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Molecular Phylogenetics of Saw-scaled Viper (*Echis carinatus*) from Pakistan

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Authors' Contribution
MRA, AN and ENS conceived and designed the project. MRA and PT conducted wet lab experiments. MRA, US, AN analyzed the result through bioinformatics software and tools. MJ helped to draft the manuscript. TY, ASH, MJ helped in discussion of results.

Key words
Saw-scaled viper, Mitochondrial genes, PCR, Phylogenetics, Diversity, Polymorphism

ABSTRACT

Snakes are one of the most dangerous animals with over 40 species of venomous snakes found in Pakistan. One such group of snakes belongs to genus Echis is saw scaled viper (*Ehcis carinatus*). Molecular techniques have made it easy to elucidate phylogenetic relationships and evolutionary histories of different groups of organisms. This study is the first attempt to find the phylogenetic relationship and diversity through mitochondrial genes in saw-scaled viper from Pakistan. Tail tip biopsies of Saw-scaled vipers were used for amplification of mitochondrial genes fragments (ND4, 16S rRNA and 12S rRNA) through Polymerase Chain Reaction (PCR). Nucleotide data was used for DNA polymorphism analyses and homology was measured among different species of genus Echis. Using the concatenated nucleotide data, Maximum likelihood and Bayesian phylogenetic trees were constructed that divided all Echis species into four groups. Saw-scaled viper in this study from Pakistan showed similarity and close relationship with Western India and UAE while showing difference from South Indian. Saw-scaled viper from South India is termed as *Echis carinatus carinatus* while that from Pakistan and Western India is *Echis carinatus sochureki*. More morphological and molecular studies are required to raise both the subspecies to separate species.

INTRODUCTION

Pakistan reports 40,000 snake bites every year resulting in 8200 fatalities (Kasturiratne *et al*., 2008). Of the 40 venomous snakes of Pakistan, cobra, krait and vipers are common (Iliyas, 1997). Pakistan has three families of venomous land snake including Elapidae, viperdae and Crotalidae: Viperdae includes *Echis carinatus sochureki*, *Echis carinatus astolae*, *E. c. multisquamatus*, *E. c. sochureki*. Saw-scaled viper is a member of genus Echis and Family Viperidae. Many phylogenetic studies conducted in previous years divide Echis into 4 groups. *Echis ocellatus*, *Echis pyramidum*, *Echis coloratus* and *Echis carinatus* are the four groups of genus (Arnold *et al*., 2009). There are two subspecies of *Echis carinatus i.e*., *Echis carinatus carinatus* and *Echis carinatus sochureki* (Arnold, 1980; Arnold *et al*., 2009; Pook *et al*., 2009). The best source of phylogenetic information among vertebrates is thought to be mitochondrial DNA. Many of the sequences are used completely while some partially are able to infer phylogenetic relationship among organisms (Macey *et al*., 2000). Mitochondrial genes have no introns which make it easier to amplify them and nuclear genes with introns should have been amplified in parts. Mitochondrial genes have high rate of substitutions but even then, they have some conserved regions that can be amplified through targeted PCR primers (Simon *et al*., 1994; Castresana, 2000).

Snakes have been the part of many phylogenetic studies for over a century, but higher-level relationships are still to be resolved. Many different phylogenies of snakes were generated a few decades ago. Morphological analysis (Lee and Scanlon, 2002a; Tchernov *et al*., 2020) showed a similar description of snake evolution but they have some opposite views about some particular taxa or regions of tree. Some molecular studies (Vidal and Hedges, 2002a; Vidal and Hedges, 2004; Lawson *et al*., 2004) have tried to resolve this issue by generating well supported clades. This study has been designed to infer biodiversity, variation and phylogenetic relationship of Saw-scaled viper from Pakistan.

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MATERIALS AND METHODS

The samples used in this study were tail tip biopsies (n= 25) of saw-scaled viper collected through reptile breeders from different cities of Pakistan. The samples were transferred to 70% ethanol before the DNA extraction. The sampling localities were plotted on the Pakistan map obtained from (https://www.ezilon.com/maps/asia/pakistan-maps.html) as shown in the Figure 1. The sample IDs and coordinates of sampling sites are shown in the Tables I. DNA was extracted through standard organic method (Sambrook and Russel, 2001) and gently dissolved in 10mM Tris (pH=8.0) storing at -20°C for further use. Polymerase Chain Reaction (PCR) primers used in previous studies were used for amplification of mitochondrial ND4 (Arevalo *et al*., 1994), 12S rRNA (Knight and Mindell, 1993), 16S rRNA (Vences *et al*., 2005) genes. Polymerase chain reactions used 0.01% bovine serum albumin and GoTaq® Flexi DNA polymerase master mix with Thermocycler Gene Amp® 9700.

Amplified DNA was detected through 1.2% agarose gel electrophoresis at 120V for 35 minutes. After agarose gel electrophoresis, the PCR products were cleaned using absolute ethanol precipitation and sent for Sanger di-deoxy DNA sequencing.

Fig. 1. Sample collection sites of Pakistan for saw-scaled viper (*Echis carinatus*).

Data analyses

Analyses of the sequence data were done through appropriate softwares. All the DNA sequences were uploaded on Sequencher v5.0 (Gene Codes, Ann Arbor, Michigan, USA). Contigs of each forward and reverse sequence chromatogram were made to get a consensus sequence making sure that there is no stop codon. Sequences with low quality than 70% were not used to avoid any ambiguity. The sequences were aligned through MEGA v6.0 (Tamura *et al*., 2013) software using Clustal W (Larkin *et al*., 2007) multiple sequence alignment tool. The aligned nucleotide data was translated into amino acids using the vertebrate mitochondrial genetic code. DnaSP v. 5.0 (Librado and Rozas, 2009) was used for DNA polymorphism analyses. The polymorphism analyses included number of mutations, singleton variable sites, parsimony Informative sites, segregating sites, number of haplotypes, haplotype diversity, and nucleotide diversity. Using Multiple Sequence Comparison by Log-Expectation (MUSCLE), homology was measured among different Echis species and presented in the form of line graph. All the contig sequences were concatenated through SequenceMatrix v1.7.8 software (Vaidya *et al*., 2011). PartitionFinder v1.10 (Lanfear *et al*., 2012) software gave best partitioning scheme and evolutionary models that were used in Bayesian Inference and Maximum Likelihood analyses.

RESULTS

Analysis of polymorphism in ND4, 16S rRNA and 12S rRNA genes included number of mutations, singleton variable and parsimony informative sites along with haplotype and nucleotide diversity as shown in Table II. Using Multiple Sequence Comparison by Log-Expectation (MUSCLE), homology was measured among different *Echis* species (Fig. 4). Maximum likelihood analyses were conducted using RaxML v8.00 (Stamatakis, 2014) on CIPRES Science Gateway server v3.2 (Miller *et al*., 2010). Nodal support was provided by bootstrapping (BS; 1,000 pseudo-replicates); with bootstrap values ≥70 or 0.7 were considered as strong supports (Hillis and Bull, 1993). MrBayes v3.3 (Ronquist and Huelsenbeck, 2012) was used for Bayesian phylogenetic analyses. Two simultaneous runs of four Markov chain Monte Carlo (MCMC) analyses with total four chains (one cold plus three incrementally heated chains) were run with trees for 5 ×3 10^6 total generations (sampled every 500 generations). Burn-in value of 25% was set that discarded 2500 generations. Trace plots and ESS value (>200) was used to examine stationarity on TRACER v1.5 (Rambaut and Drummind, 2009). Posterior probability (PP) values ≥0.95 were considered as strong supports (Mulcahy *et al*., 2011). FigTree software (Rambaut, 2007) was used to visualize the resulting maximum likelihood (ML) and Bayesian inference (BI) phylogenies.

Maximum likelihood (ML) (Fig. 2) and Bayesian (BI) (Fig. 3) phylogenies were constructed using three mitochondrial genes. *Cerastes cerastes* (Egypt) was used as an out-group for all Echis that showed a well-supported sister group relationship to Genus Echis. Maximum likelihood and Bayesian phylogenies

Table I. Saw-scaled Viper (*Echis carinatus*) samples and their location information.

Samples ID	Locality	Latitude	Longitude
EC-1	Rehmat Pura, Okara, Punjab, Pakistan	30°49'41.78"N	73°26'57.58"E
EC-2	Chak 56/2 L Okara, Punjab, Pakistan	30°49'36.05"N	73°29'26.05"E
EC-3	Noor Garden, Okara, Punjab, Pakistan	30°48'48.38"N	73°28'38.33"E
EC-4	Tiba Maqsoodpura, Bahawalnagar, Punjab, Pakistan	29°59'28.08"N	73°15'51.74"E
EC-5	Aziz Town, Bahawalnagar, Punjab, Pakistan	29°58'39.26"N	73°15'6.63"E
EC-6	Lahore Zoo, Lahore, Panjab, Pakistan	31°33'23.78"N	74°19'33.73"E
EC-7	Budla Sant, Multan, Punjab, Pakistan	30° 9'13.65"N	71°42'42.59"E
EC-8	Model Town, Multan, Punjab, Pakistan	30°14'39.35"N	71°29'58.88"E
EC-9	Changa Manga Forest, Kasur, Punjab, Pakistan	31° 4'54.19"N	73°59'53.49"E
EC-10	Lahore Zoo, Lahore, Panjab, Pakistan	31°33'23.78"N	74°19'33.73"E
EC-11	Jharianwala, Hafizabad, Punjab, Pakistan	32° 5'26.11"N	73°40'29.85"E
EC-12	Zafar Abad, Sheikhupura, Punjab, Pakistan	31°41'13.28"N	74° 3'27.19"E
EC-13	Dera Kalar wala, Sheikhupura, Punjab, Pakistan	31°43'39.77"N	73°56'50.54"E
EC-14	Jharianwala, Hafizabad, Punjab, Pakistan	32° 5'26.11"N	73°40'29.85"E
EC-15	Qadirabad, Sahiwal, Punjab, Pakistan	30°43'22.36"N	73°14'59.99"E
EC-16	Chak 225 RB Malkhanwala, Faisalabad, Punjab, Pakistan	31°21'35.97"N	73° 6'46.19"E
EC-17	Nawanpind, Faisalabad, Punjab, Pakistan	31°15'7.49"N	73° 3'13.71"E
EC-18	Dogranwala, Gujranwala, Punjab, Pakistan	32°13'22.56"N	74° 5'29.38"E
EC-19	Hiran Minar Park, Sheikhupura, Punjab, Pakistan	31°44'34.88"N	73°57'18.64"E
EC-20	Dera Kalar wala, Sheikhupura, Punjab, Pakistan	31°43'39.77"N	73°56'50.54"E
EC-21	Chak 7/4-L, Okara, Punjab, Pakistan	30°44'43.12"N	73°25'52.61"E
EC-22	Khaji wala, Multan, Punjab, Pakistan	30° 8'32.90"N	71°22'53.94"E
EC-23	Mouza Talib Chiniot, Punjab, Pakistan	31°41'18.14"N	73° 1'16.67"E
EC-24	Chak 169 P, Sadiqabad, Punjab, Pakistan	28°16'48.63"N	70° 8'54.39"E
EC-25	Tauheedabad, Chiniot, Punjab, Pakistan	31°43'37.38"N	72°59'43.84"E

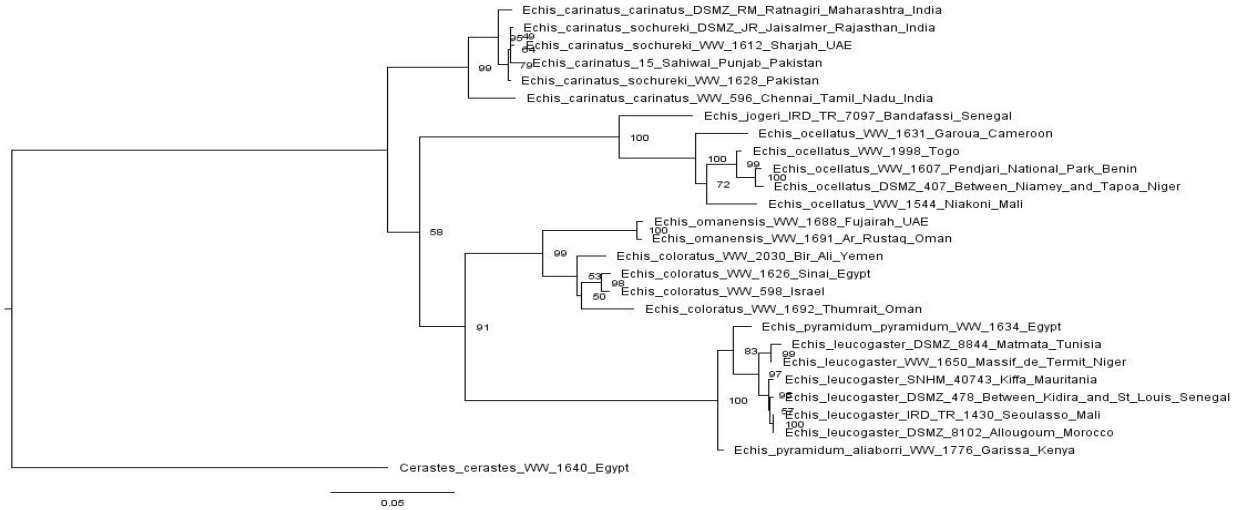

Fig. 2. Maximum likelihood (ML) phylogeny for Saw-scaled Viper (*Echis carinaus sochureki*).

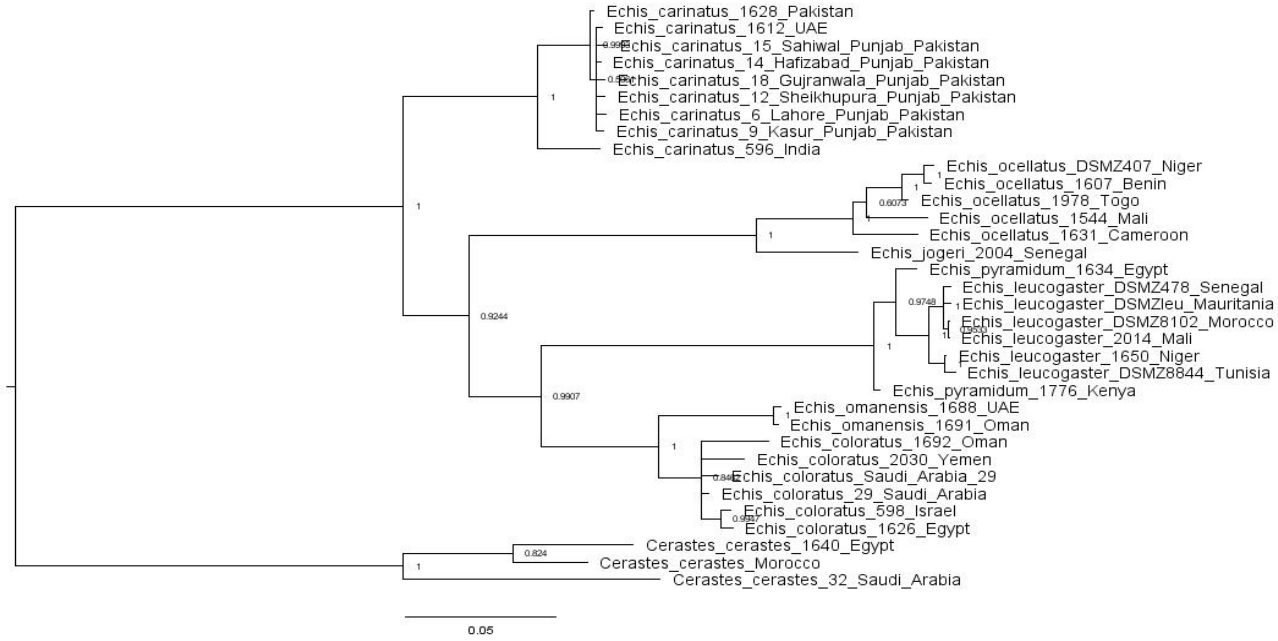

Fig. 3. Bayesian Phylogeny for Saw-scaled Viper (*Echis carinatus sochureki*).

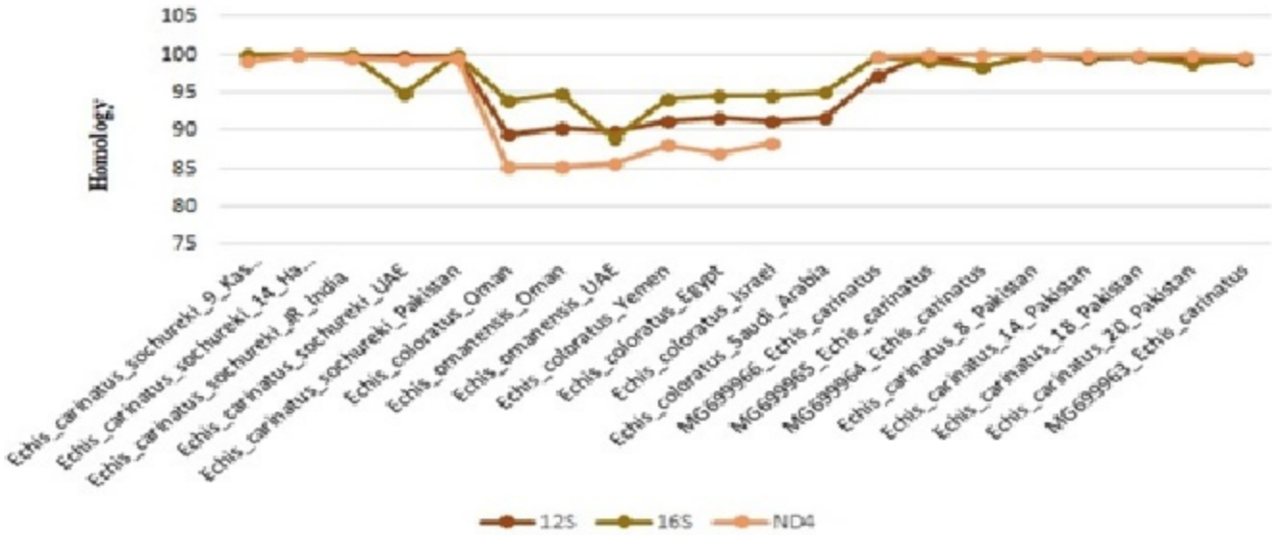

Fig. 4. Mitochondrial (ND4, 12S rRNA, 16S rRNA) gene-based homology of saw-scaled Viper (*Echis carinatus*).

showed almost similar topologies with best likelihood score (InL= -4498.85875) and (LnL= -6143.527). All *Echis* were divided into four distinct clades. These clades were *Echis carinatus*, *E. ocellatus*, *E. pyramidum* and *E. coloratus*. Although inter-relationship among the four clades was not found to be very well resolved. While *Echis pyramidum* and *Echis coloratus* groups showed well supported sister group relationship through (ML BS=91 and BI PP= >98). On the other hand, both *E. pyramidum* and *E. coloratus* did not show well supported relationship with *Echis carinatus* and *Echis ocellatus*. Barlow *et al.* (2009) used mitochondrial and nuclear genes finding the same four clades of Echis with the same interrelationship among them. They inferred Bayesian phylogenies that showed *Echis carinatus* as sister to all other three Echis clades using cytochrome b, ND4, 12S rRNA, 16S rRNA and RAG-1. Using only one representative of all four Echis species they found highly supportive posterior probability

value for monophyly of *E. coloratus*, *E. pyramidum* and *E. ocellatus*. *Echis coloratus* and *E. pyramidum* showed strong support for (ML BS=91, BI PP= 0.98) sister group relationship among them (Barlow *et al.,* 2009). Furthermore, both the groups (coloratus and pyramidum) showed low support values for sister relationship with *Echis ocellatus* (ML BS=58, BI PP= 0.5966).

Table II. Polymorphism in mitochondrial genes of saw-scaled viper (*Echis carinatus sochureki*).

Parameters	ND4	12S rRNA	16S rRNA
Total number of sites	507	343	483
Variable number of sites	121	98	63
Number of mutations	159	131	79
Singleton variable sites	30	30	23
Parsimony informative sites	91	68	40
Segregating sites	82	00	00
Number of haplotypes	26	22	21
Haplotype diversity	0.764	0.767	0.727
Nucleotide diversity	0.09451	0.06942	0.04215

DISCUSSION

Wild animals in Pakistan have always been ignored despite of being a very important part and asset of any ecosystem (Zafar *et al*., 2020). Saw-scaled vipers are found in a very large area from West Africa to India, including most of the Middle East countries. Their systematics and taxonomy has been a discussion for decades. In 1963, Klemmer recognized only two species: *Echis carinatus* in most of the range and *E. coloratus* in Arabia. Joger (1984, 1987) added *E. pyramidum* for southwestern Arabia. Cherlin (1990) described many new species and subspecies and increased the total number of *Echis* species significantly. A new species within the *E. coloratus* group was described by Babocsay (2004). Pook *et al*. (2009) using molecular genetic methods, have now clarified the complicated situation. Based on their results and additional data, they recognized the following six species in the near and Middle East. *Echis carinatus* group (Asian group): *E. (carinatus) sochureki* (Oman, UAE, Iran, Central Asia, Afghanistan, Pakistan), *Echis coloratus* group (Arabian group): *E. coloratus* (Egypt, Arabian Peninsula) *E. omanensis* (Oman, UAE) *Echis pyramidum* group **(**one of two African groups**):** *E. pyramidum* (Egypt, Sudan, East Africa) *E. khosatzkii* (western Oman, Yemen) *E.* sp. (cf. *borkini*) (Yemen, SW Saudi Arabia). *E. borkini* was originally described as a subspecies of the East African *E. varia* by Cherlin (1990).

As *Echis* bites frequently cause death and successful bite treatment depends on choosing a species-specific antivenom (if available), it is of great importance to know which species of *Echis* occur in which area. The present study inferred phylogenetic relationship of Saw-scaled viper (*Echis carinatus*) with other species of genus Echis from GenBank database. Previously, there has been confusion in taxonomy of this genus, but this study gave an important phylogenetic key for interpretation of previous hypothesis from Pakistan. Mitochondrial genes were used in this study to infer the molecular phylogenetics of Saw scaled viper (*E. carinatus*) from different parts of Pakistan. Maximum likelihood and Bayesian inference phylogenies were constructed through RaxML and MrBayes softwares. Bayesian phylogeny showed good support (PP=1) but Maximum likelihood (ML) phylogeny of the Echis did not show any support on basal node. *Cerastes cerastes* (Egypt) was used as an out-group for all Echis species included. Pook *et al*. (2009) used cytochrome b, ND4, 12S rRNA and 16S rRNA genes to infer phylogenies of medically important and taxonomically unresolved genus Echis of vipers. Maximum likelihood and Bayesian phylogenies divided all Echis into four distinct groups *i.e., Echis carinatus, E. ocellatus, E. pyramidum* and *E. coloratus* group.

Barlow *et al*. (2009) used mitochondrial and nuclear genes finding the same four clades of Echis with the same interrelationship among them. They inferred Bayesian phylogenies that showed *Echis carinatus* as sister to all other three Echis clades using Cytochrome b, ND4, 12S rRNA, 16S rRNA, and RAG-1. The present study also used mitochondrial genes and found showed good support for sister group relationship of *Echis carinatus* with other three Echis groups in phylogenetic analyses. Saw-scaled viper (*Echis carinatus*) from Pakistan and UAE was similar to northern Indian parts and stated as different subspecies (*Echis carinatus sochureki*) from south Indian saw-scaled viper (*Echis carinatus carinatus*).

The present study also agrees with Rhadi *et al*. (2016) who used two mitochondrial genes (16S rRNA and Cytochrome b) for measuring phylogenetic affinities of Iraqi population of saw-scaled viper of genus Echis. They used 1,105 bp for inferring Maximum likelihood and Bayesian inferred topologies with similar pattern as in our study. Saw-scaled viper (*Echis carinatus*) from Pakistan and UAE were found to be distantly related to that of Iraq and India.

Similarly, Arnold *et al*. (2009) inferred phylogenetic somewhat similar relationship of Echis through mitochondrial DNA sequences using 1,117bp of two genes. Genus Echis was divided into four distinct clades through Maximum likelihood (ML), Maximum parsimony

(MP) and Bayesian inference (BI) analyses. According to Arnold *et al*. (2009), *Echis carinatus* originated from Oman and Southern Iran and then dispersed to east and west of India and Iraq respectively.

On the other hand, Pook *et al*. (2009) suggested the origin from India and dispersal to north and west. *Echis carinatus* used in this study and those from NCBI database were divided into different groups. One group was from South India (Chennai Tamil Nadu), other one from Eastern and western India. Both of these groups included two haplotypes *i.e*., *Echis carinatus carinatus* (Maharashtra India) and *Echis carinatus sochureki* from Pakistan, UAE and Northern India (Rajasthan). *Echis carinatus carinatus* have also been found in southern Indian part Tamil Nadu.

Pook *et al*. (2009) found the same division of *Echis carinatus* as in our study. Their haplotypes included *E. c. carinatus* from India and *Echis carinatus sochureki* from Pakistan, Northeastern Arabian Peninsula. The haplotypes from northern part of range (Arabia, Pakistan, Central Asia and Northwestern India made a tightly packed cluster without having any clear phylogeographic structure showing recent divergence and dispersal. The basal sister groups have been shown to be originated from Maharashtra (Western India) and Tamil Nadu (Southern India).

The *Echis carinatus* group is the clade containing with minimum divergence between the populations. Cherlin identified three species *i.e., E. multisquamatus* (Central Asia, Iran), *E. carinatus* (southern India, Sri Lanka), *E. sochureki* (northern India to Pakistan), and Auffenberg and Rehman (1991) found clinal variation across the range of the group and considered all as subspecies of *E. carinatus*.

CONCLUSION

To infer more resolution in phylogenetic relationship and correct identification, more morphological and genetic studies are the required with diverse and increased number of samples from Indian subcontinent. This study is the first attempt to characterize, infer and molecular phylogenetics and genetic biodiversity in saw-scaled viper from Pakistan and will guide for further morphological and genetic studies about this genus and species which will ultimately help in designing the new local antivenom for snake bites in Pakistan as antivenom against snake bites is not as efficient as it is in India.

Statement of conflict of interest

The author(s) declare(s) that there is no conflict of interests regarding the publication of this article.

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