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RESEARCH ARTICLE

Molecular Phylogeny

Molecular phylogeography of *Echis carinatus* revisited: Insights from the Sri Lankan population of saw-scaled viper (Serpentes: Viperidae: *Echis*)

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Abstract: The saw-scaled viper (*Echis carinatus*) is a member of the Viperidae family, known for its intricate taxonomic history. Initially, it was believed to be a unique Sri Lankan subspecies called *E. c. sinhaleyus*. The subsequent clinical evidence also suggests the presence of a distinct subspecies in Sri Lanka. However, its existence was later questioned, urging the need for genetic studies. Therefore, it was aimed to unravel the molecular phylogenetic affinities of the Sri Lankan population of *E. carinatus*. For the first time, the molecular phylogenetic affinities and phylogeography of the Sri Lankan population of *E. carinatus* were explored. This study was based on sequences of samples obtained from 12 locations across the Northern Province, using mitochondrial markers *Cytb, NADH4, 16S*, and *12S*. Molecular phylogenetic analyses showed that the South Indian and Sri Lankan populations of *E. carinatus* form a reciprocally monophyletic clade, which is recovered as the sister group to the remaining groups of *E. carinatus*. Based on the divergence-time estimates, the divergence between Sri Lankan and South Indian population of *E. carinatus* is estimated to have occurred in the mid Pleistocene epoch. This study discloses that *E. carinatus* comprises two distinct subspecies namely, *E. c. carinatus* and *E. c. sochureki*. The Sri Lankan and South Indian populations belong to the subspecies *E. c. carinatus*, whereas *E. c. sochureki* comprises the remaining populations of *E. carinatus* distributed across northern, western, and eastern India (Rajasthan, Maharashtra, Odisha, and Goa), as well as in Pakistan, Sharjah, and Iraq.

Keywords: Biogeography, mitochondrial markers, molecular phylogeny, snakes, vipers.

INTRODUCTION

Sri Lanka is a tropical island, bearing distinct groups of fauna with high degree of endemism (Meegaskumbura *et al*., 2002; Bossuyt *et al*., 2004). The terrestrial snake fauna of Sri Lanka comprises at least 89 species from 11 families (Botejue, 2020) of which a minimum of 49 (>50%) are endemic (Wickramasinghe *et al*., 2019). Among them, only 5 species of snakes namely, Russell's viper (*Daboia russelii*) (Shaw & Nodder, 1797), Hump-nosed pit viper (*Hypnale hypnale*) (Merrem, 1820), Indian or common krait (*Bungarus caeruleus*) (Schneider, 1801), Sri Lankan cobra (*Naja polyocellata*) (Mehrtens ,1987) and saw-scaled viper (*Echis carinatus*) (Schneider, 1801) are considered as snakes of highest medical importance (Silva *et al*., 2023). While *Echis carinatus* is classified as a medically important snakes in Sri Lanka, instances of envenomation by this species are not considered life-threatening, with no reported fatalities (Gnanathasan *et al*., 2012). There is a divergence of opinions regarding whether *E. carinatus* merits classification in a higher tier of medical significance. Its current status will be upheld until substantiated data are published (Snakebite Expert Committee of Sri Lanka Medical Association, 2021).

Intraspecific taxonomic classification of the *E. carinatus* clade has been unstable (Escoriza *et al*., 2010),

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due to its complex taxonomic history, with several subspecies being recognized in the past (David & Ineich, 1999), including as *E. c. carinatus* (Schneider, 1801), *E. c. sochureki* (Stemmler, 1969), *E. c. multisquamatus* (Cherlin, 1981), *E. c. astolae* (Mertens, 1970), *E. c. sinhaleyus* (Deraniyagala, 1951).

The Sri Lankan population is distributed mainly in the dry and sandy coastal plains in North-Western (Kalpitiya, Wilpattu National Park), Northern (Mannar, Jaffna, Kilinochchi) and Eastern Provinces (Kularatne *et al*., 2011). It is responsible for more than 50% of the snakebite in the Jaffna District (Kularatne *et al*., 2011; Sivansuthan, 2011). Deraniyagala (1955) elevated the Sri Lankan population of *E. carinatus* as an endemic sub species based on its morphological variations compared to the northern races of India. But the taxonomic position was not studied in detail after the initial reporting by Deraniyagala in 1955. Conversely, the sub species *E. c. carinatus* and *E. c. sochureki* are considered as a medically important viperine species in South Asia. Among them the *E. c. sochureki* causes numerous bites in northern India whereas *E. c. carinatus* is regionally highly abundant and causes many bites in the western and southern India (Alirol *et al*., 2010). However, in Sri Lanka, *E. carinatus* envenomation is reported to be mild with zero mortality. Hence, it was hypothesized that the Sri Lankan saw-scaled viper could be a different subspecies that could be genetically distinct from the *E. c. carinatus* existing in India. Therefore, it was strongly suggested to explore this possibility further with genetic studies, morphological assessments, and venom profiling (Kularatne *et al*., 2011; Peranantharajah *et al*., 2012). The present phylogenetic study was designed to explore the phylogenetic relationships of the Sri Lankan population of *E. carinatus* using gene sequences from four mitochondrial gene markers.

MATERIALS AND METHODS

Tissue samples were obtained from a total number of 12 adult saw-scaled viper specimens, representing different geographical locations of the Northern Province, Sri Lanka (Supplementary Figure S1), which were collected in 2020 and 2021 and deposited in the laboratory of the Department of Parasitology, Faculty of Medicine, University of Jaffna. In addition to the sequences originated from this study, an additional 89 sequences derived from the previous studies were retrieved from GenBank and included in the analyses (Pook *et al*., 2009; Rhadi *et al*., 2016). For the out-group taxa, sequences of *Cerastes cerastes* (Linnaeus, 1758) (Wüster *et al*., 2008) were retrieved from GenBank. Information on all the samples and sequences used in this study is presented under the Supplementary Table S1. The animal study protocol was approved by the Institutional Animal Ethics Review Committee of University of Jaffna.

DNA extraction and PCR amplification

DNA was extracted from the tissue samples obtained from freshly dissected snake specimens using the Qiagen Tissue DNA extraction kit. Four mitochondrial gene markers, namely, cytochrome b (*Cytb*), NADH dehydrogenase subunit 4 (*NADH4*), small subunit of 12SrRNA (*12S*) and small subunit of 16SrRNA (*16S*) were amplified by polymerase chain reaction (PCR) using the primers and the thermal conditions as described by Pook *et al*. (2009). Details of the primers are provided in the Supplementary Table S2. Finally, the amplified PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and submitted for direct cycle sequencing by Macrogen Inc. (Korea) using the same primers. A total of 48 sequences derived from this study were deposited at the Gen-Bank under the Accession numbers of OP7339076 - OP739099 (*Cytb* and *NADH4*), OP737787 - OP737798 (*12S*) and OP737803 – OP737814 (*16S*).

Alignment of sequences

A total of 102 consensus DNA sequences (including out-group sequence) were aligned using the multiple sequence alignment tool, ClustalW (Larkin *et al.*, 2007) in Mega 11 (Tamura *et al*., 2021) with default parameters. The two protein coding genes (*Cytb* and *NADH4*) were translated into amino acid sequences as per the vertebrate mitochondrial genetic code using the translation tool available in ExPAsy server (Gasteiger, 2003) to check for stop codons or frameshift mutations.

Phylogeny

Phylogenetic analysis was carried out separately for individual mitochondrial markers and for the concatenated dataset (790 bp of *Cytb*, 645 bp of *NADH4*, 364 bp of 12S, and 481 bp of 16S) of 102 taxa using Bayesian inference (BI) and maximum likelihood (ML) methods through MrBayes v3.2.7 (Ronquist *et al*., 2012) and IQ-TREE (Nguyen *et al*., 2015), respectively. The best-fit nucleotide substitution model and the partitioning schemes

for phylogenetic inference analysis were determined for each methodological approach using PartitionFinder v2.1 (Lanfear *et al*., 2016). Each codon position of each gene was provided as the starting subset for the analyses. The optimal nucleotide substitution model and the nucleotide partitioning scheme for the Bayesian phylogenetic inference (BI) were determined by setting branch lengths as 'unlinked', model as MrBayes, model selection under the Akaike information criterion corrected (AICc) and search method as the 'greedy' algorithm. While, the optimal nucleotide substitution model and the nucleotide partitioning scheme for the maximum likelihood analysis were determined by setting branch lengths as 'linked', model selection under the AICc and search method as the 'greedy' algorithm (Lanfear *et al*., 2012).

Maximum likelihood analysis was conducted in IQ-TREE with 1,000 ultrafast bootstrap (BP) iterations (Minh *et al*., 2013), implementing the partitioning scheme obtained from PartitionFinder 2. BI was carried out in MrBayes v3.2.7 using Markov Chain Monte Carlo (MCMC) randomization in three parallel runs of four chains (3 heated and 1 cold) of 20 million generations with a sampling frequency of 200 and a diagnostic frequency of 5000. Convergence of the three parallel runs was assessed using Tracer v1.7.2, and the first 25% of trees were discarded as burn-in. BI statistical support for respective clades was evaluated as Bayesian posterior probability values (PP) (Huelsenbeck *et al*., 2001) using the trees remaining after the burn-in. Posterior probability (PP) values of 0.95 above were considered as an indication for strong support (Mulcahy *et al*., 2011; Wilcox, 2002). FigTree software (V1.4.4) was used to visualize the trees resulting from the BI and ML (*http://tree.bio.ed.ac.uk/software/figtree/)*. Intraspecific genetic distances between each pair of individual taxa within the *E. carinatus* clade and interspecific genetic distances between each pair of *Echis* species were calculated using Mega 11 (Tamura *et al*., 2021) based on uncorrected p-distances parameters.

Genetic diversity and haplotype network

The genetic diversity and demographic history of population of *E. carinatus,* were explored by estimating the number of haplotypes (h), polymorphic sites (S), parsimony informative sites (P), Nucleotide diversity (π), haplotype diversity (Hd), Tajima's D (Tajima, 1989), and Fu and Li's F (Fu and Li, 1993) neutrality tests using DnaSP v. 6.12 (Rozas *et al*., 2017). The haplotype network for *Cytb* and *NADH4* of the *E. carinatus* clade were constructed using PopART v1.7 (Leigh and Bryant, 2015) through a median-joining network (Bandelt *et al*., 1999). The sequences generated by the current study and the sequences obtained from the GenBank (Table 1) were used for this analysis.

Divergence-time estimation

The combined mtDNA dataset was used in BEAST 2 (Bouckaert *et al*., 2014) to estimate the divergence timings among major lineages of *Echis.* The divergence-timing analyses were carried out separately on both the concatenated dataset of 120 taxa and condensed dataset of 24 taxa, representing unique haplotypes. Each lineage was represented by a selected individual referred to as a Molecular Operational Taxonomic Unit (MOTU) (Blaxter *et al*., 2005)). The optimal substitution model for each gene subset was determined using PartitionFinder 2. The model selection was based on the 'Akaike information criterion corrected (AICc)' using 'unlinked' branched length, and 'Beast' with 'greedy' schemes search. The pairwise measures of divergence, assuming a Poisson distribution for the accumulation rate of substitutions, indicate that the substitution rate of Cytb and ND4 in Viperidae is 1.4% my^{-1} (with 95% confidence limits ranging between 1.09-1.77% my^{-1}). Therefore, the mean substitution rate per lineage per site for *Cytb* was calculated as 0.7% my^{-1}. To calibrate the *Cytb* clock rate, the average Viperidae *Cytb* substitution rate of 0.007 substitutions per site per million years was employed (Ursenbacher *et al*., 2006; Wüster *et al*., 2002). A Yule pure-birth model and a relaxed clock under lognormal distribution were used as the tree and clock prior, respectively. The substitution rate for other markers was estimated relative to *Cytb*. Two independent runs, each consisting of 20 million generations, were conducted with a sampling interval of 5000 generations for the Markov Chain Monte Carlo (MCMC) method. Convergence of the runs and an effective sample size (ESS) greater than 200 for the combined run were assessed using Tracer. The post-run diagnostic parameters of Tracer v1.7.2 revealed significantly high effective sample sizes (ESS). The initial 10% of generations were discarded as burn-in (Pook *et al*., 2009), and the LogCombiner tool was used to combine the two runs. Finally, maximum clade credibility (MCC) tree was constructed from the posterior sample of trees using TreeAnnotator and visualized with FigTree v1.4.4.

Table 1: Details of the samples considered for construction of haplotype network in PopART v1.7, including localities, voucher reference, and GeneBank accession numbers.

Species	Sample/Isolate	Locality	CYTB	Haplotype	NADH4	Haplotype
E. c. sochureki	DSMZ JR	Jaisalmer, Rajasthan, India	GQ359434	H13	GQ359522	H12
E. c. carinatus	DSMZ TCTN	Tuticorin, Tamil Nadu, India	GQ359435	H1	GQ359523	H5
E. c. carinatus	DSMZ RM	Ratnagiri, Maharashtra, India	GQ359439	H18	GQ359527	H11
E. c. multisquamatus	AJ Coll. Göran Nilson.,	Turkmenistan (cm1)	AJ275702	H20	-	
E. c. sochureki	DSMZ 2	Pakistan	GQ359441	H15	GQ359528	H11
E. c. carinatus	WW 596	Chennai, Tamil Nadu, India	GQ359433	H10	GQ359521	H6
E. c. sochureki	WW 1612	Sharjah, UAE (cs1)	GQ359436/		GQ359524/	H9
E. c. sochureki	WW 1613	Sharjah, UAE (cs1)	GQ359437/	H12	GQ359525/	H8
E. c. sochureki	WW 1627	Pakistan	GQ359440/	H16	EU624223/	H10
E. c. sochureki	WW 1628	Pakistan	GQ359438/	H17	GQ359526/	H7
E. c. sochureki	WW 1668	Al Wasit, Sharjah, UAE (cs1)	EU852295/	H12	EU852301/	H8
E. carinatus	V31	Tamil Nadu, Vadanemmeli	MG995822.1	H11	MG995837.1	H5
E. carinatus	V6	India: Odisha, Talcher	MG995811.1	H19	MG995831.1	H11
E. carinatus	isolate 5117	Iraq	KX233705.1	H22	-	
E. carinatus	isolate 5118	Iraq	KX233707.1	H23	-	
E. carinatus	isolate 5119	Iraq	KX233706.1	H21	-	
E. carinatus	isolate 5120	Iraq	KX233708.1	H21	-	
E. carinatus	isolate 5121	Iraq	KX233712.1	H23	-	
E. carinatus	isolate 5122	Iraq	KX233709.1	H24	-	
E. carinatus	isolate 5123	Iraq	KX233710.1	H21	-	
E. carinatus	isolate 5308	Iraq	KX233719.1	H25	-	
E. carinatus	Isolate 5309	Iraq	KX233718.1	H23	-	
E. carinatus	Isolate 5310	Iraq	KX233717.1	H28	-	
E. carinatus	isolate 5311	Iraq	KX233716.1	H27	-	
E. carinatus	isolate 5312	Iraq	KX233715.1	H26	-	
E. carinatus	SL001	Jaffna,Ariyala	OP739076	H9	OP739088	H1
E. carinatus	SL002	Kilnochchi,Pooneryn	OP739077	H6	OP739089	H2
E. carinatus	SL003	Delft Island	OP739078	H4	OP739090	H3
E. carinatus	SL004	Kilinochchi,Veravil	OP739079	H7	OP739091	H2
E. carinatus	SL005	Kilinochchi,Palai	OP739080	H3	OP739092	H4
E. carinatus	SL006	Kilinochchi,Visvamadu	OP739081	H8	OP739093	H2
E. carinatus	SL007	Kilinochci,Iyakkachchi	OP739082	H2	OP739094	H4
E. carinatus	SL008	Jaffna,Analaithivu	OP739083	H4	OP739095	H3
E. carinatus	SL009	Jaffna,Passaiyoor	OP739084	H5	OP739096	H4
E. carinatus	SL010	Kilinochchi,Malayalapuram	OP739085	H5	OP739097	H4
E. carinatus	SL011	Jaffna,Allaipitty	OP739086	H4	OP739098	H3
E. carinatus	SL012	Jaffna,Kaithady	OP739087	H3	OP739099	H4

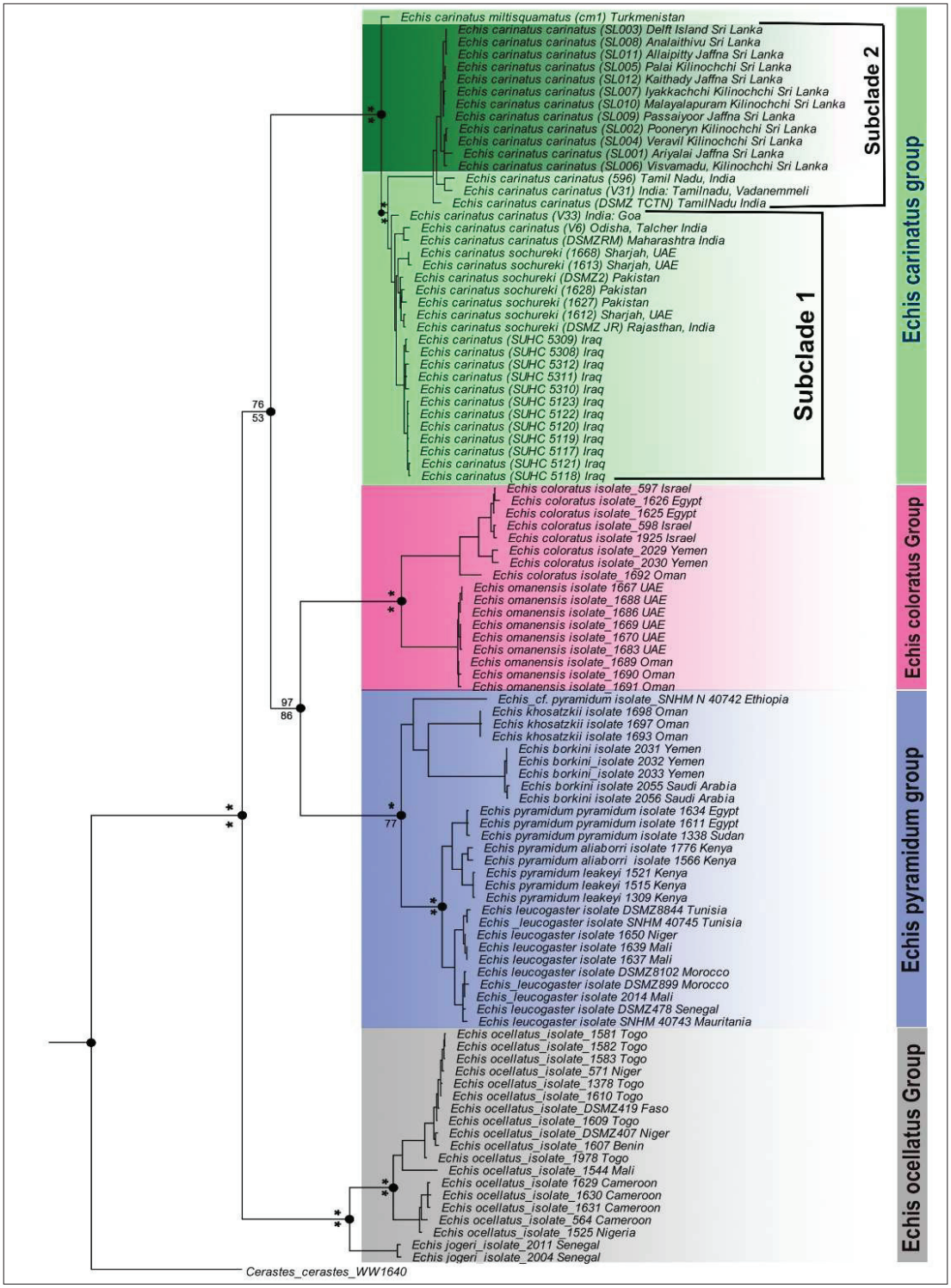

Figure 1: Molecular phylogenetic relationships of genus *Echis*, based on Bayesian inference of the concatenated sequence alignment of the *Cytb + NADH4 +16S + 12S* (2280 bp) mitochondrial-gene markers. Asterisks (*) above and below nodes indicate Bayesian posterior probabilities (BPP) ≥ 0.95 and maximum likelihood ultrafast bootstrap values, respectively. Values below 60% for both Bayesian posterior probabilities and maximum likelihood ultrafast bootstrap are not displayed. The scale bar corresponds to the number of substitutions per site. The numbers in parentheses correspond to the sample isolate numbers listed in Supplementary Table S1.

RESULTS AND DISCUSSION

Saw-scaled vipers are considered as one of the most dangerous snakes in the world and kill more people than any other venomous snakes in their vicinity (Einterz and Bates, 2003; Pitman, 1972; Warrel *et al*., 1977). However, the taxonomic classification of genus *Echis* has a long history of controversy and confusions (Escoriza *et al*., 2010; Lenk *et al*., 2001; Rhadi *et al*., 2015). Based on the morphological analysis, there were many species and sub species reported in the past, under this genus (Babocsay, 2003, 2004; Cherlin, 1990). Among them, a wide range of morphological variation was reported in *E. carinatus* clade (Arnold *et al*., 2009). Although there were five subspecies reported under the *E. carinatus* clade, the subsequent molecular phylogenetic studies have focused primarily on three subspecies. Two sub species namely *E. c. astolae* and *E. c. sinhaleyus* (Deraniyagala, 1951) were not incorporated in any of these studies due to the unavailability of specimens from the Astolae Island in Pakistan and Sri Lanka (Pook *et al*., 2009). This phylogenetic analysis primarily relied on the 2280 bp of concatenated *mt*-DNA sequence alignment (*Cytb*: 790 bp; *NADH4*: 645 bp; 12S: 364 bp; 16S: 481 bp) from 102 individual DNA sequences. The final alignment of mitochondrial protein-coding genes was free of indels, frameshifts or non-sense codons. The optimal nucleotide substitution models for each partition, as determined by PartitionFinder 2, are presented in Supplementary Table S3.

The ML (Supplementary Figure S2) and BI (Figure 1) analysis of this molecular phylogenetic analysis retrieved mostly concordant trees with similar topologies. However, the topological differences, which were observed among the trees belong to individual mitochondrial (*Cytb* [Supplementary Figure S3]*, NADH4* [Supplementary Figure S4]) and concatenated (*Cytb+NADH4+16S+12S* [Figure 1]) datasets. Discrepancies between the individual data sets and the concatenated data set are highlighted where necessary. The main emphasis of these results lies on the concatenated data set, which exhibited the well-resolved tree, displaying high node support in both the BI and ML methods. Phylogenetic analysis retrieved four main monophyletic clades within genus *Echis,* with strong node support. The main four clades correspond to the *E. ocellatus, E. carinatus, E. coloratus,* and *E. pyramidum* (Figure 1). For convenient reference, aforementioned clades are introduced as (a) 'ocellatus clade', (b) 'carinatus clade', (c) 'coloratus clade', and (d) 'pyramidum clade'. The ocellatus clade was recovered as the sister group to the carinatus + coloratus + pyramidum group with strong node support (PP = 1.00, BP = 100) in both BI tree and ML tree for concatenated dataset (Figure 1 and Supplementary Figure S1). The pyramidum clade is recovered as the sister group to rest of the three groups with strong node support (PP=1.00) in BI tree for *Cytb* (Supplementary Figure S3). However, the BI tree for *NADH4* (Supplementary Figure S4) retrieved carinatus clade as the sister group for rest of the three clades with high node support (PP = 1.00).

The carinatus clade is recovered as the sister group to the remaining monophyletic clade of coloratus + pyramidum group, with mixed and weak node support (PP = 0.76, BP = 53, Figure 1) implying the unresolved evolutionary relationship. The BI for *Cytb* also recovered an unresolved sister group relationship between carinatus clade and coloratus clade, with weak node support (PP= 0.72, Supplementary Figure S3). The intra sister-group relationships of carinatus clade are not congruent among phylogenetic analyses. The *E. c. multisquamatus* is recovered as the sister group to the remaining species in the carinatus clade with high node support (PP = 1.00, BP = 100, Figure 1), in the BI and ML for *Cytb + NADH4 + 16S + 12S*, while the BI for *Cytb* did not recover *E. c. multisquamatus* as the sister group to the remaining species (Supplementary Figure S3) of the carinatus clade. The monophyletic clade comprising both Sri Lankan and Tamil Nadu populations of *E. c. carinatus* is recognized as the sister group to remaining terminal taxa of *E. carinatus, E. c. carinatus, and E. c. sochureki* (PP = 1.00, BP = 100). But the sub species *E. c. carinatus* is recovered as a paraphyletic group within the carinatus clade among all the phylogenetic analysis.

The pyramidum and coloratus clades were recovered as sister groups to each other with strong node support in both BI for *Cytb + NADH4 + 16S + 12S* (PP = 1.00, Figure 1) and BI for *NADH4* (PP = 0.97, Supplementary Figure S4). However, weak node support (BP = 86, Figure 1) observed in ML for *Cytb + NADH4 + 16S + 12S* suggests the unresolved evolutionary relationship. In contrast to that, coloratus and carinatus are recognized as sister groups for each other with unresolved sister group relationships due to the weak node support in BI for *Cytb* (PP = 0.73, Supplementary Figure S3).

The genetic diversity data for *E. carinatus* are shown in Supplementary Table S4. According to analysis conducted to examine the genetic diversity and phylogeographic structure for *Echis carinatus*, the numbers of haplotypes (h) between Sri Lanka and the populations of Maharashtra + Odisha + Rajasthan + Pakistan + UAE were similar for *Cytb* gene, while numbers of haplotypes between Sri Lanka + Tamil Nadu and Maharashtra + Odisha +

Rajasthan + Pakistan + UAE populations were similar for *NADH4* gene. The number of parsimony informative sites was similar between Sri Lankan and Maharashtra + Odisha + Rajasthan + Pakistan + UAE, while haplotype diversity between Iraq and Pakistan + UAE populations was similar for *NADH4* gene. None of the neutrality tests were significant in any of the populations except the Fu and Li's F test which retrieved a negative significant value for Cytb in the Pakistan+UAE population.

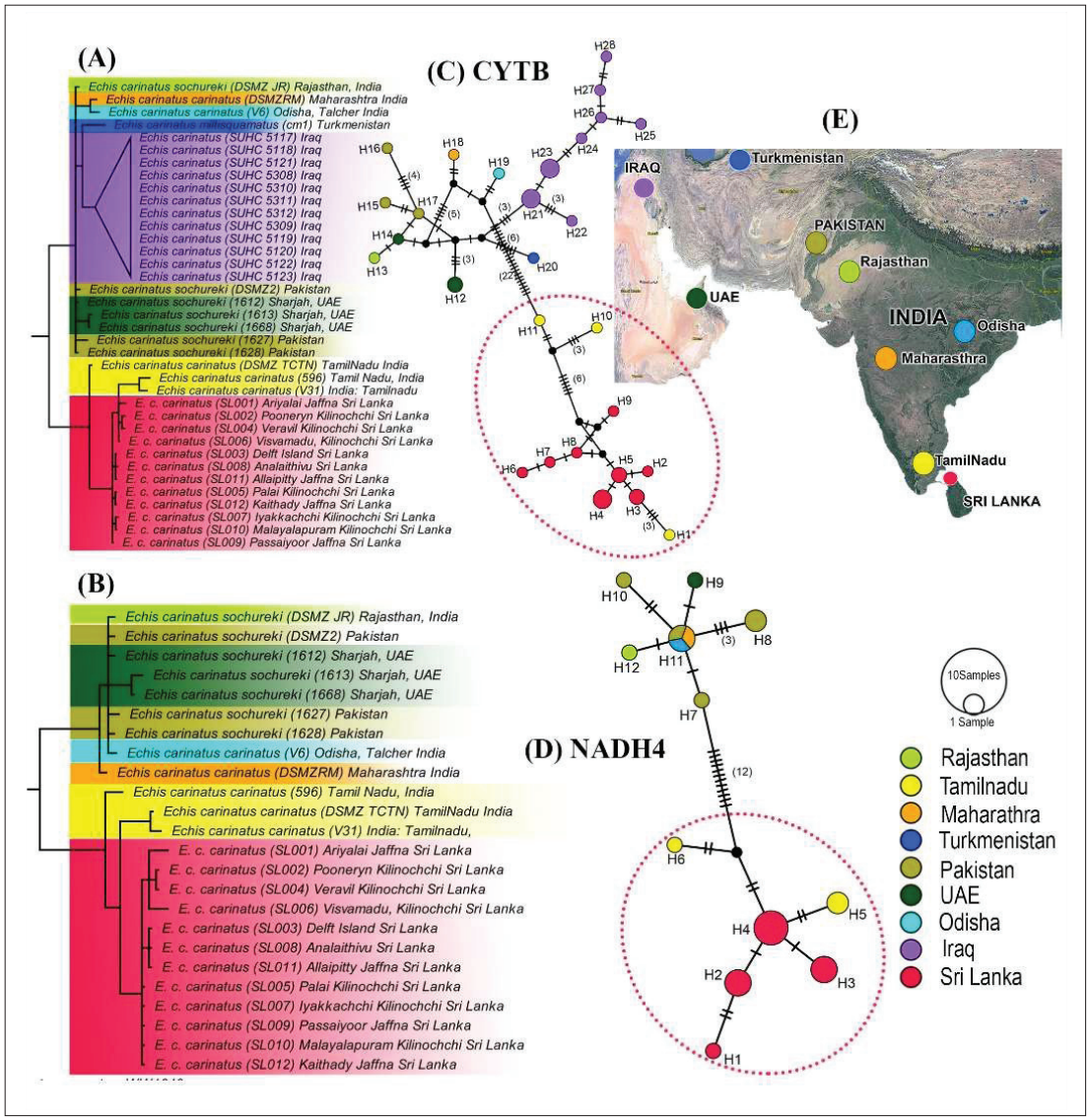

Figure 2: The haplotype network using PopART v1.7, for *Cytb* and *NADH4* mitochondrial-gene markers of *Echis carinatus*. (A) *Echis carinatus* clade of Bayesian inference tree for *Cytb* (790 bp) gene marker. (B) *Echis carinatus* clade of Bayesian inference tree for *NADH4 (*645 bp) gene marker. (C) Median joining haplotype network for 790bp fragment of *Cytb* gene marker. (D) Median joining haplotype network for 645 bp fragment of *NADH4* gene marker. (E) Map showing the sample localities. The areas of the circles proportionally represent the number of individuals sharing a specific haplotype, while the number of mutational steps > 3 is shown in parentheses. Hypothetical nodes are represented by black circles. The colors in the legend correspond to different sample localities. Haplotypes depicted by red circles represent subclade 2 in Figure 1, while the remaining haplotypes represent subclade 1 in Figure 1.

The median-joining network splits *E. carinatus* populations into two phylogeogarphic structures through 22 mutational steps in the *Cytb* haplotype network (Figure 2C) and 12 mutational steps in the *NADH4* haplotype network (Figure 2D). The separation of these two phylogeographic structures corresponds to the subclade 1 and subclade 2, in Figure 1. The Sri Lankan and Tamil Nadu populations of *E. c. carinatus* are confined to the corresponding phylogeographic structure of subclade 2 (Figure 1, Figure 2C and 2D), while the remaining part corresponds to the subclade 1, which comprises *E. carinatus, E. c. carinatus, E. c. sochureki* from various geographical regions (Figure 1, Figure 2C and 2D). In the median-joining network for *NADH4*, a single haplotype is shared among each of the samples from Rajasthan (DSMZ JR), Odisha (V6), and Pakistan (DSMZ 2) (Figure 2). However, in both the median-joining network for *Cytb* and *NADH4*, no haplotypes are shared between the Sri Lankan and Tamil Nadu populations of *E. c. carinatus* (Figure 2C and 2D).

Uncorrected pairwise genetic distances (p-distance) computed for the *Cytb* and *NADH4* genes for the species of *Echis* are presented in (Table 2). Inter-specific p-distances among ten different species of *Echis* are given in Table 2A. The minimum interspecific p-distance for the *Cytb* was 2 – 6, while for *NADH4* it was 8 – 10. The maximum interspecific p-distance was observed for *Cytb* and *NADH4* were 16 – 18 and 25 – 27 respectively. The intra-specific p-distance within *E. carinatus* was estimated using the sequences of eight different populations from various geographical locations (Table 2B). The maximum intra-specific p-distances were 5.2 - 6.4 for *Cytb* and 6.2 – 7.3 for *NADH4*, while the minimum values were 0.4 – 1.4 for *Cytb* and 0.3 for *NADH4*. The p-distances for Sri Lankan population of *E. carinatus* showed genetically distant relationship to the UAE population (5.2 – 5.6 for *Cytb* and 5.3 – 7.0 for *NADH4*) and genetically proximal affinity to the Tamil Nadu population (1.5 – 2.3 for *Cytb* and 1.8 – 2.9 for *NADH4*).

Table 2: Inter-specific and intra-specific uncorrected pairwise genetic distances for Cytb and NADH4 gene markers in *Echis* species.

(A) Inter-specific uncorrected pairwise genetic distances (%)

	Cytb *NADH4*	1								
1	*E. ocellatus*	- -	2							
2	*E. pyramidum*	15 - 17 25 - 27	- -	3						
3	*E. leucogaster*	14 - 17 24 - 28	3 - 10 4 - 27	- -	4					
4	*E. omanensis*	16 - 17 21 - 25	14 - 16 18 - 24	15 - 16 18 - 20	- -	5				
5	*E. khosatzkii*	14 - 16 24 - 27	9 10 - 27	9 - 10 11 - 13	14 - 14 19 - 21	- -	6			
6	*E. jogeri*	8 - 10 11 - 12	15 - 17 10 - 24	14 - 15 23 - 25	17 23	16 23 - 24	- -	7		
7	*E. coloratus*	16 - 17 19 - 24	15 - 17 17 - 23	15 - 16 18 - 20	7 - 9 8 - 10	14 - 15 18 - 21	16 - 17 20 - 22	- -	8	
8	*E. borkini*	15 - 18 24 - 26	10 - 16 10 - 25	10 - 15 10 - 11	14 - 16 19 - 20	9 - 15 10 - 11	16 - 18 22 - 23	15 - 17 17	- -	9
9	*E. carinatus*	15 - 18 20 - 25	14 - 17 15 - 24	14 - 17 18 - 25	14 - 17 15 - 19	15 - 16 19 - 23	15 - 16 18 - 21	14 - 17 12 - 16	19 18 - 21	- -
10	*E. multisquamatus*	16 - 18 -	16 - 16 -	16 -	16 -	15 -	15 - 16 -	15 - 17 -	2 - 18 -	2 - 6 -

(B) Intra-specific uncorrected pairwise genetic distances (%).

	Cytb *NADH4*	1						
1	UAE	- -	2					
2	Pakistan	0.5 - 1.9 0.3 - 1.5	- -	3				
3	Iraq	1.5 - 2.4 -	1.7 - 2.2 -	- -	4			
4	Rajasthan	0.4 - 1.4 0.3 - 1.3	0.6 - 1.3 0.3 - 0.5	1.5 - 2.4 -	- -	5		
5	Odisha	1.8 - 2.3 0.5 - 1.5	1.8 - 2.3 0.5 - 0.6	2.5 - 3.2 -	1.9 0.5	- -	6	
6	Maharastra	1.6 - 2.2 0.5 - 1.3	1.6 - 2.3 0.3 - 0.8	2.2 - 3.0 -	1.8 0.5	1.0 0.3	- -	7
7	Tamil Nadu	4.2 - 5.5 5.0 - 7.0	1.8 - 2.3 4.7 - 6.4	5.1 - 6.1 -	3.4 - 4.7 5.0 - 6.3	3.8 - 4.1 4.8 - 6.1	4.1 - 4.7 6.2 - 7.3	- -
8	Sri Lanka	5.2 - 5.6 5.3 - 7.0	3.5 - 5.1 5.1 - 6.2	5.2 - 6.4 -	4.7 - 5.1 5.3 - 5.9	3.8 - 4.7 5.1 - 5.9	4.8 - 5.2 6.2 - 7.3	1.5 - 2.3 1.8 - 2.9

The divergence-timing analysis for the concatenated dataset (*Cytb + NADH4 + 16S + 12S)* of 102-taxa, using a *Cytb* substitution rate in BEAST yielded congruent tree topologies (Supplementary Figure S5) with those from the BEAST analysis of 24-taxa concatenated dataset (*Cytb + NADH4 + 16S + 12S,* is shown in Figure 3). The estimated ages for the selected nodes in both analyses were remarkably similar. The phylogenetic relationships among *Echis* species, as well as the resulting topology, in the BEAST analysis predominantly consistent with those obtained from the BI and ML analyses of the same dataset. Estimated divergence timing based on the 24-taxa dataset is presented here. The basal split between *Echis* and *Cerastes cerastes* was dated at 21.2 million years ago (Ma), in the early Miocene (95% highest posterior denisity (HPD): 18.1-24.9 Ma), while the split between ocellatus clade and its sister group was dated at in the early Miocene 18.2 (95% HPD: 16.2-20.2) Ma, (Figure 3). The divergence between carinatus clade and its sister group was dated at 16.4 in the mid Miocene (96% HPD: 14.6-18.3) Ma, while the split between pyramidum and coloratus clades was dated at 14.4 Ma in the mid Miocene. The first split within the ocellatus clade, pyramidum clade and coloratus clade was estimated to have occurred 7.6 Ma (95% HPD: 5.6-8.6 Ma), 8.2 Ma (95% HPD: 7.2-9.5 Ma), and 6.8 Ma (95% HPD: 5.4-8.1 Ma), respectively in the late Miocene (Figure 3). The subsequent diversification of *E. carinatus* was estimated to have occurred 4.2 Ma (95% HPD: 3.3-5.1 Ma) in the early Pliocene. The divergence between Sri Lankan and South Indian (Tamil Nadu) population of *E. carinatus* was dated at 1.7 Ma (95% HPD: 1.2-2.3 Ma), in the mid Pleistocene epoch (Figure 3). However, a discrepancy in the absolute values of estimated divergence times was observed in the current study compared to the study by Pook *et al*. (2009), particularly towards the root of the tree. Pook *et al*. (2009) estimated cladogenesis within the *Echis* using fossil calibration information with secondary calibration points. The divergence-timing estimates in the current study were primarily based on the *Cytb* substitution rate for Viperidae and are younger than those estimated by Pook *et al*. (2009). The primary focus of the precent study was to understand the sequence of divergence of the Sri Lankan population of *E. c. carinatus*

from their most recent common ancestor. Although fossil evidence-based divergence-timing estimates result in older divergence estimates towards the root of the tree, current study retrieved approximately similar estimations for *Echis cariantus* lineage compared to the study of Pook *et al*. (2009).

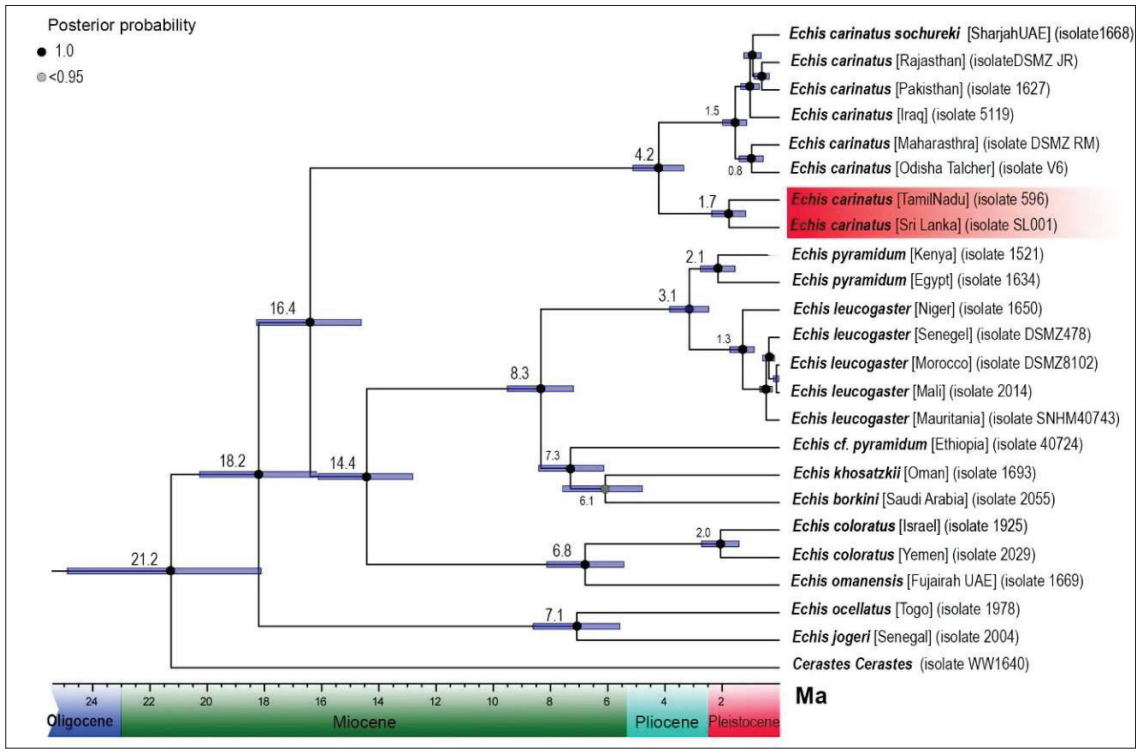

Figure 3: Bayesian divergence-times estimated for *Echis* (saw-scaled viper) for 24-taxon dataset using *Cytb* substitution rate in Beast v2.6.7 for the concatenated sequence alignment of the *Cytb+NADH4+16S+12S* (2280bp) mitochondrial-gene markers. The node values represent the mean ages for divergence estimates and node bars denote 95% highest probability density for divergence-time estimates.

Mitochondrial markers are maternally inherited and typically lack recombination. They possess a faster substitution rate than nuclear markers, making them valuable for revealing genetic variations within closely related species and populations. Therefore, these markers find extensive application in studies focused on the geographic distribution of evolutionary lineages, known as phylogeography (Pethiyagoda & Sudasinghe, 2021). Considering the potential of using mitochondrial markers to determine the genetic structure, Pook *et al*. (2009) conducted the first comprehensive molecular investigation based on four mitochondrial gene sequences and recognized all the species and subspecies of the genus *Echis* under four distinct clades. The subsequent molecular phylogenetic studies have also identified the same four major clades, despite some variation among the sister group relationships of these four clades (Arnold *et al*., 2009; Barlow *et al*., 2009; Escoriza *et al*., 2010; Rhadi *et al*., 2016; Ashraf *et al*., 2020). Aligned with the prior reporting, the present phylogenetic study also retrieved the genus *Echis* into four distinct clades and clustering all the Sri Lankan samples under the monophyletic clade of *E. carinatus*. Given the enhanced resolution achievable through the inclusion of a sufficient number of phylogenetic informative sites supporting a consistent tree structure (Rokas and Carroll, 2005; Heath *et al*., 2008) in addition to the samples incorporated by Pook *et al*. (2009), new samples from distinct geographical areas of Iraq, Tamil Nadu, Odisha, and Goa, with the twelve samples from Sri Lanka, were integrated to increase the resolution of the *E. carinatus* clade in the present study.

However, discrepancies can be observed between traditional subspecies classification and resulted phylogenetic tree. According to the traditional

classification of *E. carinatus*, Indian population of saw-scaled viper belongs to the subspecies *E. c. carinatus,* except the Rajasthan population which belongs to the *E. c. sochureki*. Our phylogenetic analysis has resolved this misclassification, exclusively by introducing new sequences to the carinatus clade which was not incorporated by Pook *et al*. (2009). The resulted phylogenetic analysis retrieved two distinct monophyletic subclades for the carinatus clade, implying that the clade of *E. carinatus* is composed mainly of two sub species namely, *E. c. carinatus* and *E. c. sochureki.* The subclade *E. c. carinatus* comprises Sri Lankan and South Indian (Tamil Nadu) populations, while the subclade *E. c. sochureki* comprises rest of the populations (Iraq, Pakistan, UAE, Rajasthan, Maharashtra, Odisha, and Goa) of *E. carinatus* (Figure 3). The resulted classification based on the phylogenetic tree is also supported by the results obtained from the analysis; uncorrected pairwise genetic distance of *E. carinatus* clade (Supplementary Table S5) and the median-joining network split for *E. carinatus* populations (Figure 2C, D).

Indeed, the clear demarcation in the geographical boundaries of these two sub clades, a very close genetic distance between the Sri Lanka and Tamil Nadu populations within the subclade 2 compared to more northern species (subclade 1), and the divergence time estimation indicating a split of Tamil Nadu and Sri Lankan population during the Pleistocene around 1.7 Ma, strongly support the dispersal of the Sri Lankan saw-scaled viper from South India. Sri Lanka is separated from the southern tip of the Indian mainland by the narrow and shallow Gulf of Mannar and Palk Strait (Chauhan, 2008). The separation of Sri Lanka from the main land of India occurred, as a result of marine transgression event in early Miocene (Cooray, 1991; Sahni and Mitra, 1980). In the Messinian age, the two landmasses were reconnected during the Miocene-lower Pliocene possibly due to the reduced see level in the Messinian age (Aharon *et al*., 1993). This landmass connectivity occurred from time to time in the Quaternary period and the most recent separation occurred during the early Holocene sea level rise between 12000 and 7000 years ago (Gunatilaka, 2000). Based on the fossil evidence, it was assumed that during the past 1 million years, the two lands were one landmass for most of the time. The recurrent connections between these landmasses facilitated biotic exchange between India and Sri Lanka (Biswas and Pawar, 2006). The subsequent complete separation of Sri Lanka from the Indian mainland enabled the island to have a combination of many uniquely endemic species as well as a subset of the Indian biota within its overall fauna (Bossuyt *et al*., 2004).

The divergence time estimated by the molecular dating in the present study also suggests that the subclade 2 including the South Indian (TN) and Sri Lankan *E. carinatus* populations diverged from the Northern *Echis carinatus* population (Subclade1) during Pliocene around 4.2 Ma. Subsequently, the split between Sri Lankan and India occurred around 1.7 Ma during the Pleistocene epoch. Furthermore, it's worth noting that the dry zones in northwestern Sri Lanka and southeastern India share similar arid climates where the *E. c. carinatus* subspecies is found (Pethiyagoda and Sudasinhe, 2021). The closely connected landmass of South India and Northern Sri Lanka for longer period which experience the same tropical humid and dry climate may facilitate the dispersal of this subspecies into the Northern Sri Lanka and eventually to the west and east coastal directions, which is evident by the relatively high number of saw-scaled viper bite cases reported from these areas (Kularatne *et al*., 2011; Pirasath *et al*., 2021; Sivansuthan, 2011). Previously, Sri Lankan saw-scaled viper was designated as a distinct subspecies endemic to Sri Lanka based on phenotypic characters by Deraniyagala (1955). However, the present study on the genotypic characters shows shallow genetic divergence between the *Echis* populations of Sri Lanka and Southern India along with the observations from phylogenetic analysis, uncorrected p-distance analysis, haplotype network, and divergence-timing analysis. Therefore, the Sri Lankan saw-scaled viper population can be synonymized with the *E. c. carinatus* of South Indian (Tamil Nadu) population.

On the other hand, based on the clinical observations, Sri Lankan saw-scaled viper population was hypothesized to be a different subspecies of *E. carinatus* (Kularatne *et al*., 2011; Peranantharajah *et al*., 2012). But the present study and the clinical observations of the envenomed victims reported from the different geographical locations of India strongly suggest that, *E. c. carinatus* distributed in Sri Lanka and the Southern India is less toxic when compared with *E. c. sochureki* distributed in North India, which leads to more severe form of hemo-nephrotoxic envenomation (Chauhan and Thakur, 2016). Kochar *et al*. (2007) has also described the ineffectiveness of the polyvalent antivenom produced in Southern India for neutralizing the envenoming effect of saw-scaled viper bites of Northern Indian population suggesting a genetically distant relationship between Southern Indian and Northern Indian populations of saw-scaled viper. Therefore, our phylogeographic study infers that the Sri Lankan population of *E. carinatus* has a genetically proximal affinity to the South Indian population, suggesting that the Sri Lankan saw-scaled viper population dispersed from South India during the Plio-Pleistocene dispersal event.

CONCLUSION

In conclusion we suggest that the *E. carinatus* clade comprises two distinct subspecies; *E. c. carinatus* and *E. c. sochureki*. The Sri Lankan and South Indian populations belong to the subspecies *E. c. carinatus*, whereas *E. c. sochureki* comprises the remaining populations of *E. carinatus* distributed across northern, western, and eastern India (Rajasthan, Maharashtra, Odisha, and Goa), as well as in Pakistan, Sharjah, and Iraq.

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Supplementary Materials

Supplementary Table S1: Primers used in the amplification of mitochondrial gene sequences (Pook *et al.* 2009)

Primer name	Position*	Sequence 5'- 3'
CYTB		
GludgMod2 EchR	14902	CTGAAAAACCACCGTTGT
	15787	GCTCCDCCBAGTTTRTT
ND4		
NADH4	11677	CACCTATGACTACCAAAAGCTCATGTAGAAGC
HIS12763V	12594	TTCTATCACTTGGATTTGCACCA
12S		
L1091	478	AAACTGGGATTAGATACCCCACTAT
H1557	980	GTACACTTACCTTGTTACGACTT
16S		
L2510	1828	CGCCTGTTTATCAAAAACAT
H3059	2376	CCGGTCTGAACTCAGATCACGT

* The position refers to the position in the mitochondrial genome of *Dinoden semicarinatus* (Kumazawa *et al.* 1998) at which the 5' end of the primer aligns.

Supplementary Table S2: The best-fit nucleotide substation model and the partitioning schemes used for the phylogenetic inference analysis as determined by PartitionFinder 2

Analysis	Number of sequences	Number of partitions (Subsets)	Partitions	The best model
Bayesian inference: MrBayes		1	Cytb cp1, Cytb cp2, NADH4 cp2, NADH4 cp2, 16S, 12S	GTR+I+G
		2	Cytb cp3, NADH4 cp1	GTR+I+G
Maximum likelihood inference: IQ-TREE		1	Cytb cp1, NADH4 cp2, 12S	TVM+I+G
		2	Cytb cp2	HKY+I
		3	Cytb cp3	TIM+I+G
		4	NADH4 cp1	GTR+I+G
		5	NADH4 cp3	TRN+I+G
		6	16S	GTR+I+G
Divergence timing analysis based on Cytb substitution rate: BEAST2		1	Cytb, NADH4	GTR+I+G+X
		2	16S, 12S	GTR+I+G+X

cp, codon position

Supplementary Table S3: Genetic diversity of *Echis carinatus* for *Cytb* and *NADH4* mitochondrial gene markers. Number of sequences (N), number of haplotypes (h), polymorphic sites (S), parsimony-informative sites (P), nucleotide diversity (π), haplotype diversity (Hd).). None of the test results of Tajma's D test and Fu/ Li's F test values was significant.

Cytb NADH4	N	h	S	P	π	Hd	Tajima's D test	Fu and Li's F test
Sri Lanka	12	08	10	07	0.00476	0.92424	-0.77046	-0.83266
	12	05	07	03	0.00464	0.78788	-1.27046	-1.61307
Sri Lanka + Tamil Nadu	15	11	37	19	0.00888	0.95238	-0.98155	-0.54892
	15	08	38	13	0.01767	0.86667	-1.94618	-2.47595
Maharashtra + Odisha + Rajasthan + Pakistan + UAE	09	08	23	14	0.01201	0.97222	-0.57536	-0.49572
	09	08	10	03	0.00732	0.97222	-0.29572	-0.22291
Pakistan + UAE	06	05	11	09	0.00755	0.93333	-0.09078	-0.02322
	06	06	08	05	0.00878	1.00000	-1.21100	-1.18979
Iraq	12	09	14	05	0.00665	0.93939	-0.64103	-1.17168
	-	-	-	-	-	-	-	-

Supplementary Table S4: Inter-species and intra-species uncorrected pairwise genetic distance for *Cytb* and *NADH4* gene markers in *Echis* species. Minimum and maximum values are highlighted in blue and green colours, respectively.

(a) Inter-specific uncorrected pairwise genetic distances

	Cytb *NADH4*	1	2	3	4	5	6	7	8	9
1	*E. ocellatus*	-								
		-								
2	*E. pyramidum*	0.15-0.17	-							
		0.25-0.27	-							
3	*E. leucogaster*	0.14-0.17	0.03-0.10	-						
		0.24-0.28	0.04-0.27	-						
4	*E. omanensis*	0.16-0.17	0.14-0.16	0.15-0.16	-					
		0.21-0.25	0.18-0.24	0.18-0.20	-					
5	*E. khosatzkii*	0.14-0.16	0.09	0.09-0.10	0.14-0.14	-				
		0.24-0.27	0.10-0.27	0.11-0.13	0.19-0.21	-				
6	*E. jogeri*	0.08-0.10	0.15-0.17	0.14-0.15	0.17	0.16	-			
		0.11-0.12	0.10-0.24	0.23-0.25	0.23	0.23-0.24	-			
7	*E. coloratus*	0.16-0.17	0.15-0.17	0.15-0.16	0.07-0.09	0.14-0.15	0.16-0.17	-		
		0.19-0.24	0.17-0.23	0.18-0.20	0.08-0.10	0.18-0.21	0.20-0.22	-		
8	*E. borkini*	0.15-0.18	0.10-0.16	0.10-0.15	0.14-0.16	0.09-0.15	0.16-0.18	0.15-0.17	-	
		0.24-0.26	0.10-0.25	0.10-0.11	0.19-0.20	0.10-0.11	0.22-0.23	0.17	-	
9	*E. carinatus*	0.15-0.18	0.14-0.17	0.14-0.17	0.14-0.17	0.15-0.16	0.15-0.16	0.14-0.17	0.19	-
		0.20-0.25	0.15-0.24	0.18-0.25	0.15-0.19	0.19-0.23	0.18-0.21	0.12-0.16	0.18-0.21	-
10	*E. multisquamatus*	0.16-0.18	0.16-0.16	0.16	0.16	0.15	0.15-0.16	0.15-0.17	0.02-0.18	0.02-0.06
		-	-	-	-	-	-	-	-	-

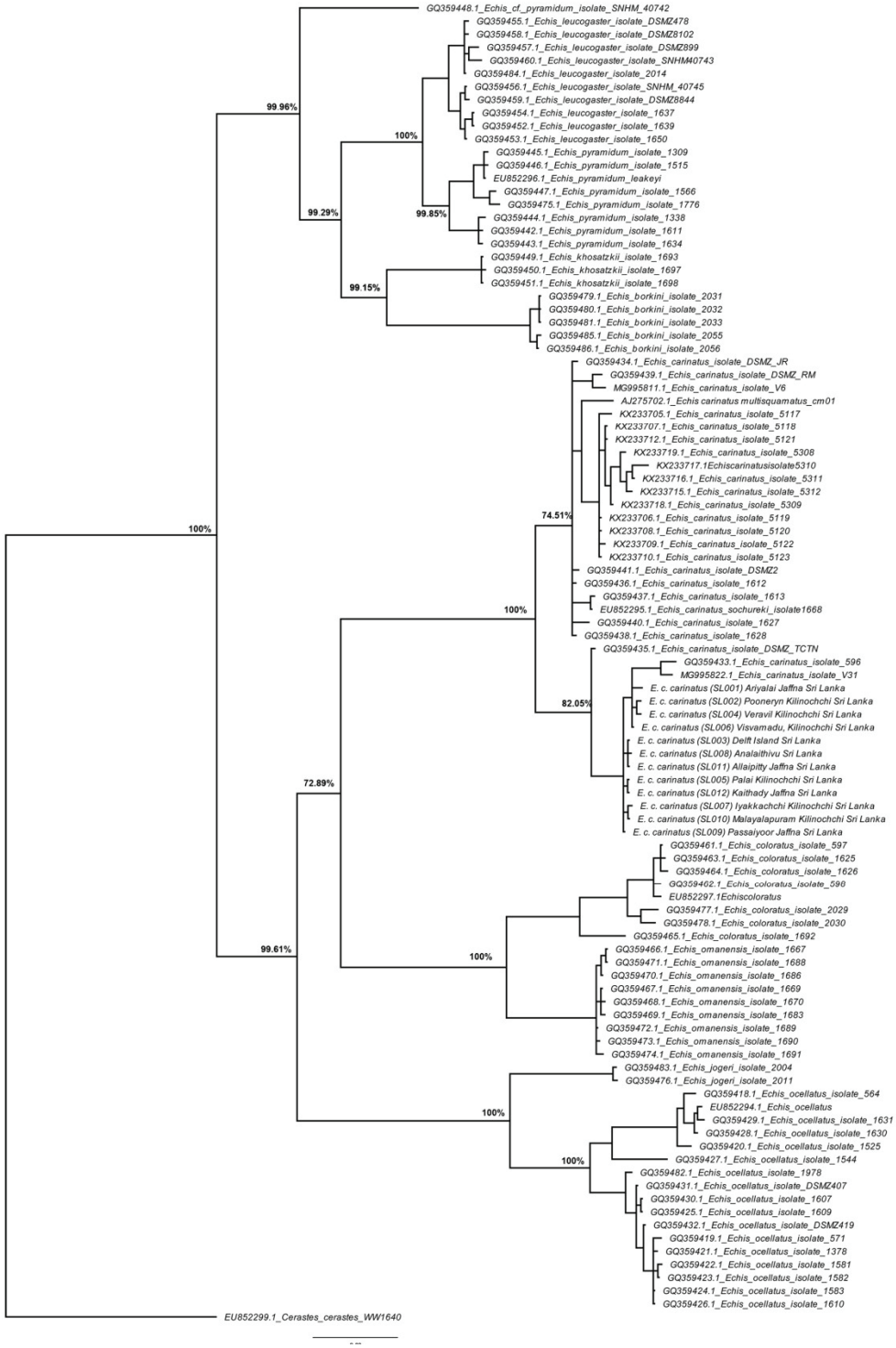
(a) Intra-specific uncorrected pairwise genetic distances.

	Cytb / *NADH4*	1	2	3	4	5	6	7
1	UAE	-						
		-	2					
2	Pakistan	0.005-0.019	-					
		0.003-0.015	-	3				
3	Iraq	0.015-0.024	0.017-0.22	-				
		-	-	-	4			
4	Rajasthan	0.004-0.014	0.006-0.013	0.015-0.024	-			
		0.003-0.013	0.003-0.005	-	-	5		
5	Odisha	0.018-0.023	0.018-0.023	0.025-0.032	0.019	-		
		0.005-0.015	0.005-0.006	-	0.005	-	6	
6	Maharastra	0.016-0.022	0.016-0.023	0.022–0.030	0.018	0.010	-	
		0.005-0.013	0.003-0.008	-	0.005	0.003	-	7
7	Tamil Nadu	0.042-0.055	0.018-0.023	0.051-0.061	0.034-0.047	0.038-0.041	0.041-0.047	-
		0.050-0.070	0.047-0.064	-	0.050-0.063	0.048-0.061	0.062-0.073	-
8	Sri Lanka	0.052-0.056	0.035-0.051	0.052-0.064	0.047-0.051	0.038-0.047	0.048-0.052	0.015-0.023
		0.053-0.070	0.051-0.062	-	0.053-0.059	0.051-0.059	0.062-0.073	0.018-0.029

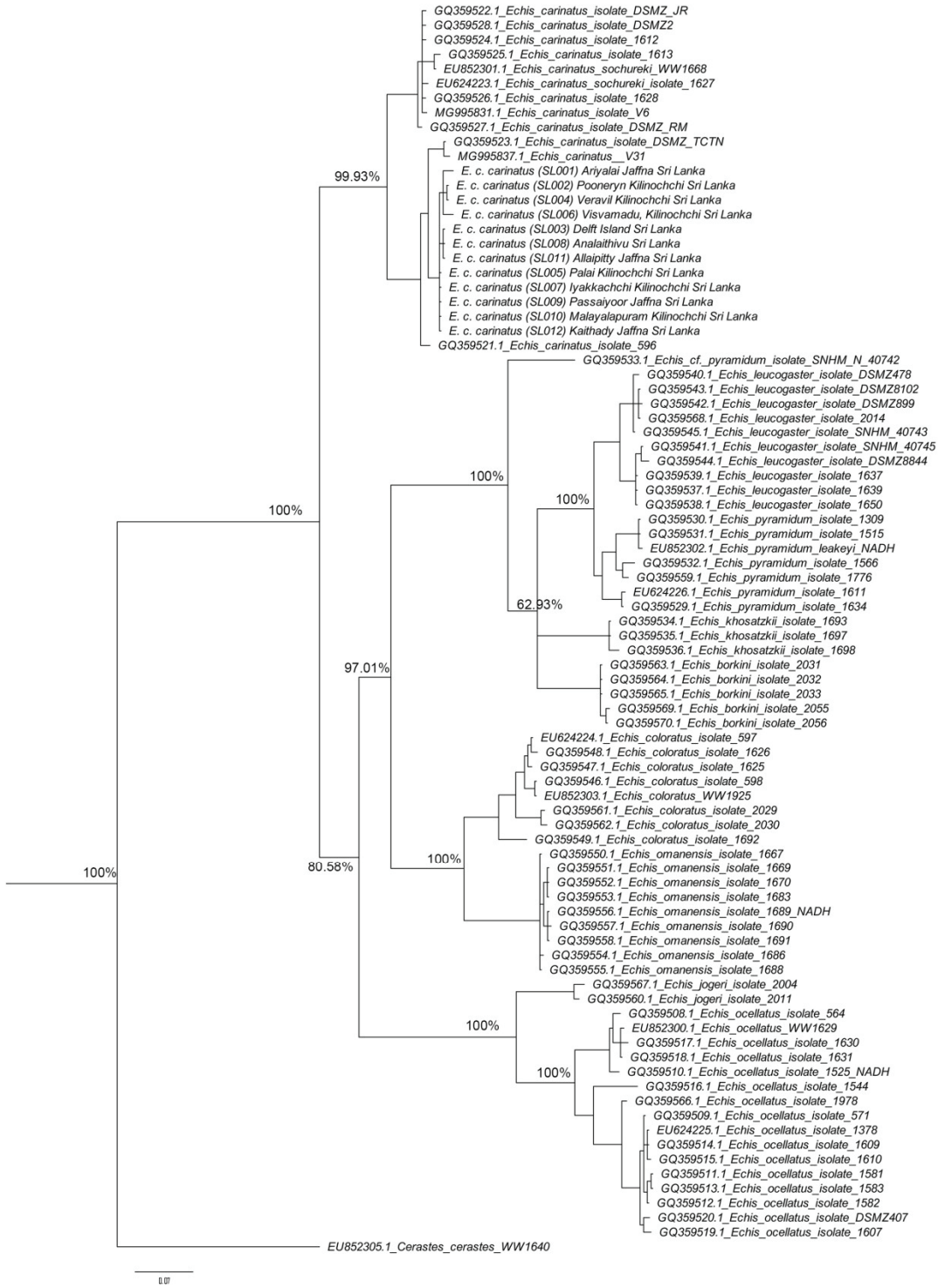
Supplementary Figures

Supplementary Figure S1: Molecular phylogenetic relationships of genus *Echis*, built upon Maximum Likelihood (ML) of the concatenated sequence alignment of the *Cytb + NADH4 +16S + 12S* (2280 bp) mitochondrial-gene markers in IQ-TREE. The node values indicate 100% maximum likelihood ultrafast bootstrap values. Values below 60% maximum likelihood ultrafast bootstrap are not displayed. The scale bar corresponds to the number of genetic changes per site. The numbers in parentheses correspond to the sample isolates numbers listed in Table 1.

Supplementary Figure S2: Molecular phylogenetic relationships of genus *Echis*, built upon Bayesian inference (BI) of the sequence alignment of the *Cytb* (790 bp) mitochondrial-gene markers in MrBayes. The node values indicate 100% Bayesian posterior probabilities values. Values below 60% Bayesian posterior probabilities are not displayed. The scale bar corresponds to the number of genetic changes per site. The numbers in parentheses correspond to the sample isolates numbrs listed in Table 1.



Supplementary Figure S3: Molecular phylogenetic relationships of genus *Echis*, built upon Bayesian inference (BI) of the sequence alignment of the *NADH4* (645 bp) mitochondrial-gene markers in MrBayes. The node values indicate 100% indicate 100% Bayesian posterior probabilities values. Values below 60% Bayesian posterior probabilities are not displayed. The scale bar corresponds to the number of genetic changes per site. The numbers in parentheses correspond to the sample isolates numbrs listed in Table 1.



Supplementary Figure S4: The complete Bayesian divergent-time estimated tree of *Echis* (saw-scaled viper) for 102-taxon dataset, using *Cytb* substitution rate in Beast v2.6.7 for the concatenated sequence alignment of the *Cytb+NADH4+16S+12S* (2280bp) mitochondrial-gene markers. The node values represent the mean ages for divergence estimates and node bars denote 95% highest probability density for divergence-time estimates.