and reference is presented in Table 2. The nucleotide sequences of the amplified DNA fragments were obtained using Capillary Electrophoresis Sequencing (CES) (Macrogen Inc., Seoul, South Korea). The obtained sequences were double-checked with the ABI chromatogram files and aligned with homologous sequences from the National Centre for Biotechnology Information (NCBI) database using ClustalW (Thompson *et al*., 1994) in MEGA XI® (Tamura *et al*., 2021). Considering the availability of homologous sequences in NCBI, only the isolates with both *Cytb* and *ND2* were concatenated. A total of 33 sequences were used for *Cytb* gene; while 19 concatenated sequences of both *Cytb* and *ND2* were used for available localities to build a further accurate result. *Daboia mauritanica*, which is a sister species of *D. russelii* was used as the outgroup in all cases. The number of parsimony informative sites were determined using DNAsp v6 (Rozas *et al*., 2017). Phylogenetic reconstruction was carried out using maximum likelihood (ML) and Bayesian inference (BI) analysis. The best fit nucleotide substitution model and the partitioning scheme for the BI were determined using the PhyML (Guindon *et al*., 2010) and greedy (Lanfear *et al*., 2012) algorithms via PartitionFinder 2 software (Lanfear *et al*., 2017) under the Corrected Akaike Information Criterion (AICc). A similar software was used to determine the best fit nucleotide substitution models for ML as well. Two phylogenetic trees were constructed for *Cytb* and concatenated *Cytb+ND2* sequences by Bayesian Inference using Markov Chain Monte Carlo (MCMC) randomization in MrBayes 3.2.7 (Ronquist *et al*., 2012). Two parallel runs of four chains (3 heated and 1 cold) were performed for 1 million generations in *Cytb* and 1.5 million generations in *Cytb+ND2* respectively (significant value of the standard deviation of split frequency < 0.01). The runs were sampled at every 500th generation. The point of convergence (burnin) and the average estimated sample size for each parameter were estimated in Tracer v1.7.2 (Rambaut *et al*., 2018). The first 25% generations of burnin were discarded and the consensus trees were calculated from the remaining 75% of the posterior distribution. The Ultrafast bootstrap (BP) with 1,000 iterations (Minh *et al*., 2013) in IQ-TREE and the partitioning scheme obtained from PartitionFinder

2 were used to determine the statistical support in the ML trees (both *Cytb* and *Cytb+ND2* trees). The output consensus trees were viewed using FigTree v1.4.4. The different evolutionary best fit nucleotide substitution models, partitioning scheme, and number of sequences used in the analysis are provided in Supplementary Table S1. The uncorrected pairwise genetic distances for various populations of *Daboia* for the two genes were calculated using MEGA XI®. Finally, the haplotype network reconstruction for *Cytb* gene of various populations of *Daboia russelii* was inferred by Median Joining Network (Bandelt *et al*., 1999) using PopArt (Leigh & Bryant, 2015). The sequences generated by the current study and the sequences obtained from the GenBank (Supplementary Table 2) were used for this analysis.

Table 2: NCBI accession numbers of the gene sequences used for phylogenetic analysis (NA- not given)

Source	Isolate	Organism	Locality	*Cytb*	*ND2*
NCBI	RA-1961.325, DM02	*Daboia mauritanica*	Morocco	MF140584	MT232999
(Thorpe *et al*., 2007)	*NA*	*D. russelii*	Thayur, Tamil Nadu, India	AY165087.1	AY165075.1
(Thorpe *et al*., 2007)	*NA*	*D. russelii*	Gampola, Sri Lanka	AY165088.1	AY165076.1
(Thorpe *et al*., 2007)	*NA*	*D. russelii*	Pakistan	AJ275723.1	AY165074.1
This Study	RV001	*D. russelii*	Jaffna, Sri Lanka	MW771366.1	MW881135.1
This Study	RV002	*D. russelii*	Jaffna, Sri Lanka	MW771367.1	MW881136.1
This Study	RV003	*D. russelii*	Jaffna, Sri Lanka	MW771368.1	MW881137.1
This Study	RV006	*D. russelii*	Jaffna, Sri Lanka	MW771369.1	MW881138.1
This Study	RV007	*D. russelii*	Jaffna, Sri Lanka	MW771370.1	MW881139.1
This Study	RV008	*D. russelii*	Jaffna, Sri Lanka	MW771371.1	MW881140.1
NCBI	DR11	*D. russelii*	Pakistan	MZ711546.1	-
NCBI	DR18	*D. russelii*	Pakistan	MZ711545.1	-
NCBI	DR15	*D. russelii*	Pakistan	MZ711544.1	-
NCBI	DR20	*D. russelii*	Pakistan	MZ711543.1	-
NCBI	DR4	*D. russelii*	Pakistan	MZ711542.1	-
NCBI	DR3	*D. russelii*	Pakistan	MZ711541.1	-
NCBI	DR1	*D. russelii*	Pakistan	MZ711540.1	-
NCBI	DR2	*D. russelii*	Pakistan	MZ711539.1	-
NCBI	*NA*	*D. russelii*	Pakistan	HM179463.1	-
NCBI	AIWC 074	*D. russelii*	Tamil Nadu, India	MZ029432.1	-
NCBI	V30	*D. russelii*	Vedenemmeli, Tamil Nadu, India	MG995824.1	-
NCBI	V27	*D. russelii*	Goa, India	MG995821.1	-
(Thorpe *et al*., 2007)	*NA*	*D. siamensis*	Fong Shan, Pingtung, Taiwan	AY165089.1	AY165077.1
(Thorpe *et al*., 2007)	*NA*	*D. siamensis*	Tuban, W of Gresik, East Java, Indonesia_1	AY165083.1	AY165070.1
(Thorpe *et al*., 2007)	*NA*	*D. siamensis*	Tuban, W of Gresik, East Java, Indonesia_2	AY165084.1	AY165071.1
(Thorpe *et al*., 2007)	*NA*	*D. siamensis*	Tonggurambang, Mbay, Flores, Indonesia_1	AY165085.1	AY165072.1
(Thorpe *et al*., 2007)	*NA*	*D. siamensis*	Tonggurambang, Mbay, Flores, Indonesia_2	AY165086.1	AY165073.1
(Thorpe *et al*., 2007)	*NA*	*D. siamensis*	Htauk Kyant, Myanmar	AY165080.1	AY165067.1
(Thorpe *et al*., 2007)	*NA*	*D. siamensis*	Thailand (southern central)_1	AY165090.1	AY165078.1
(Thorpe *et al*., 2007)	*NA*	*D. siamensis*	Thailand (southern central)_2	AY165091.1	AY165079.1
(Thorpe *et al*., 2007)	*NA*	*D. siamensis*	Poipet, Sisophon, Cambodia	AY165081.1	AY165068.1
(Thorpe *et al*., 2007)	*NA*	*D. siamensis*	Guangdong, China	AY165082.1	AY165069.1
NCBI	S-05	*D. siamensis*	China	KF913330.1	-
NCBI	S-04	*D. siamensis*	China	KF913329.1	-

RESULTS AND DISCUSSION

Until 2007, the systematics of the Russell's viper species complex was deduced solely based on the utilization of morphological characters and envenomation symptoms. Owing to its discontinuous distribution across the regions of South and Southeast Asia, these populations were designated as discrete subspecies. This designation was prompted by observed divergences in morphology, as well as differences in envenomation patterns. Smith (1943) in his description, identified two distinct colour forms *Vipera russelli russelli* and *V. r. siamensis*. Later, the Russell's viper was reclassified under the genus *Daboia* due to its isolated position under albumin immunology and blood serum electrophoresis (Herrmann *et al*., 1992).

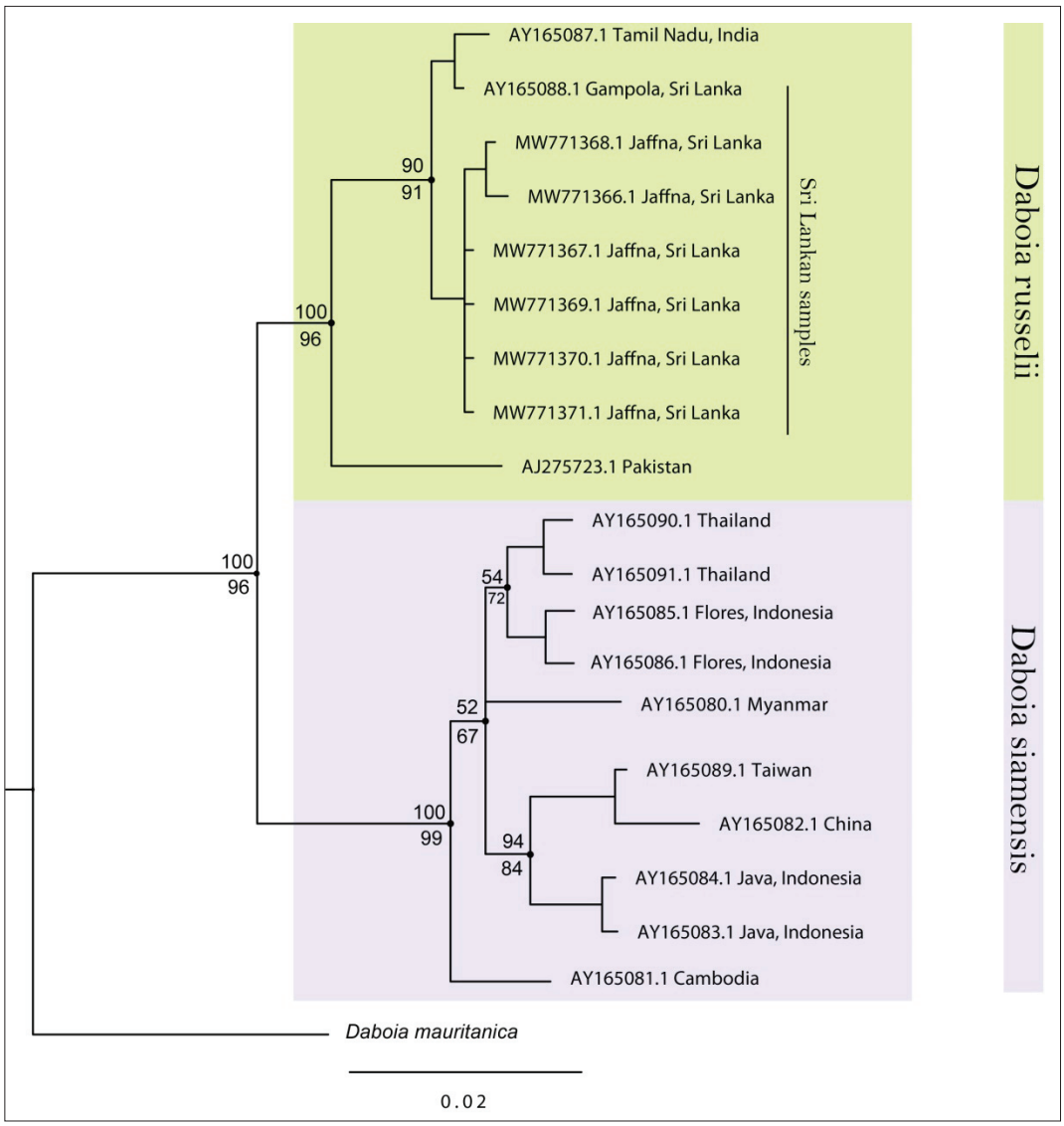

Figure 2: Molecular phylogenetic relationships of Russell's viper, built upon Bayesian inference of the concatenated sequence alignment of the *Cytb + ND2* (846 bp) mitochondrial-gene markers. The numerical values above and below the nodes indicate Bayesian posterior probabilities and maximum likelihood ultrafast bootstrap values respectively. The scale bar corresponds to the number of genetic changes per site. The accession number at the branch tip corresponds to the *Cytb* gene, listed in Table 2.

The work of Wüster *et al*., (1992) showed variation in the Russell's viper taxonomy by performing multivariate analysis of morphological characters only. Later, Thorpe *et al*. (2007), explained the genetic variation of the Russell's viper by studying the phylogeography of the species in relation to its colour pattern and envenomation symptoms and classified them into two species, namely, *Daboia russelii* and *Daboia siamensis.* The molecular phylogenetic analysis revealed their distinction at the species level, attributed to significant genetic divergence, as well as discernible morphometric and geographic distinctions (Thorpe *et al*., 2007). This classification remains upheld to the present day (Wallach *et al*., 2014; Boundy, 2020).

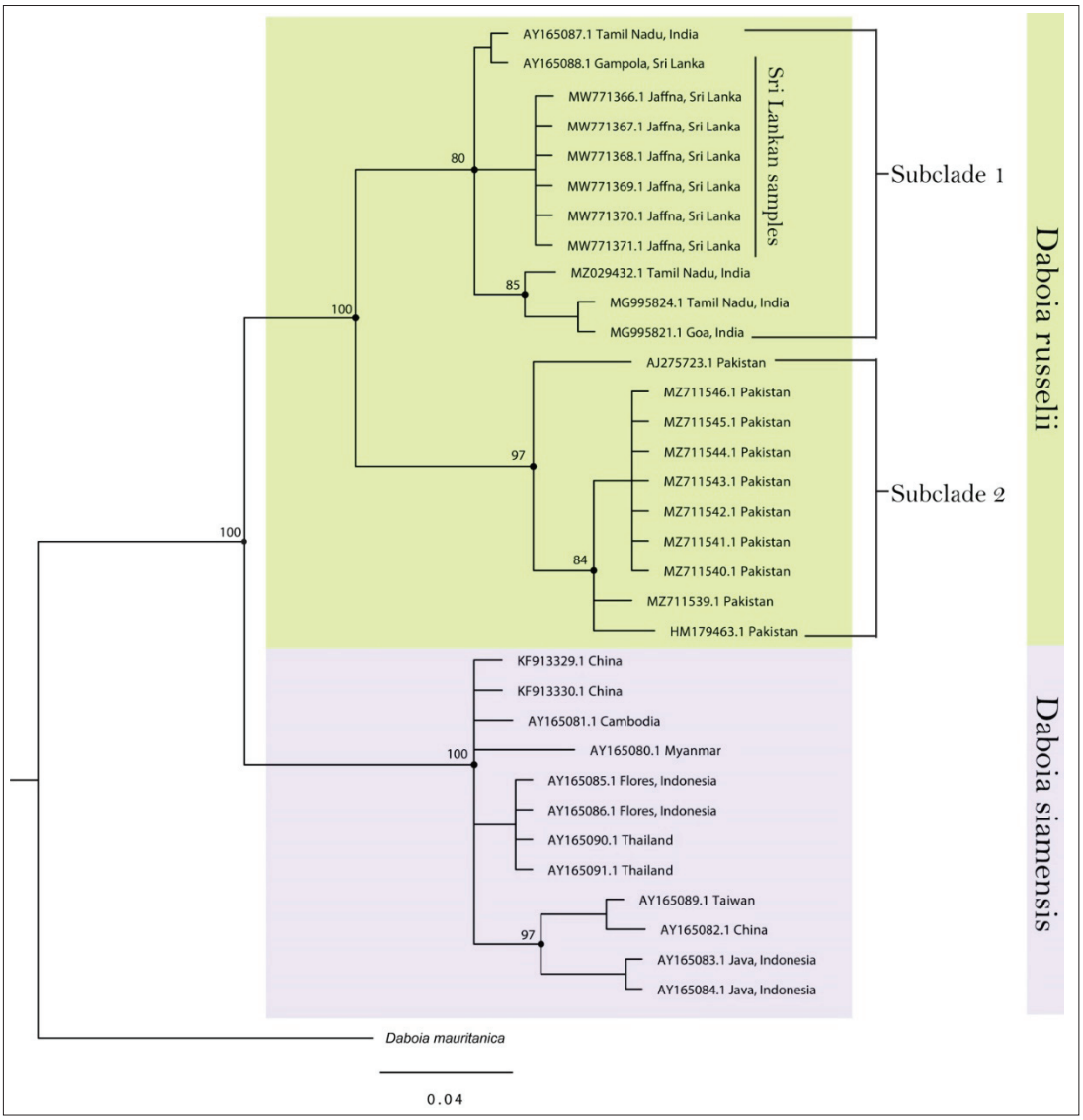

Figure 3: Molecular phylogenetic relationships of Russell's viper, built upon Bayesian inference of the multiple sequence alignment of the *Cytb* (576 bp) mitochondrial-gene markers. The numerical values above the nodes indicate Bayesian posterior probability values. The scale bar corresponds to the number of genetic changes per site. The accession number at the branch tip corresponds to the *Cytb* gene, listed in Table 2.

Above study (Thorpe *et al*., 2007) utilized three mitochondrial genes (cytochrome b, *NADH* dehydrogenase subunit 2 and 4) of Russell's viper from the Southern and South-east Asian countries; in which the position of Sri Lanka has been analyzed with a single specimen, despite the availability of Russell's viper from a range of geographical locations according to the bite records (Kasturiratne *et al*., 2005; Das & Silva, 2016).

In the present study, the final alignment of *Cytb* consisted of 576 base pairs while the concatenated *Cytb+ND2* alignment consisted of 846 base pairs (*Cytb*: 576bp and *ND2*: 270bp). The pseudogenes were confirmed to be absent with the absence of indels and stop codons within the coding genes (Zhang & Hewitt, 1996). The maximum parsimony informative sites for the sequences analysed are *Cytb*: 71, *Cytb+ND2*: 90. The best evolutionary models for each partition determined by PartitionFinder 2 (Lanfear *et al*., 2017), are given in Supplementary Table S1. The standard deviations of split frequencies in BI analysis for *Cytb* and *Cytb+ND2* datasets are 0.007 and 0.003 respectively. The results consist of two BI phylogenetic trees (Figure 2 and 3) and two ML trees (Supplementary Figure S1 and S2). The Bayesian posterior probability (PP) and Ultrafast bootstrap (BP) of each node has been given at the tip of each node. Interspecific and intraspecific uncorrected pairwise genetic distance (p-distance) computed for the *Cytb* and *ND2* genes for the various populations of genus *Daboia* is presented in Table 3 and 4, respectively.

Table 3: Interspecific uncorrected pairwise genetic distances (%), for the *Cytb* and *ND2* genes among *Daboia* species

Cytb *ND2*	*Daboia mauritanica*	*Daboia russelli*
Daboia mauritanica		
Daboia russelii	14.5-10.9 12.2-10.2	
Daboia siamensis	14.3-13.6 12.8-12.5	11.6-5.0 9.9-8.2

The BI (Figure 2 and 3) and ML (Supplementary Figure S1 and S2) analysis retrieved concordant trees with similar topologies. However, the topological differences were observed in the few substructures of the *Daboia siamensis* clade. The major variations were observed between the *Cytb* BI tree (Figure 3) and *Cytb* ML tree (Supplementary Figure S2). Our phylogenetic analysis retrieved two primary clades, i.e., *D. russelii* clade and *D. siamensis* clade, with strong node support, while *Daboia mauritanica* is recovered as the basal in both the BI and ML trees. Our interspecific uncorrected pairwise distances values (Table 3) also support the aforementioned divergence between the primary clades.

The *D. siamensis* clade consists of four main lineages, viz 1) Cambodia, 2) Myanmar, 3) Thailand / Flores, 4) China / Taiwan / Java. This clade exhibits a "star" phylogeny in all four trees with minor differences (Figure 2, 3 and Supplementary Figure S1, S2), as the aforementioned lineages are recovered with poorly supported nodes, similar to the previous finding of Thorpe *et al*., (2007). This type of phylogeny shows a population expansion event from a common ancestor (the founder lineage) in the recent past. The Cambodian sample is recovered as the basal group in all trees despite the slight changes in BI tree for *Cytb* (Figure 3). A similar finding was also reported by Thorpe *et al*. (2007), although their BI phylogeny retrieved the Myanmar sample as the basal lineage. The *D. russelii* clade includes samples confined to Pakistan, India and Sri Lanka. The Pakistan lineage (subclade 1) is recovered as sister group to the India/ Sri Lanka lineage (subclade 2), with high node support, in both BI (Figure 3) and ML (Supplementary Figure S2) tree for *Cytb.* However, in both the BI tree (Figure 2) and the ML tree for the concatenated sequence (Supplementary Figure S1), the Pakistan group is identified as the basal group within the *D. russelii* clade. This basal position for the Pakistan group within the *D. russelii* clade was also reported in a previous study (Thorpe *et al*., 2007).

The present study represents the first phylogenetic analysis of the Russell's viper from the Jaffna peninsula, Sri Lanka, having used the mitochondrial gene sequences obtained from the specimens of Jaffna, and the sequence data from NCBI (Table 2).The initial intention was to use the fragments of the same genes as Thorpe *et al*. (2007), but due to the lack of amplification yield with the provided *NADH* dehydrogenase subunit 4 (*ND4*) primers, it was decided to proceed with the study using *Cytb* and *ND2* genes only. The present study included six individual samples of Russell's viper from Jaffna; excluding two of the total eight samples due to putrifaction. The primers used for the amplification were identified from the published literature (Table 1). The phylogenetic analysis of the current study utilized mitochondrial gene sequences from 33 different isolates, including the six samples from Jaffna, Sri Lanka, generated by current study (Table 2).

Thirteen isolates were previously reported by Thorpe *et al*. (2007), and the remaining 14 were downloaded from the GeneBank database. These additional fourteen isolates have sequences only for the *Cytb* gene region (Table 2). Therefore, in this study a separate tree has been drawn for the *Cytb* gene (Figure 3) for which the highest number of sequences are available from multiple localities within South Asia. All the trees represent the deviation between *Daboia siamensis* (Node B) and *Daboia russelii* (Node A) with strong node support. Furthermore, in addition to the results of previous studies, the specimen from Pakistan also shows mild divergence from the South Indian specimen (including Sri Lanka) with strong node support showcased with BI tree for *Cytb* (Figure 3). Sri Lanka shows genetic proximity to the South Indian specimen which is clearly observed in BI tree for *Cytb* (Figure 3), but with poor node support. This is also supported by the results of intraspecific uncorrected pairwise genetic distance analysis (Table 4).

Table 4: Intraspecific uncorrected pairwise genetic distances (%), for the *Cytb* and *ND2* genes among *Daboia russelii* populations from various localities

Cytb ND2	Pakistan	Goa, India	Tamil Nadu, India	Jaffna, Sri Lanka
Pakistan				
Goa, India	2.8 NA			
Tamil Nadu, India	3.3-2.6 2.0	0.9-0.4 NA		
Jaffna, Sri Lanka	3.1-2.2 1.7	1.0 NA	1.0-0.8 0.7	
Gampola, Sri Lanka	2.9-2.4 1.6	0.7 NA	0.7-0.2 0.3	0.6-0.5 0.6

The haplotype network generated for *Cytb* gene (Figure 4) also resembles this shallow divergence between the Pakistan and India/Sri Lanka lineages (Figure 4B), while demonstrating the genetic proximity between Indian samples and Sri Lankan samples. However, a contradiction arises with the specimen from Gampola, Sri Lanka which has been clustered together with Tamil Nadu specimen (*Cytb* p-distance: 0.7 - 0.2, *ND2* p-distance: 0.3) and not with the specimen from Jaffna, Sri Lanka (*Cytb* p-distance: 0.6 - 0.5, *ND2* p-distance: 0.6) in all four phylogenetic trees. This is a completely opposite outcome from what we expected to observe.

The Sri Lankan affinity to the South Indian fauna has also been published in previous studies (Bossuyt *et al*., 2004; Crusz, 1973; Gunatilleke *et al*., 2017). The Western Ghats and Sri Lanka display considerable diversity in both landscape and climate, which gives rise to a rich variety of plant life and distinct animal communities. However, Sri Lanka's wet zone has a more pronounced wet and seasonal climate. The evergreen forests in both regions boast unique animal species, many of which belong to specific, localized groups. In contrast, the lowland dry forests show more similarities in terms of the plants and animals they host. This difference might be due to the possibility that connections between wet zones were less frequent during ice ages compared to the connections between dry zones (Gunawardene *et al*., 2007). However, the expected genetic proximity between South India and the dry-zone in Sri Lanka (Jaffna) was not observed in the outcome of our phylogenetic analysis. However, this underscores the necessity for a study to be conducted using authentically collected samples that represent all the geographical locations in South Asia where the population of Russell's viper is abundant. Furthermore, incorporating an additional number of genes (both mitochondrial and nuclear) may influence the accuracy of the phylogenetic analysis outcomes (Rokas & Carroll, 2005). This approach has been successfully implemented in a previous study as well (Pyron *et al*., 2013a; 2013b). Therefore, it could be helpful in resolving the unresolved phylogenetic relationship of the India/Sri Lanka subclade within the *Daboia russelii* clade. Under the current availability of data, a regional comparison for both areas of the mitochondrial genome

is impossible due to the lack of adequate sequences. Of all two genes, Cytochrome b (*Cytb*) can be proposed as the most effective barcode to construct the phylogeny for its availability of sequences. Also, this study could not completely address the variations in envenomation symptoms and the composition of venom to fully evaluate the status of the Jaffna population. Therefore, as a future suggestion, such analysis would be helpful to discuss the results of our phylogenetic analysis.

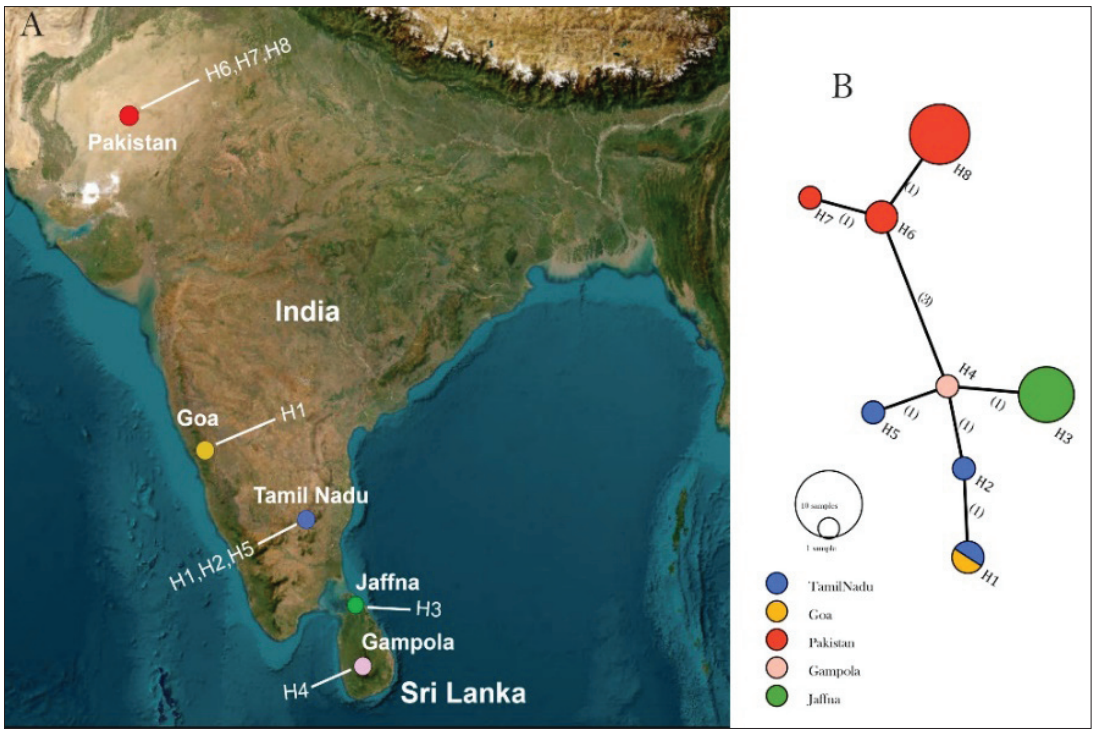

Figure 4: The haplotype network using PopART v1.7, for *Cytb* mitochondrial-gene markers of *Daboia russelii*. (A) A map represents the sample localities. (B) Median joining haplotype network for the 576 bp fragment of the *Cytb* gene marker. The mutational steps are shown in parentheses.

Discussion

Sri Lanka is a continental island connected to the Indian subcontinent under the seabed (Crusz, 1973). In the Pleistocene glacial ages, Sri Lanka and mainland India periodically connected until around 10,000 years ago due to rising sea levels. Comparing faunal components across the Palk Strait reveals morphological similarities, suggesting a recent exchange of plant and animal life with Southern India (Bossuyt *et al*., 2004). However, evergreen forests in Sri Lanka and the Western Ghats hold unique animal species, many specific to localized groups. This distinction may arise due to wet zones having fewer connections during ice ages (Bossuyt *et al*., 2004; Gunawardene *et al*., 2007). While the differentiation between the primary subclade of *D. russelii* in Pakistan and India/Sri Lanka is clear, the phylogenetic relationship within the India/Sri Lanka subclade remains unresolved. This is evidenced by the BI tree, ML tree, and p-distance analysis. The current phylogenetic analysis recovered the Russell's viper population in Sri Lanka (Gampola and Jaffna) as a paraphyletic group in all analyses. Although we expect the genetic affinity of the Jaffna population to be more towards Tamil Nadu, the Gampola population shows more proximal genetic relatedness to the Tamil Nadu population than Jaffna.

Fluctuating sea levels have separated the island from the Indian sub-continent, creating a narrow strip of sea known as the "Palk Strait" (Arasaratnam & Peiris, 1999), which limits the migration of terrestrial animals between the two landmasses. Thus, geographical isolation of organisms has driven the way to allopatric speciation (Cabej, 2012) creating new species and subspecies of

reptiles and other terrestrial animals of Sri Lanka. This underscores the necessity for a study to be conducted using authentically collected samples that represent all the geographical locations in South Asia where the population of Russell's viper is abundant. Incorporating more taxa and more gene markers (nuclear + mitochondrial) to the phylogenetic study may improve the resolution among the phylogenetic analysis, and a reliable outcome can be expected (Rokas & Carroll, 2005).

Russell's viper can be considered as a keystone species due to its ecological contributions in maintaining the ecological balance and controlling populations of pests such as rodents (Beaupre & Douglas, 2009; Glaudas, 2021a; Martin, 2021). Although it has been listed as a least concerned (LC) species in the national red list of Sri Lankan fauna and flora (MOE, 2012), its survival is threatened by deforestation and intentional killing by humans out of fear. Therefore, understanding its exact phylogenetic position and relationships is critically important. This understanding will not only help resolve uncertainties related to evolutionary biology but also aid in assessing the conservation status of *D. russelii* in Sri Lanka, laying a platform for which future conservation efforts may be initiated.

CONCLUSION

The current study confirms a sister group relationship between the Indian/Sri Lankan lineage and the Pakistani lineage of the Russell's viper (*D. russelii*). Additionally, it discloses the presence of an incipient genetic divergence between Russell's viper populations in Jaffna and South India.

Acknowledgements

The Jaffna Teaching Hospital (JTH) and Mr. S. Arthiyan (Department of Zoology, Faculty of Science, University of Jaffna) are acknowledged for their support in obtaining specimens, and the Parasitology Laboratory (Faculty of Medicine, University of Jaffna) is acknowledged for their support in genetic analysis. The "Staff Research Grant" of the University of Jaffna is acknowledged for the financial support rendered in sequencing the DNA products.

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SUPPLEMENTARY MATERIALS

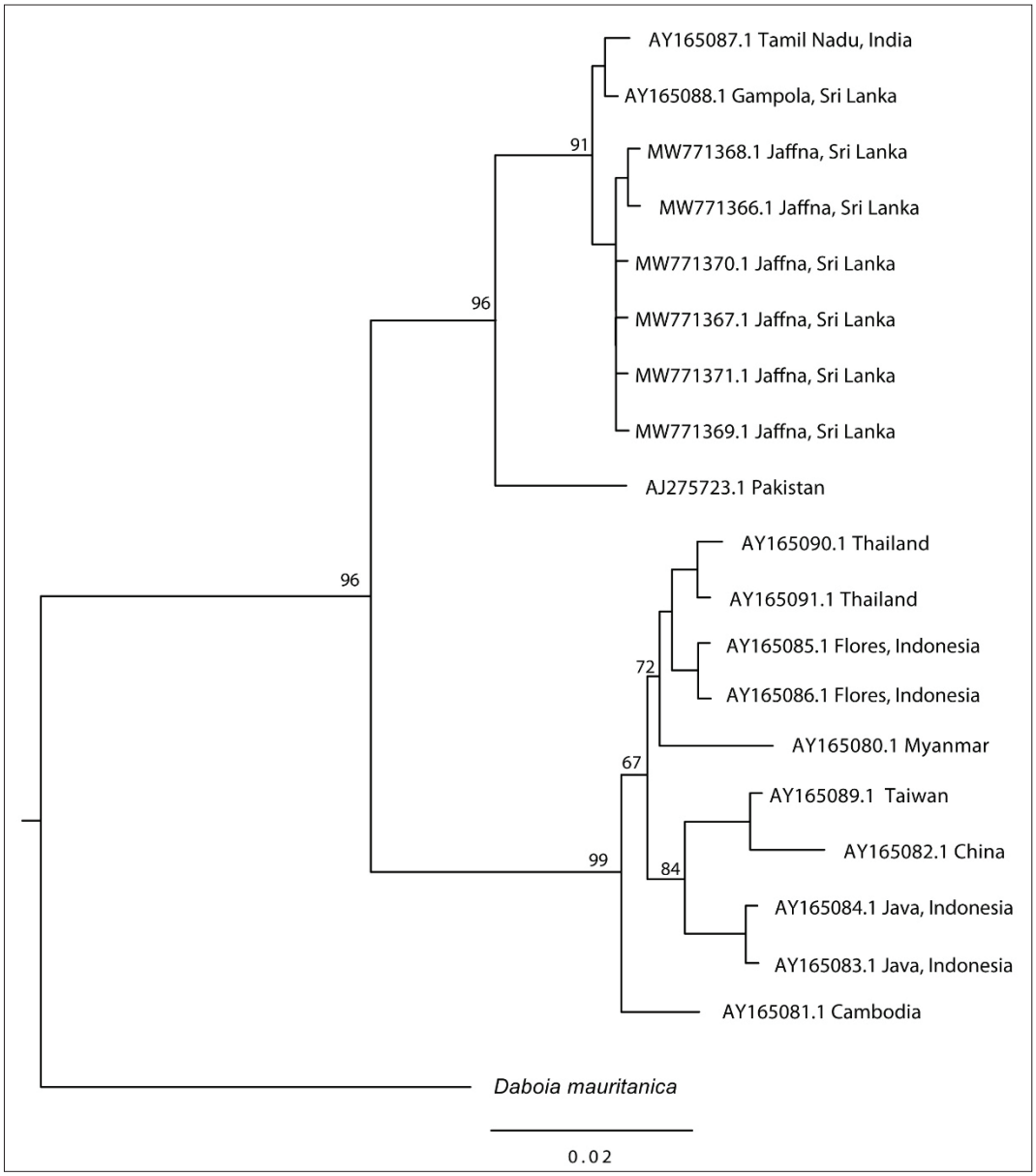
Supplementary Table S1: The best-fit nucleotide substation model and the partitioning schemes used for the phylogenetic inference analysis as determined by PartitionFinder 2

Subsequent Analysis	Genes (Number of Taxa)	Number of partitions (Subsets)	Partitions	The best model
Bayesian inference using MrBayes	Cytb (34)	1	Cytb cp1	SYM
		2	Cytb cp2	HKY
		3	Cytb cp3	GTR
	Cytb + ND2 (18)	1	Cytb cp1	SYM
		2	Cytb cp2	HKY
		3	Cytb cp3	GTR
		4	ND2 cp1	HKY+G
		5	ND2 cp2	HKY+I
		6	ND2 cp3	HKY+G
Maximum likelihood inference using IQ-TREE	Cytb (34)	1, 2, 3	Cytb cp1, Cytb cp2, Cytb cp3	TRN
	Cytb + ND2 (18)	1, 2, 3	Cytb cp1, Cytb cp2, Cytb cp3	TRN
		4	ND2 cp1	K81UF+G
		5	ND2 cp2	HKY+I
		6	ND2 cp3	TRN

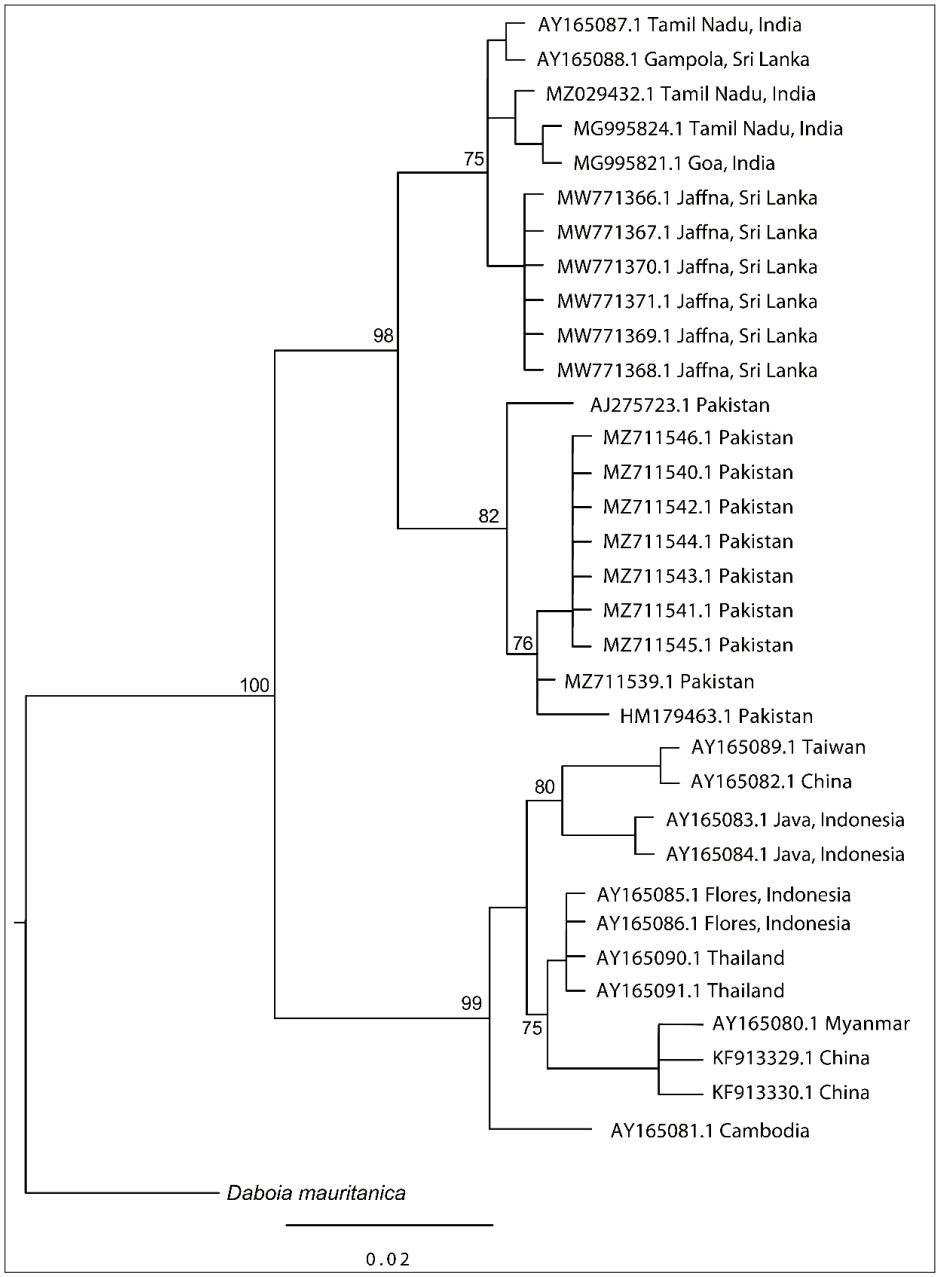
cp, codon position

Supplementary Table S2: Details of the samples considered for construction of haplotype network in PopART v1.7, including localities, voucher reference, and GeneBank accession numbers.

Sample/Isolate	Organism	Locality	*Cytb*	Haplotype
NA	*D. russelii*	Thayur, Tamil Nadu, India	AY165087.1	H5
NA	*D. russelii*	Gampola, Sri Lanka	AY165088.1	H4
NA	*D. russelii*	Pakistan	AJ275723.1	H7
RV001	*D. russelii*	Jaffna, Sri Lanka	MW771366.1	H3
RV002	*D. russelii*	Jaffna, Sri Lanka	MW771367.1	H3
RV003	*D. russelii*	Jaffna, Sri Lanka	MW771368.1	H3
RV006	*D. russelii*	Jaffna, Sri Lanka	MW771369.1	H3
RV007	*D. russelii*	Jaffna, Sri Lanka	MW771370.1	H3
RV008	*D. russelii*	Jaffna, Sri Lanka	MW771371.1	H3
DR11	*D. russelii*	Pakistan	MZ711546.1	H8
DR18	*D. russelii*	Pakistan	MZ711545.1	H8
DR15	*D. russelii*	Pakistan	MZ711544.1	H8
DR20	*D. russelii*	Pakistan	MZ711543.1	H8
DR4	*D. russelii*	Pakistan	MZ711542.1	H8
DR3	*D. russelii*	Pakistan	MZ711541.1	H8
DR1	*D. russelii*	Pakistan	MZ711540.1	H8
DR2	*D. russelii*	Pakistan	MZ711539.1	H6
NA	*D. russelii*	Pakistan	HM179463.1	H6
AIWC 074	*D. russelii*	Tamil Nadu, India	MZ029432.1	H2
V30	*D. russelii*	Vedenemmeli, Tamil Nadu, India	MG995824.1	H1
V27	*D. russelii*	Goa, India	MG995821.1	H1



Supplementary Figure S1: Molecular phylogenetic relationships of Russell's viper, built upon maximum likelihood method of the concatenated sequence alignment of the Cytb + ND2 (846 bp) mitochondrial-gene markers. The numerical values above and below nodes indicate maximum likelihood ultrafast bootstrap values. The scale bar corresponds to the number of genetic changes per site. The accession number at the branch tip corresponds to the Cytb gene, listed in Table 2.



Supplementary Figure S2: Molecular phylogenetic relationships of Russell's viper, built upon maximum likelihood method of the multiple sequence alignment of the Cytb (576 bp) mitochondrial-gene markers. The numerical values above nodes indicate ultrafast bootstrap values. The scale bar corresponds to the number of genetic changes per site. The accession number at the branch tip corresponds to the Cytb gene, listed in Table 2.