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PHYLOGENETIC RELATIONSHIPS OF MEDICALLY IMPORTANT VIPERS OF PAKISTAN INFERRED FROM CYTOCHROME *B* SEQUENCES

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ABSTRACT

The present study principally comprises the phylogenetic comparison of the three medically important vipers (*Echis carinatus sochureki, Daboia russelii russelii* and *Eristicophis macmahoni*) based on their molecular studies. In Pakistan, No comprehensive phylogenetic studies have so far been undertaken to collect molecular information by deciphering the cytochrome *b* gene (complete or partial) for the three species of interest. Keeping in mind the significance and nuisance of these deadly vipers of Pakistan, a molecular phylogeny was elaborated by successfully translating the cytochrome *b* gene sequence data for the three taxa of interest. Snakes for the said studies were collected through extensive field surveys conducted in Central Punjab and Chagai Desert of Pakistan from 2004 to 2006. The genetic data obtained werefurther elucidated statistically through maximum parsimony and bootstrap analysis for knowing the probable relationships among the species of interest. A comprehensive resolution of their phylogeny should be brought about for medical reasons as these lethal vipers are significant sources of snakebite accidents in many urban and rural areas of Pakistan

Key words: Cytochrome b gene, Morphological difference, Mitochondrial DNA, Phylogeny, Vipers.

INTRODUCTION

The major families of venomous snakes in the Indian subcontinent are Elapidae, Viperidae and Hydrophiidae (Philip, 1994). Venomous snakes of the same three families, viz. Elapidae, Viperidae and Hydrophiidae are found in Pakistan. (Underwood, 1979; Oehme *et al*, 1980; Tan, 1991). Out of a total of 80 snake species found in Pakistan, 15 are venomous. The venomous species include sea snakes (15 species); elapids (5 species) and vipers (7 species / sub species) (Minton, 1966).

Most vipers have stocky bodies, large heads, and long fangs. The morphological differences among vipers are associated with a dichotomy in foraging behavior. Vipers usually are sit-and-wait "ambush" hunters (Klauber, 1972).

Family Viperidae include asps, moccasins, rattlesnakes, and true vipers and comprises about 260 species in four subfamilies, viz, Azemiopinae, Causinae, Crotalinae and Viperinae (McDiarmid *et al*, 1999). Based on morphology (Liem *et al*, 1971) and mitochondrial DNA, Viperidae appear to be monophyletic (Heise *et al*, 1995; Kraus and Brown, 1998; Gravlund, 2001).

Vipers of subfamily Viperinae are commonly called true vipers, Old world vipers or pitless vipers. Most of the true vipers are found in the tropical and subtropical areas of the world. Viperinae are distinguished by the lack of the heat-sensing pit organs which the members of the subfamily Crotalinae comprise (Mallow *et al* 2003). All three snake species included in the present study, i.e., *Echis carinatus sochureki, Daboia russelii russelii* and *Eristicophis macmahoni*, belong to the subfamily Viperinae of the Family Viperidae.

The phylogenetic approach can be generally used to locate solutions to a variety of queries and riddles pertaining to evolutionary biology. In addition to resolving the taxonomic relationships of species, phylogenetics is used to study the evolution of gene families (Zhang & Nei, 1996; Johnston *et al*, 1998). Phylogenetics helps in evaluating evolutionary rates in different lineages (Kocher *et al*, 1989, Pamilo and O'Neill, 1997).

Elucidation of phylogenetic relationships among closely-related taxa benefits us by contributing to our understanding of the evolution of biodiversity. This elucidation of phylogenetic relationships is also important to correctly infer patterns of community structure. Various patterns of biogeography, and character evolution are also inferred fittingly (Arnold, 1993; Eggleton and Vane-Wright, 1994; Riddle, 1995; Harvey *et al*., 1996; Losos, 1996; Ortolani and Caro, 1996; Zamudio, *et al* 1997; da Silva and Patton, 1998; Roderick and Gillespie, 1998).

Information on the genetic relationships of organisms can help managers to focus their efforts on truly unique and distinctive species. Owing to the increasingly popularity of this approach over the recent years, methods have been developed to take phylogenetic

distinctiveness into account when setting various conservation priorities and policies (Moritz, 1994, 1995; Moritz and Faith, 1998).

Most DNA- based phylogenetics have proven very effective fundamentally on account of the rapid rate of sequence evolution characteristic of this genome (Brown *et al*., 1979; Caccone *et al*., 1997; Vidal *et al*, 1999), yielding greater proportions of potentially informative (variable) sites. Variation in mitochondrial DNA (mtDNA) has also been broadly used to map genetic variation of natural populations (Avise, 2000b).

Dessauer *et al*. (1987) reviewed the available molecular evidence for relationships among snakes and also presented some additional data. Cadle (1988) studied the phylogenetic relationships of advanced snakes, using micro-complement fixation. Four major clades of advanced snakes were recognized, which include viperids, elapids, colubrids, and Atractaspis.

Knight and Mindell (1994) sequenced portions of the mitochondrial 12s and 16s ribosomal RNA genes to address the relationships of the Colubrinae, Elapidae, and Viperidae.

Mitochondrial DNA has thus emerged as the most extensively used molecular marker in animal systematics, mainly at low taxonomic levels, owing to its ease of isolation and interpretation (Avise *et al*., 1987).

Mitochondrial DNA can also be very helpful in determining phylogenetic relationships between closely related taxa (Moritz *et al*. 1987).

Cytochrome *b* (cyt *b*) is one of the cytochromes implicated in the electron transport in the respiratory chain of mitochondria. It has eight transmembrane helices linked by intramembrane or extramembrane domains (Esposti *et al*., 1993). Cytochrome *b* is the only cytochrome coded by mitochondrial DNA.

The Cyt b gene has also been widely used for inference of phylogenetic relationships among numerous vertebrate clades (e.g., fish (Farias *et al*., 2001), snakes (Vidal & Lecointre, 1998; Lenk *et al*., 2001), and birds (Helbig & Seibold, 1999).

Characteristics of complete snake cytochrome *b* sequences have previously been considered by Slowinski and Keogh (2000) and Campbell (1997). Drawing on cytochrome *b* sequences from a variety of snakes, in general, most of which were henophidian snakes, Campbell described that the gene in snakes is between 1113 and 1116 bp long. Using an arrangement against other tetrapods, he established that the reason why the gene is shorter in snakes than in other vertebrates is due to a number of codon deletions near the ends of the gene. In another study, a greater variation was found in the total length of the cytochrome b gene of elapids than was reported by Campbell, with the gene ranging between 1101 and 1122 bp long (Slowinski & Keogh. 2000).

In one study four protein-encoding mitochondrial genes including cytochrome *b*, NADH-dehydrogenase subunits 1, 2 and 4 and one nuclear (c-mos) gene were sequenced to infer phylogenetic relationships among Old and New World representatives of racers and whipsnakes, *Coluber* (sensu lato) (Nagy *et al*., 2004).

Portions of cytochrome *b* or its complete sequence have been amplified using the polymerase chain reaction from a few species of viperidae during the recent past. A phylogeny of a taxonomically wide variety of taxa within the colubroidea using the nucleotide sequences of c-mos gene and the mitochondrial cytochrome *b* gene was inferred (Lawson *et al*., 2005). In another imperative study by Lenk *et al*., 2001, nucleotide sequences of mitochondrial cytochrome *b* (partial) and 16S rRNA genes, totaling 946 bp, have been used to reconstruct a molecular phylogeny of 42 species of the subfamily Viperinae representing 12 of the 13 recognized genera in order to further elucidate and clarify phylogenetic relationships in the viperine complex. These also include *Echis carinatus, Daboia russelii & Eristicophis macmahoni*, the three taxa of interest in the present study.

In another study, molecular phylogeny of *Vipera Laurenti,* 1768 and the related genera Macrovipera (Reuss, 1927) and *Daboia* (Gray, 1842) was elucidated (Garrigues *et al*., 2005). Phylogeography of the Russell's viper (*Daboia russelii*) complex in relation to variation in the colour pattern and symptoms of envenoming was also studied in which fragments of cytochrome *b* comprising 758 bp were amplified (Thorpe *et al*., 2007).

Sequence of complete cytochrome *b* from Russell's viper (*Daboia russelii*) was included among the cytochrome sequences studied for myriad of Colubroidea (Lawson *et al*., 2005). Partial sequence of cytochrome *b* from Russell's viper (*Daboia russelii*) was also studied while suggesting a modified taxonomy for Asian pitvipers (Malhotra & Thorpe, 2004). In another study, fragments of cytochrome *b* comprising 636 bp from Russell's viper (*Daboia russelii*) were amplified and studied (Castoe & Parkinson, 2006).

Problem: In Pakistan, though some scattered work, as cited above, has been done regarding the natural history and ecology of the three taxa of interest along with their significant morphological data, no comprehensive studies have been undertaken whereupon the phylogenetic studies based on their molecular information are discussed for the said taxa. The deciphering of thecytochrome *b* gene (complete or partial) for the three species of interest has also not been taken up in any indigenous studies so far. Keeping in mind the significance and nuisance of these deadly vipers of Pakistan, a molecular phylogeny for three taxa of interest using cytochrome *b* gene sequence data through maximum parsimony and bootstrap analysis for elucidating the probable relationships among the species of interest is therefore much required.

The present study principally comprises the

phylogenetic comparison of the three medically important vipers i.e., *Echis carinatus sochureki, Daboia russelii russelii* and *Eristicophis macmahoni* based on their molecular studies.

A resolution of their phylogeny should also be brought about for medical reasons as these lethal vipers are significant sources of snakebite accidents in different urban and rural areas of Pakistan (Alam *et al*., 1997; Minton, 1962, 1974, 1988) and a thorough phylogenetic framework is therefore also indispensable for research into venom composition, both for academic and applied purposes (Daltry *et al*., 1996; Fry *et al*., 2003).

The present study was designed to decipher the cytochrome *b* gene (complete or partial) for the three species of interest. a molecular phylogeny for three taxa of interest using cytochrome *b* gene sequence data through maximum parsimony and bootstrap analysis for elucidating the probable relationships among the species of interest shall also be attempted.

MATERIALS AND METHODS

Study Area of Central Punjab: The study area was located in part of the plains lying between the rivers Chenab and Ravi. It is the Central Punjab, part of the Indus plain that lies between 32° 04′ and 34° 38′ north latitudes and 32° 27′ and 73° 41′ east longitudes encompassing the main study area which comprises the six districts of the province Punjab viz. Faisalabad, Jhang, Toba Tek Singh, Sheikhupura and Hafizabad that lie in the land tract between the rivers Ravi and Chenab while Okara is located in the tract between the rivers Bias and Ravi. Some of the coordinates of the sampling sites at Central Punjab are given in Table 1.

Study Area of Chagai Desert: The Chagai Desert extends for more than 500 km to the Northwestern side of Balochistan and the Southern desert regions of Afghanistan. It is traversed by small and widely spaced mountain ranges in an open expand of basin. Small stones and sand dunes generally cover this basin. Climate of the Chagai Desert ranges from extreme hot in the summer to severe cold in winter. Coordinates of sampling sites at Chagai are given in Table 2.

Species of interest: *Echis carinatus sochureki*: Saw-scaled viper is a species very widely distributed from northern Africa to India and Sri Lanka. *Echis* has been split into 12 species (20 taxa) in 3 subgenera (Cherlin, 1981, Cherlin and Borkin, 1990), 3 subspecies of which occur in Pakistan (Khan, 1983, 2002). The subspecies under study *Echis carinatus sochureki* Stemmler, 1964, (Sind Valley saw-scaled viper), commonly called Sindi khappra saamp is the most widely distributed race of saw scaled viper in Pakistan (Bhat, 1974; Sanford 1966).

***Daboia russelii russelii*,** Russell's viper is a large, stocky, tan-colored snake, with very distinct, large, black-bordered ocelli on the back and a smaller set laterally. Dorsum is light yellowish brown to sandy, with 3 longitudinal series of (22-32), large oblong or oval chestnut blotches. (Wüster 1998b). It is found in some areas and absent in others, from Afghanistan near the Kabul River, east and southern through all of Pakistan, throughout the Indus Valley, from Karachi to Rawalpindi, at low altitudes, except the driest parts. (Wüster, 1998b).

***Eristicophis macmahoni*,** Leaf-nosed viper can be distinguished from all other Pakistani vipers by the shape of a typical rostral scale, which is projected laterally on each side to The dorsum is light reddish brown to khaki, with a series of small dark brown lateral spots, each surrounded in its upper half by light dots. It is well adapted to live in the fine loose sand of shifting dunes. This snake has been found to be distributed from Seistan province in the extreme east of Iran into Afghanistan south of the Helmand River and southwestern Balochistan, between the Chagai Hills and the Siahn Range, east to Nushki.

Table 1: Coordinates of the major sampling sites in Central Punjab selected for the present study

Major sampling sites	Province	Coordinates
Sheikhupura	Central Punjab	(31° 42′N 73° 30′E)
Hafizabad	Central Punjab	(32° 04′N 73° 41′E)
Faisalabad	Central Punjab	(31° 25′N 73° 07′E)
Okara	Central Punjab	(30°48′N 73°27′E)
Toba Tek Singh	Central Punjab	(30°57′N 72°28′E)
Bahawalangar	Central Punjab	(29°59′ N 71°42′E)
Hamun e Mashkel	Balochistan	(N 28 33 05.7, E 061 56 40.8)
Naushki	Balochistan	(N 28 33 05.2 E 061 56 393)
Chagai	Balochistan	(N 28 21 35.5, E 062 50 35.1)
Chagai	Balochistan	(N28 21 49.2, E 062 50 35.1)
Tahlab	Balochistan	(N 28 29 40.3, E 062 55 24.0)

Table 2: Viper species collected along with the coordinates of the sampling sites

Coordinates	Species collected
(31○42′N, 73○30′E)	*Echis carinatus*
(32○04′N, 73○41′E)	*Daboia russelii*
(31○25′N, 73○07′E)	*Echis carinatus*
(30○48′N, 73○27′E)	*Echis carinatus*
(30○57′N, 72○28′E)	*Echis carinatus*
(29○59′ N, 71○42′E)	*Daboia russelii*
(N 28 33 05.7, E 061 56 40.8)	*Eristicophis macmahoni*
(N 28 33 05.2 E 061 56 393)	*Eristicophis macmahoni*
(N 28 21 35.5, E 062 50 35.1)	*Eristicophis macmahoni*
(N28 21 49.2, E 062 50 35.1)	*Eristicophis macmahoni*
(N 28 29 40.3, E 062 55 24.0)	*Eristicophis macmahoni*

Table 3: Tally and description of species encountered and captured from 2004 to 2006 field surveys

Species	Visual encounter	Snake clutch	Stone turning	pitfall trap	Total
Echis carinatus	27	6	2	4	39
Daboia russelii	14	4	3	0	21
Eristicophis macmahoni	12	1	2	1	16
Total	53	11	7	5	76

Table 4: Tally of species encounter records from 2004 to 2006 field surveys

Taxa	Species Encounters	Species captured
Echis carinatus	27	12
Daboia russelii	14	7
Eristicophis macmahoni	12	4

Table 5: Some ecological records of the three species from study areas during 2004-06

Museum number	Species	Locality	Life stage	Sex	Province	Collector's name	Date
1	*Echis carinatus*	Okara	Adult	M	Punjab	Ahsan	21-07-04
2	*Echis carinatus*	Faisalabad	Adult	F	Punjab	Riaz	20-03-05
3	*Echis carinatus*	Faisalabad	Adult	M	Punjab	Khalid Baig	09-07-05
4	*Echis carinatus*	Bahawalangar	Adult	M	Punjab	Ahsan	05-04-06
5	*Echis carinatus*	Sheikhupura	Adult	M	Punjab	Khalid Baig	29-07-06
6	*Dabioa russselli*	Sheikhupura	Adult	M	Punjab	Khalid Baig	24-07-04
7	*Dabioa russselli*	Sheikhupura	Subadult	F	Punjab	Ahsan	18-03-05
8	*Dabioa russselli*	Hafizabad	Adult	M	Punjab	Khalid Baig	12-07-05
9	*Dabioa russselli*	Faisalabad	Adult	F	Punjab	Ahsan	03-04-06
10	*Dabioa russselli*	T. T. Singh	Adult	M	Punjab	Khalid Baig	28-07-06
11	*Eristicophis macmahoni*	Chagai	Adult	M	Balochistan	Ataullah	21-04-04
12	*Eristicophis macmahoni*	Naushki	Adult	M	Balochistan	M. Rafique	09-04-05
13	*Eristicophis macmahoni*	Naushki	Adult	M	Balochistan	Khalid Baig	12-04 -05
14	*Eristicophis macmahoni*	Chagai	Subadult	F	Balochistan	Ahsan	28-06-06
15	*Eristicophis macmahoni*	Chagai	Adult	M	Balochistan	M. Rafique	02-07-06

Table 6: Sequence and sources of primers used for PCR and sequencing in the present study

Name	Sequence 5'-3'	Reference
L14724	TGACTTGAAGAACCACCGTTG	Palumbi *et al*. 1991
L15162	ATAGCHACCGCCTTCTTCGG	Vidal and Lecointre 1998
L15584	TCCCATTYCACCCATACCA	de Queiroz *et al*. 2002
H15175	CCCTCAGAATGATATTTGTCCTCA	Palumbi *et al*. 1991
H15366	TATGGGTGGAAKGGGATTTT	Vidal and Lecointre 1998
H16064	CTTTGGTTTACAAGAACAATGCTTTA	Burbrink *et al*. 2000

The field surveys for both *Echis* and *Daboia* were conducted simultaneously and in mostly the same localities of Central Punjab, while separate surveys were conducted for *Eristicophis macmahoni* of Balochistan. These surveys were conducted during the early summer and early monsoon period from 2004 to 2006. Tally of species encounter records for these three species from surveys during 2004 to 2006 is given in Table 4. Tally and description of species encountered and captured from 2004 to 2006 field surveys is given in Table 5.

Certain morphological measurements were made from a number of snakes belonging to species of interest. These were photographed digitally and released in the wild without harming them. Only adult specimens were captured and preserved. All the captured specimens from the three species were brought to the Pakistan Museum of Natural History (PMNH) and added to the reptile repository of the Zoological Sciences Division (ZSD) of the Museum. Some ecological records of the three species from study areas during 2004-06 are elucidated in Table 6.

MOLECULAR ANALYSES

DNA extraction: DNA was extracted from living specimens or tissue samples removed from freshly dissected or ethanol preserved animals using Gentra kits (Gentra, Minneapolis, MN). For all three species of

interest, the samples comprised of muscle tissues obtained from various portions of the snake's body including the tails. Tissue samples were stored in 97% ethanol at -20°C.

Table 8: Sequences used in Analyses (Figures 4.3-4.6)

Abbreviation	Taxon	Locality	Accession No.
D rus1	*Daboia russelii*	This study	
D rus2	*Daboia russelii*		DQ305459
D rus3	*Daboia russelii*	Myanmar	AF471076
D rus4	*Daboia russelii*	Pakistan	AJ275723
D rus5	*Daboia russelii*	Thailand	AY165090
E mac1	*Eristicophis macmahoni*	This study	
E mac2	*Eristicophis macmahoni*		AJ275711
EC car1	*Echis carinatus*	Pakistan	AJ275706
EC oce1	*Echis ocellatus*		AF292568
EC oce2	*Echis ocellatus*	Mali	AJ275710
EC oce3	*Echis ocellatus*		AF191579
EC mul1	*Echis multisquamatus*		AJ275702
EC pyr1	*Echis pyramidum*		AJ275709
EC pyr2	*Echis pyramidum*		AJ275707
EC col1	*Echis coloratus*		AJ275708
A nit1	*Atheris nitschei*		AF471070
A squ1	*Atheris squamigera*		AJ275684
M des1	*Macrovipera deserti*		AJ275712
M lep1	*Macrovipera lebetina*	Turkmenistan	AJ275713
M mau1	*Macrovipera mauritanica*	Morocco	AJ275714
M sch1	*Macrovipera schweizeri*		AJ275715
V asp1	*Vipera aspis aspis*	France	AY321098
V lat1	*Vipera latastei*	Spain	AY321094
V kaz1	*Vipera kaznakovi*	Turkey	AY321093
V ber1	*Vipera berus*	France	AY321091
V amm1	*Vipera a. ammodytes*	Yugoslavia	AY311381
V pal1	*Vipera palaestinae*	Isreal	AJ275722
V alb1	*Vipera albizona*	Turkey	AJ275727
V rad1	*Vipera raddei*	Turkey	AJ275730
P per1	*Pseudocerastes persicus*	Pakistan	AJ275717
P fie1	*Pseudocerastes fieldi*		AJ275716
C res1	*Causus resimus*		AY223555

Approximately 250 mg of ethanol-preserved tissue was washed with several volumes of distilled water and blotted dry. Each sample was placed in a mortar containing liquid nitrogen and ground into a fine powder once frozen. This fine powder was placed in a micro centrifuge tube and processed following the directions provided with the Gentra kits, including cell lysis and Proteinase K digestion, protein precipitation and removal, and DNA precipitation. Air dried DNA precipitate was rehydrated in 50 ul of sterile distilled water.

The DNA concentration for each extraction was determined on a NanoDrop 1000 spectrophotometer (Nanodrop, Bethesda, MD) and between 80 and 120 ng of DNA was used as a template for PCR amplification. Ready-To-Go PCR Beads (GE Healthcare, Piscataway, NJ) were used with 25 µl PCR reactions containing 2.5 µl of a 10 µM solution of the forward primer and 2.5 µl of a 10 µM solution of the reverse pr1991r.

Amplification of Cytochrome *b* gene: Sequence and sources of primers used for PCR and sequencing in the present study are given in Table 7. The entire cytochrome *b* gene was amplified using primers L14724 (Palumbi *et al*. 2001) and H16064 (Burbrink *et al*. 2000) and shorter fragments were amplified with primer pairs L14724 and H15175 from Palumbi *et al*. (1991), L15162 and H15366 from Vidal and Lecointre (1998), and L15584 (de Queiroz *et al*. 2002) and H16064. Most PCR reaction were conducted with an initial denaturing at 95^{o}C for 5 minutes followed by 35 cycles (denaturing at 95^{o}C for 30 seconds, annealing at 50^{o}C for 30 seconds and elongation at 72^{o}C for 1 minute) and a final elongation at 72^{o}C for 10 minutes. Amplification of a cytochrome b fragment with primer pair L15162 and H15162 was conducted with an annealing temperature of 55^{o}C. PCR reaction were prepared for sequencing by treatment with exonuclease I to digest any unincorporated primer DNA and shrimp alkaline phosphatase to digest unincorporated nucleotide triphosphates using ExoSAP-IT (USB Corporation, Cleveland, OH).

Direct double-stranded sequencing of a segment of mitochondrial cytochrome *b* gene from the PCR reaction was performed using the ABI PRISM Dye Terminator Cycle Sequencing Kit (Perkin Elmer) with primers L14724, L15162, L15584, H15175, and H15366. Sephadex columns were used to remove excess dyes and the reaction were fractionized and visualized on an ABI 3100 DNA sequencer.

Sequencing of the gene: The sequence of the cytochrome b fragment was aligned visually against the published (Lawson *et al*. 2005) cytochrome *b* sequence of *Atheris nitschei* (AF471070).

Statistical Analyses: Two methods of phylogenetic analysis were exercised for all data sets and their results compared. These were maximum parsimony (MP) and bootstrap analysis. Phylogenetic relationships inferred from DNA sequences were examined in a maximum parsimony framework using PAUP* (Swofford 1998) and nodal support was examined by bootstrapping (Felsenstein 1985). These extensively applied phylogeny reconstruction methods are persuaded by diverse philosophies and therefore warrant satisfactorily different perspectives in data analyses.

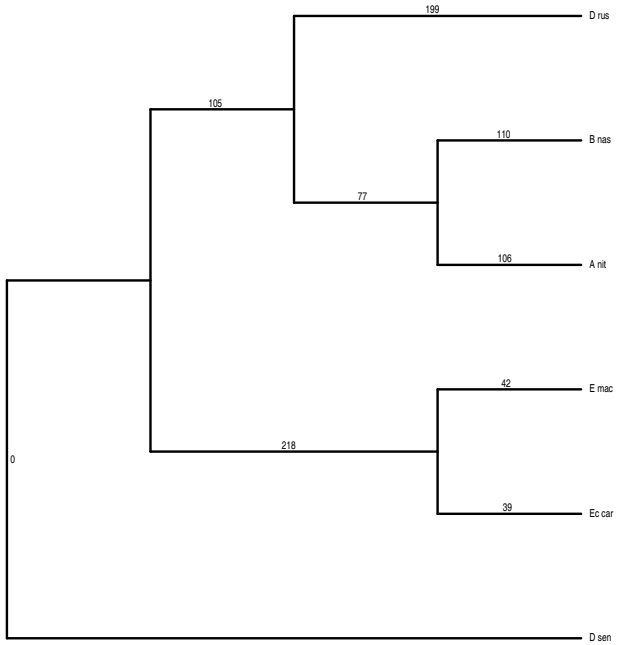

Figure 1. Maximum Parsimony Analysis, Complete Cytochrome b Sequence (1117 bp), Taxa: D rus = *Daboia russelii*, B nas = *Bitis nasicornis* (AY188009), A nit = *Atheris nitschei* (AF471070), E mac = *Eristicophis macmahoni*, Ec car = *Echis carinatus*, and D sen = *Dinodon semicarinatus* (NC001945)

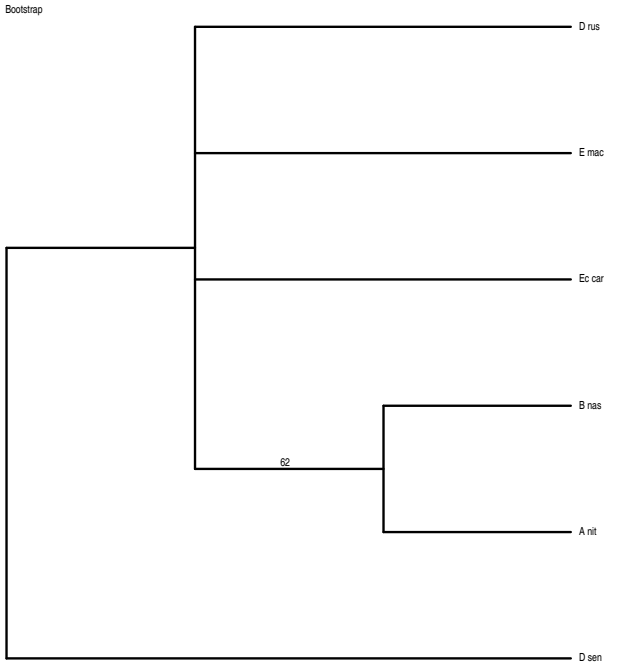

Figure 2. Bootstrap Analysis, Complete Cytochrome b Sequence (1117 bp), Taxa: D rus = *Diaboia russelii*, B nas = *Bitis nasicornis* (AY188009), A nit = *Atheris nitschei* (AF471070), E mac = *Eristicophis macmahoni*, Ec car = *Echis carinatus*, and D sen = Dinodon semicarinatus (NC001945)

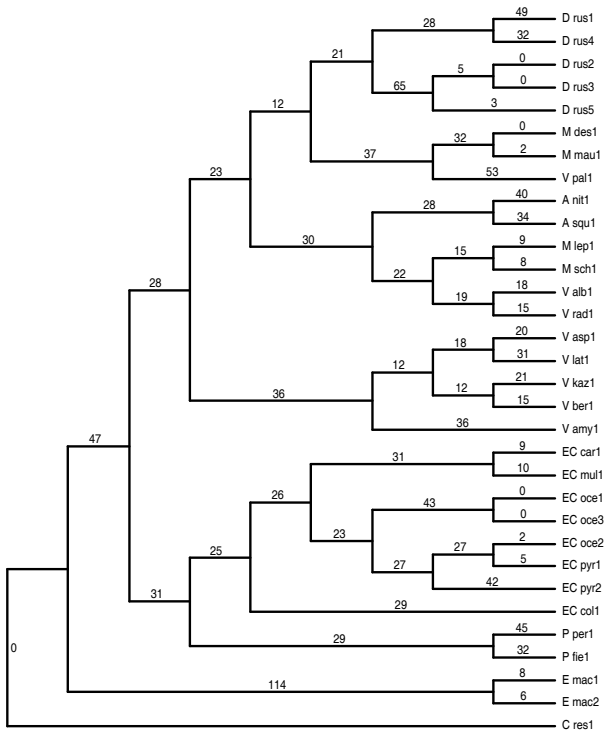

Figure 3: Maximum Parsimony Analysis, Partial Cytochrome b Sequence (672 bp)

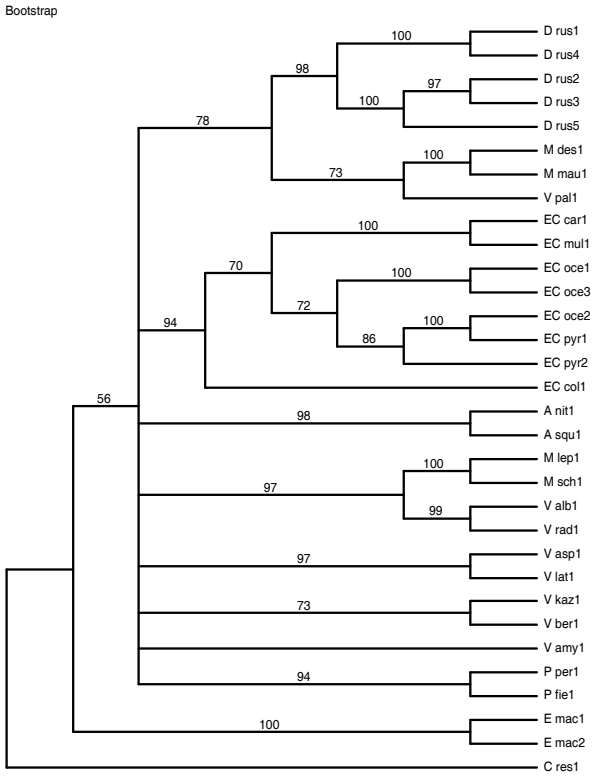

Figure 4: Bootstrap Analysis, Partial Cytochrome b Sequence (672 bp),
Taxa see Table 8.

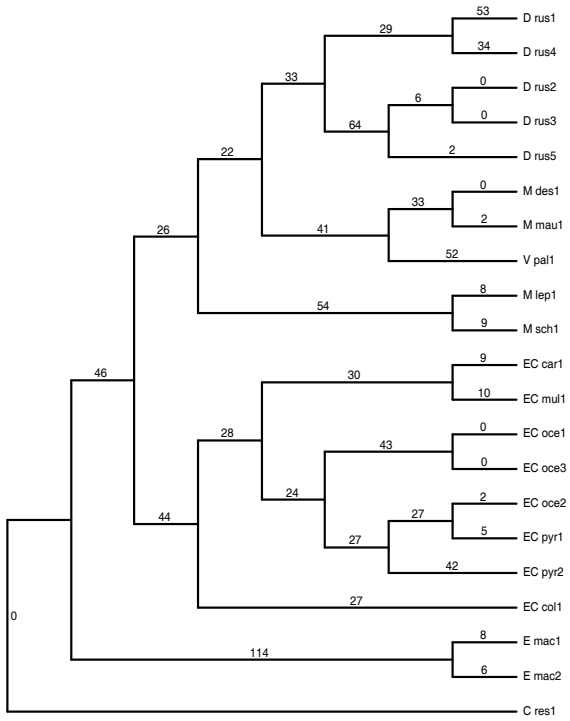

Figure 5: Maximum Parsimony Analysis, Partial Cytochrome b Sequence (672 bp), Taxa see Table 8.

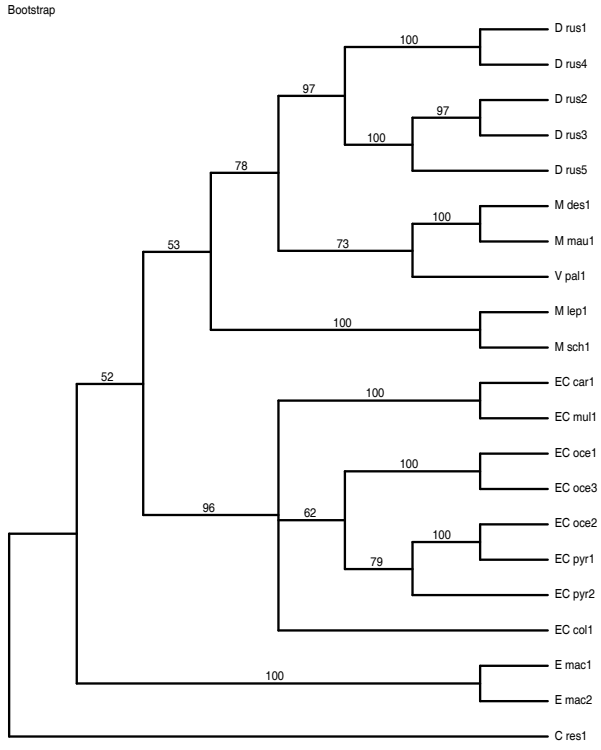

Figure 6: Bootstrap Analysis, Partial Cytochrome b Sequence (672 bp), Taxa see Table 8. – small number of taxa used

RESULTS AND DISCUSSION

The analysis included the comparative cytochrome *b* gene sequencing (partial and complete) from mitochondrial DNA for each species to determine whether there is evidence of independent phylogenetic lineages within these snakes. The molecular analyses elucidated in the Figures 1- 6 are all cladistic (character based analyses) in a maximum parsimony framework. Figures 1 & 2 show the analyses of complete cytochrome b sequence for 6 taxa of the Viperidae. Table 8 shows the Sequences used in analyses (Figures 3-6). Maximum parsimony tree (Figure 1) is rooted with *Dinodon semicarinatus.*

Maximum Parsimony (MP) analysis suggests that *Eristicophis macmahoni* and *Echis carinatus* are more closely related than either is to *Daboia russelii* based on the number of mutational differences. The number of mutational differences is 81 between *Eristicophis macmahoni* & *Echis carinatus.* It is 564 between *Eristicophis macmahoni* & *Daboia russelii* and 567 between *Echis carinatus* & *Daboia russelii*. Figure 2 however, shows there is no nodal support for any relationship other than that between B nas & A nit and even this is very low (62% = not well supported). These analyses suggest that all three taxa of interest are not closely related. Missing data for *Echis carinatus* also contributes substantially to the lack of resolution of these analyses. There is a very small number of taxa of the Viperidae for which data is available for the entire cytochrome b gene. Sequence for three taxa (*Aitheris nitschei*, *Bitis nasicornuis*, & *Dinodon semicarinatus*) is available in GeneBank and the present study has added two others.

In figures 3 and 4, analyses of a 672 base pair fragment of the first part of the cytochrome b gene was conducted for data from 32 samples representing 24 taxa of Viperids. Figure 3 is a maximum parsimony tree rooted with *Causus resimus*. This analysis confirms that the three taxa of interest are not closely related and each species seems to be more closely related to some other taxa than to one another. This distinctiveness relates to mixing of zoogeographic faunas as has been discussed by some of the other workers who have published molecular systematic studies of the Viperidae. Figure 4 shows that the strongest bootstrap support is at the level of genera and the analysis shows little support of resolving the relationships among genera.

Figures 5 and 6 elucidate the analyses of a 672 base pair fragment of the first part of the cytochrome b gene conducted for a data set containing a smaller number of taxa (21 samples representing 13 taxa). Figure 5 is a maximum parsimony tree rooted with *Causus resimus*. This analysis confirms that the three taxa of interest are not closely related and each seems to be more closely related to some other taxa than to any other.

Although both this set of analyses and Figures 3 & 4 suggest that *Daboia* is more closely related to *Echis* than either is to *Eristicophis*, this relationship is not strongly supported (52%= Figure 6). This limited sequence data not able to resolve the relationships of these three divergent genera of Viperids.

Overall the molecular data doesn't show any strong support for a closer relationship between either of the taxa of interest. Although that is a relationship between *Echis carinatus* and *Eristicophis macmahoni* shown in maximum parsimony tree of Figure 1, it is not supported by the bootstrap analysis and thus no such closer relationship should and can not be inferred from the molecular data. Likewise, the molecular analyses of the partial sequence data suggest a closer relationship between *Daboia russelii* and *Echis carinatus* (Figures 3 & 5), but again this relationship is also not supported by the bootstrap values (Figures 4 & 6). Thus, the molecular data can not resolve which of these three taxa are most closely related and strongly suggests that the three taxa are quite divergent probably each evolving from a different group.

MP tree in figure 1 lacks phylogenetic information between species included in analysis. Bootstrap analysis (Figure 2) also shows low bootstrap values between *Daboia*, *Eristicophis* and *Echis*, making relationships unresolved. Even bootstrap value between *Bitis* and *Atheris* is low.

In MP tree shown in Figure 3 the numbers of taxa used for analysis allows getting better results for relationships within genus level (*Daboia, Echis, Macrovipera* + *Montivipera*). *Causus* is used for outgroup.

Two main clades bring out of the MP rebuilding method:

- *Daboia, Vipera* and related genera are clustered with *Atheris* in one clade
- *Echis* and *Pseudocerastes* are clustered in the other

All these relationships are, however, not much supported by bootstrap analysis.

The clade of *Daboia* including *D. russelii* + ancient (ex) *M. deserti* and *M. mauritanica* + ancient *V. palaestinae* is well supported with 78% bootstrap, confirming the taxonomic revision of Lenk *et al* (2001) and Garrigues *et al*. (2005). This group is monophyletic clade. Thus, species of the *Daboia* genus are *D. russelii*, *D. deserti, D. mauritanica* and *D. palaestinae.*

The sister group of *Daboia* is formed by a clade including *Montivipera* (*V. raddei; V. albizona) +Macrovipera* + *Atheris*, but Bootstrap value supporting this relationship is weak (under 50%). Only the clade including *Montivipera* and *Macrovipera* is strongly supported (97% btp) that is consistent with the results of Lenk *et al*. (2001) and Garrigues *et al*. (2005). Nevertheless, position of *Atheris* as sister group of *Macrovipera* + *Montivipera* is surprising according to the results of Lenk *et al*. (2001).

The clade of *Vipera* genus remains monophyletic but is not well supported by bootstrap. In the same way the relationship between the clade containing *Echis* species and the clade of *Pseudocerastes* is not supported by bootstrap.

The *Echis* group is monophyletic and well supported (94% bootstrap). However, one *E. ocellatus* from Mali is clustered with *E. pyramidum*. *E. coloratus* forms the basal lineage of the clade (94% bootstrap). *E. carinatus* and *E. multisquamatus* are sister species (100% bootstrap). *E. ocellatus* and *E. pyramidum* are sister species (moderately supported 72% bootstrap).

Position of *Pseudocerates* is unexpected compared with the results of lenk *et al*. (2001) showing a cloth relationship with *Eristicophis*. In the present study, *Eristicophis* form the basal lineage of this phylogeny. Phylogenetic relationships for *Echis* are better supported in fig 4.

Taking into account their limitations and advantages, both the molecular data and morphological information should be brought in use together for such studies. It is not prudent to count solely on only one of these two major sources of information as none of these is independently efficient or sufficient to present an independent phylogenetic analysis for the taxa of interest. There is no compelling morphological evidence that links any two of the three species. Although there are several areas where morphological phylogenetics can be ameliorated, perhaps the finest solution to these predicaments is to keep using morphological data along with the molecular data by using clear methodology, development and application of new methods, and precise testing of these methods using simulations and congruence studies.

The present study is an archetype in Pakistan focusing on molecular techniques to examine genetic structure and distinctiveness within the three species of Viperidae. With this knowledge, these medically important viper species of Pakistan will likely continue to prove a more productive subject for investigating patterns of evolutionary and ecological divergence among the reptile predators. This study also concentrates on perking up our understanding of the phylogenetic relationships and aspects of the evolutionary ecology of a conspicuous group of important Pakistani snakes.

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