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Molecular Identification of Family Colubridae from Punjab, Pakistan

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ABSTRACT

The present one year study was extended from May 2019 to March 2020 was conducted in selected sites of Punjab. This study focuses on the molecular identification and morphometric analysis of two snake species, *Platyceps rhodorachis* (Cliff Racer) and *Amphiesma stolatum* (Buff Striped Keelback) collected from selected sites in Punjab, Pakistan. Each captured specimen was tagged with specific number and identified using morphological keys. Total genomic DNA was extracted from preserved tissues and amplified using COI primers set. After trimming ambiguous bases, the obtained COI fragments of *Platyceps rhodorachis* was 668 bp while fragments of *Amphiesma stolatum* was 639 bp. The COI fragments aligned with available sequence from NCBI comprised of 620 bp. The DNA sequences have shown reliable and exact species identification. The mean intraspecific identities of *Platyceps rhodorachis* (Cliff Racer) and *Amphiesma stolatum* (buff striped keelback) was 0.11±0.01 and respectively based of p-distance. Different molecular markers have been used in this study. *Platyceps rhodorachis* and *Amphiesma Solatum* successfully amplified using COI gene. It can be concluded that, identification of reptiles on morphological basis is still considered authentic. However, large scale molecular analysis of these taxa is required for exact species identification and to report any new species from area.

Introduction

Roughly 0.01 per cent of the world's approximate total of 15 million species has been described so far. Among the species described till now include 8,734 reptiles. Reptiles are believed to be paraphyletic group and they are used to describe all non-avian surviving taxa of the Testudines, Crocodylia, Sphenodontia, and Squamata (Uetz *et al*., 2010). They act as an excellent biological indicator, control many insect populations, play vital role in food chain as predator-prey relationship ending up as a conspicuous part of an ecosystem (Heywood and Watson 1995). Population of reptiles is greatly being effected by factors such as climate change, disease outbreaks, invasive species, habitat destruction and exclusion, hunting and biodiversity loss (Gibbons *et al*., 2000; Nijman *et al*., 2012). For certain cases, rodenticide application also triggers its decline by inducing secondary poisoning (Kim *et al*., 2011). In addition, road mortality has become one of the causes why vertebrates, such as reptiles, are decreasing in our ecosystem (Trombulak and Frissel 2000). Although squamates (59% lizards, 35% snakes and 2% amphisbaenians) are the

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second most abundant group of amniotes but they are more diverse than turtles (3.4%), crocodilians (0.3%) and tuataras (0.01%) (Donoso *et al*., 2013).

Morphological keys based identification system required a group of 15000 taxonomists to describe all the species of globe (Chambers and Hebert 2016). Sometime phenotypic characters lead to incorrect identification of the species. In addition, many cryptic species remain hidden under the term subspecies or common species (Waugh, 2007). Despite the fact that species recognition is mostly based on biological research, taxonomic proficiency is declining. First of all as one genotype can produce more than one phenotypes when exposed to different environments can cause improper identifications. Secondly, there are a variety of elusive sister species that can also contribute to misidentification. Thirdly, morphology can be studied at a particular stage of life, hence many individuals are unable to be identified. Lastly, dominant advances require such skillfulness that incorrect identification is common (Vieites *et al*., 2009). Since the discovery of DNA Barcoding, it is being utilized in many research projects like records of uncontrived regions of biodiversity (Hajibabaei *et al*., 2007; Janzen et *al*., 2009), specie recognition using databases for barcodes (Ratnasingham *et al*., 2007), pest management (Ball and Armstrong 2006), Regulation of species (Armstrong and Ball 2006) and health impacts (Lowenstein *et al.,* 2010). The use of DNA barcoding up in a particular region for a preparatory molecular characterization of a given organism group would be amongst the most popular examples in biodiversity studies. This can extent from very confined representative groups to variety of species in wide areas (Hausmann, 2011).

For any conservation strategy or for biological study a great amount of knowledge related to biodiversity is very crucial. The scientists not only well aware about major extinction events but also about the species that disappeared the world event unknown to science. From the last few years a large amount of data has been obtained which heled us in getting knowledge about biological diversity and for species recognition genetics tools has been used. Molecular data is efficiently used for species recognition. Although, due to high level of intraspecific variations, COI marker has ambiguous barcoding gap. The analyzed individuals of the same species clustered together. Also, another mtDNA gene often termed as 16S rRNA gene is a choice for barcoding of amphibians. Genetic distance analysis via Kimura 2-parameter (K2P) analysis indicates the mean intra-specific variations for 16S rRNA and COI genes concluded that COI is best DNA barcoding marker for reptiles.

Under the specifications of barcoding of DNA or DNA taxonomy, the usage of sequences of short DNA for the purpose of standard organism identification has gathered much recognition. The assignment of undiscovered life-history phases to adult organisms, the wide-scale identification of organisms in research either related to ecology or genomics and the most contentious exploratory surveys to identify possibly undiscovered "target" species are some of the favorable and dominant uses of this technique (Hebert *et al*., 2004;Venter *et al*., 2004). Several things should be given importance while barcoding amphibians and reptiles such as species and complexes of species are ancient and may have cryptic successors. The tropics appear to be encountered with such situation commonly. Fresh cryptic lines occurs less commonly as species are better researched, in temperate areas as well as on the other side these species have often extended from frozen shelters in the Pleistocene so that specie deviations are less profound and comparable mitochondrial haplotypes can be found in large geographical areas (Vences *et al*., 2005a; Zhang *et al*., 2005).

Using the DNA barcoding advent a peculiar short sequence of DNA is checked with a reference database (Hajibabaei *et al*., 2007). For the facilitation of sequence alignment, the barcode should fulfill requirements such as universality to ease the amplification from diverse species. Moreover, it must have some insertions or deletions. Not just this, but the rate of mutation should be enough to bring about a barcoding gap. This signifies that minimum interspecific variation is greater than maximum intraspecific variation (Hebert *et al*., 2003). Owing to the reason of high potential for DNA Barcoding in vertebrates' nuclear ribosomal DNA genes such as 18S and 28S were recommended as standard markers for all vertebrates. It is hard to amplify and sequence nuclear DNA through high profile approaches (Tautz *et al*., 2003), even though it has the great convenience of being inherited by both parents, mitochondrial DNA (mt DNA) was also proposed for the barcoding of DNA in amphibians (Vences et al., 2005b). COI serves the purpose of universal gene which is a compulsion for practical DNA Barcoding (Hebert et al., 2003). COI is much more effective in identifying cryptic species in contrast to 16S because of its relatively high rates of evolution (Xia et al., 2012).

COI is constantly being used for multiple applications which include assessments of biodiversity because of its productive and steady outcomes. In DNA barcoding of reptiles, the gene COI is in early stages because of amplification problems. With the help of 12S rRNA and Cytb unknown vertebrate species are being identified. Furthermore, 12S rRNA, 16S rRNA and COI genes are mostly used in reptiles identification and phylogenetic analysis (Clause et al. 2016). To conserve the amphibian and reptilian species, there is need to assessor evaluate the composition and diversity of the herpetiles in populated areas (Tsai et al. 2012).

Amphibians and reptiles are declining on global scale and more than 40% species have been extinct so far (Venues et al. 2012). Amphibians are of great concern in conservation as these vertebrates are declining at alarming rate. Lack of genetic variability can be a serious cause of reduced fitness and decreased survival rate. (Hebert et al., 2003).

The inner mitochondrial membrane consists of a protein known as cytochrome c oxidase. It plays a primary role in ETC. And hence has a major part in the metabolic activities of multicellular organisms utilizing oxygen. Mitochondrial genome codes for catalytic cytochrome c oxidase subunit I which is among the several subunits of cytochrome c oxidase. Due to this central role in metabolism the COI gene was chosen and also because it is present in almost all eukaryotes. Moreover evolutionary rates are higher for mitochondrial genes than genes coded by nucleus (e.g. small subunit rRNA) and are best adapted to differentiate among similar taxa (Mueller, 2006). The sequence of COI is used as the reliable barcode for almost all specie' taxa. Furthermore, it can also be used for molecular taxonomy and systematics of all living organism. For the identification many fishes, aves and insects including flies, butterflies COI has proven highly efficient. COI uses only 600-650 bp of whole genome to exactly identify species without ambiguity (Hebert *et al*., 2004).

The eukaryotic mitochondrion consists of a protein called cytochrome b. It is an important subunit of b6f complexes and works as part of the electron transport chain. Due to its sequence variability Cytb is usually used as a region of mtDNA for the determination of phylogenetic relationships between organisms. To determine family and genera relationships it is considered most useful. New classification schemes have been developed which involves cytochrome b and its comparative studies. Moreover for studying evolutionary relationships and to assign a genus to newly described species cytochrome b is used. Phylogenetic studies mainly focused on different mitochondrial genes such as Cytb, COI, 12S rRNA and 16S rRNA but insertions and deletions are majorly constrained in taxonomic studies (Doyle and Gaut, 2000). For the identification of animals' globally mitochondrial cytochrome c oxidase I can be used as a basis. It can not only minimize the misidentifications but also provide the genomic information. The prokaryotic ribosome has a 16S ribosomal RNA (or 16S rRNA) component which is present on the 30S small subunit. It binds to the Shine-Dalgarno sequence. The genes that code for it are termed as 16S rRNA gene. Because of the gradual evolution of this genomic region they are used for reconstruction of phylogenies (Nagy *et al*. 2012)

Reptilian species of Pakistan are mostly overlooked and are rarely studied. A total of 195 reptilian species have been known from Pakistan. Among these, 13 species are considered as endemic from country (Khan, 2006). The most threatened groups for vertebrates are reptiles and amphibians. Furthermore, because discovery of cryptic species and rate of new species remains high the specie richness is undervalued. Methods utilizing the morphology for the identification of species and their characterization are time taking and mostly lead to ambiguous results. There has never been an effort to apply a genomic identification system for reptiles in the Pakistan. The present study was therefore planned to identify members of family colubridae from Punjab, Pakistan.

Materias and Methods

Study Area

Specimens were collected from selected sites of Pattoki, Kasur, Okara and Lahore from Punjab province. The temperature starts to rise by mid-February and temperature of the study area varies from 0 °C to 45 °C, but in summer it can reach 50 ° C (122 ° F) (Ali *et al.* 2016; Ali W et al 2024).

Sample collection and preservation

Each captured specimen was tagged with specific number and identified using morphological keys following Ali *et al.* 2016. A few specimen of each species (n=5) were euthanized and preserved in 75% ethanol for molecular analysis.

DNA extraction

Total genomic DNA was isolated from less than 25mg of preserved liver and skin tissues by DNeasy Tissue Kits (Qiagen, Switzerland) as per the manufacturer's instructions. In addition, DNA extraction was also be performed through salt extraction method. Purity of DNA was analyzed through 1% agarose gel electrophoresis in the post-graduate lab, Department of Wildlife and Ecology, University of Veterinary and Animal Sciences, Pattoki.

PCR amplification and Sequencing

Two different mitochondrial DNA fragments were amplified using one set of Cytochrome b (700-750bp) and Cytochrome oxidase subunit I (COI) (1000-1200) primers (Table 1). PCR amplification was done in 25 µL volume reaction with 1-5 µl of DNA. The PCR amplification comprised of 94 °C for 3 min; 40 cycles at 94 °C for 30 seconds, 42–48 °C for 30 seconds depending on the primers anneal-

ing temperature and 72 °C for 1 min; and a final 10 min at 72 °C. The PCR products were checked on 1% agarose gels. Unsuccessful amplified were re-amplified up to two times. Purification of PCR products was accomplished using the Qiagen purification kit and all the samples were sequenced in both directions using dideoxy chain termination direct Sanger sequencing on ABI3730XL DNA Analyzer (Applied Biosystems) from Central Laboratory Complex (CLC), University of Veterinary and Animal Sciences, Ravi Campus Pattoki. The newly obtained DNA sequences were submitted to Genbank for accession numbers.

Data analysis

The newly produced DNA sequences were checked in Bioedit software 7.2 and sequences were aligned in Clustal X (Ali W et al 2024). All the related DNA seqeucnes were downloaded from NCBI for N-J tree analysis using bootstrap value of 100 replicates in MEGA 10. Genetic variations within and between species were calculated in Mega 10.0 based on p-distance.

Results

The present one year study was extended from May, 2019 to March , 2020 was conducted in selected sites of Punjab, Pakistan (Figure 1). During the study, *Platyceps rhodorachis* (Cliff Racer) and *Amphiesma stolatum* (buff striped keelback) were captured. 5 specimens were taken for morphometric measurements and only one specimen of each species was euthanized and preserved in 75% alcohol for molecular characterization.

Snakes captured from study area

The description of captured species captured from study area is below.

Genus Platyceps

Platceps belongs to the largest snake family Colubridae. Its members can be found all around the world except Antarctica.

i. *Platyceps rhodorachis:*

Table 1: List of primers used in the study.

Marker	Primer Pair	Sequence (5–3')	Annealing condition	Reference
Cytb	L14910	GACCTGTGATMTGAAAAACCATCGTTGT	48 °C for 30 seconds	Vences *et al.* 2005a
	H16064	CTTTGGTTTACAAGAACAATGCTTTA		
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	42°C for 30 seconds	
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA		

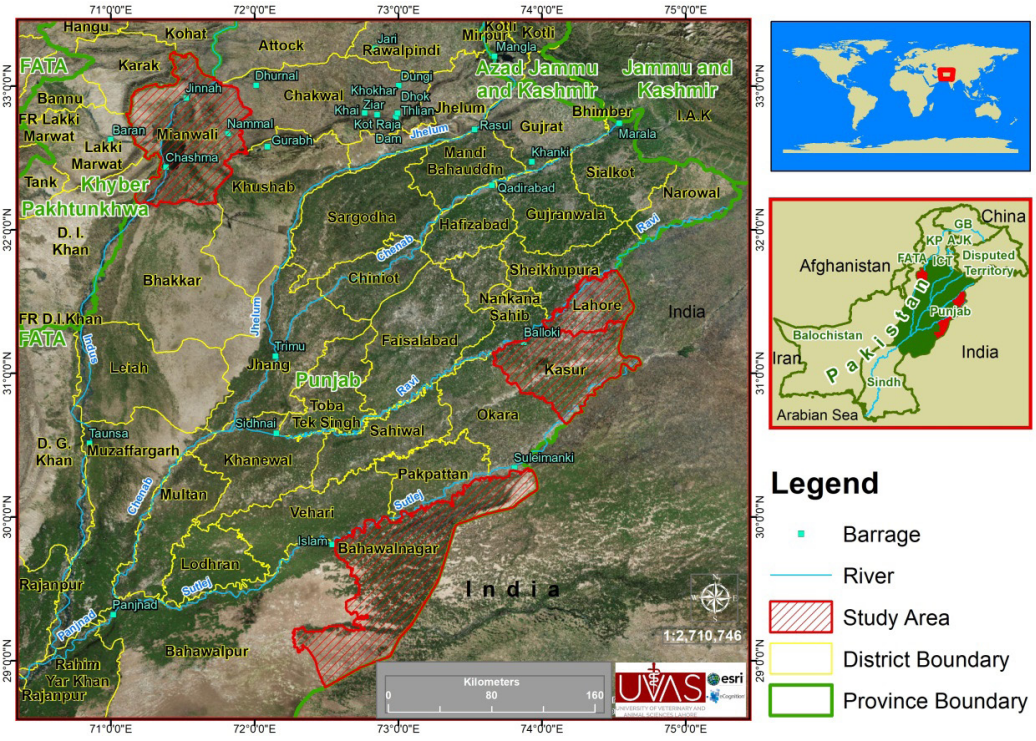

Fig. 1: Map of the study area

Taxonomic position

Reptilia Laurenti, 1768
Squamata Oppel, 1811
Colubridae Oppel, 1811
Platyceps Blyth, 1860
Platyceps rhodorachis (Jan, 1865)

Coloration

Platyceps rhodorachis ranges from dull shades such as dark grey to brown. The body is covered with circular spots at varying distance. Head is usually brown with both oculars (pre and post) being a bit lighter. Lower body is ivory in color.

Morphology

It has a frail body with head being recognizable. It has nine supralabials while 9-10 infralabials are present. The pre and post ocular stripes are present and are quite distinct. The specimens captured had an average snout to vent length of 584.88±93.99 mm. The mean tail length was 176.85±41.65 mm. Mean head length and head width was 18.92±3.71 and 11.53±2.32 mm respectively. Mean body width was 15.26±2.58 mm and mean body weight was 57.40±13.84g. The average total length of captured specimen was 589.74±13.84 mm. The average trunk length was measured to 589.74±91.22 mm (Table 2).

Natural history

It is commonly referred as cliff racer It inhabits rocky places with light vegetation. It occupy narrow spaces such as cracks and cervices of rocks. Being diurnal in nature it roams the areas occupied by birds and rats. It feeds on small lizards, rats, nestlings, small birds and sometimes insects. When cornered it hisses and can bite. The breeding season starts from April and lasts till May. The eggs are laid in rock breaches and are somewhat outstretched. Cliff racer is widely spread in Middle East in countries such as Syria, Iran, Saudi Arabia and Somalia. In Pakistan it can be found in Baluchistan, Karachi and some parts of Punjab.

Genus *Amphiesma*

Amphiesma stolatum is the only specie of genus Amphiesma. It is a non-venomous snake and is closely related to water snake.

i. *Amphiesma stolatum*

Taxonomic position:

Reptilia Laurenti, 1768
Squamata Oppel, 1811
Colubridae Oppel, 1811
Amphiesma Bibron, & Duméril, 1854
Amphiesma stolatum (Linnaeus, 1758)

Coloration

The upper body is uniformly colored greenish to gray with no distinction of head. Both the sides have numerous black spots while the underside is fairly cream colored. The ventral head is cream to bright yellow while the ventral surface of neck region is yellow. Tongue has black stem and gray fork tips. Two yellow or buff stripes extend along the sides upper body.

Morphology

Amphiesma solatum commonly known as buff striped keel back is a small and slender snake. As mentioned before the, it is covered in yellow stripes that run along its length. Body is covered with keeled scales. The supraocular is mildly well evolved forming an obverse ridge and a short anterior distance to the eye.

The specimen captured had an average snout to vent length of 335.37±109.9 mm and a tail length of 74.32±27.44mm. The average head length and head width was found to be 15.47±3.72 and 9.52±2.96 mm, respectively. The mean body width was 13.88±5.43. The average weight of specimen was concluded to be 37.56±14.23 g. The average total length and trunk length was 409.69±137.42 and 339.14± 103.13 mm, respectively (Table 2).

Table 2: Comparison of mean body weight (g) and external body measurements (mm) of species captured from study area.

	Body Parameters							
Species	**SVL**	**TAL**	**HL**	**HW**	**BW**	**W**	**TL**	**T**
Platyceps rhodorachis	584.88±93.99	176.85±41.65	18.92±3.71	11.53±2.32	15.26±2.58	57.40±13.84	761.73±135.64	589.74±91.22
Amphiesma stolatum	335.37±109.98	74.32±27.44	15.47±3.72	9.52±2.96	13.88±5.43	37.56±14.23	409.69±137.42	339.14±103.13

SVL, Snot to Vent Length; TAL, Tail Length; HL, Head Length; HW, Head Width; BW, Body Width; W, Weight; TL, Total Length; T, Trunk Length

Natural history

It is commonly known as striped keel back. Although it is a terrestrial snake but it prefers habitat that are well drenched. Other places include small hills and plains. It is a diurnal snake that feeds on small amphibians such as frogs and toads, but fish, geckos and earthworm are also a part of its diet. Female lay eggs from May till September. They are laid in underground holes and are white in color. Females tend to stay with eggs until they are hatched. Buff striped keel back aestivates in hot weather and can be seen at the end of summer. It expands its body causing exposure to the vivid colors between scales if frightened. *Amphiesma solatum* is distributed through Southeast Asia within countries such as Philippines, Nepal, Cambodia, Indonesia Sri Lanka, India, and Pakistan. In Pakistan it is found in Punjab and lower parts of Sindh.

Amplification and Sequencing

During the study, the DNA of *Platyceps rhodorachis* (Cliff Racer) and *Amphiesma stolatum* (buff striped keelback) were successfully amplified using universal COI primer set. Table 2 summarizes the successfully amplified species and the GenBank accession numbers. After trimming ambiguous bases, the obtained COI fragments of *Platyceps rhodorachis* was 668 bp while fragments of *Amphiesma stolatum* was 639 bp. The COI fragments aligned with available sequence from NCBI comprised of 620 bp.

Phylogenetic relationship

The DNA sequences have shown reliable and exact species identification. Newly produced sequences of *Platyceps rhodorachis* were submitted to GenBank and accession numbers were obtained. Platyceps rhodorachis and closely related sequences were clustered together in the Neighbor-joining tree. N-J tree based on COI sequences of *Platyceps rhodorachis* clearly separated as out-group with other members of family colubridae (Figure 2).

Genetic diversity and variation

The mean intraspecific identities of *Platyceps rhodorachis* (Cliff Racer) and *Amphiesma stolatum* (buff striped keelback) was 0.11±0.01 and respectively based of p-distance. Table 2 summarizes the comparison the genetic identities of family colubridae.

DISCUSSION

Reptiles are distributed in every continent except Antarctica. They inhabit a wide range of ecosystems such as deserts, grasslands, forests and aquatic bodies. Snakes can be considered as somewhat common reptiles after lizards. There are almost 195 reptilian species living in Pakistan. Among these approximately 97 species belong to order serpents. *Platyceps rhodorachis* is distributed throughout Pakistan, Iran, and Afghanistan to central Asia and Northern Africa whereas *Amphiesma stolatum* distribution ranges from East Asia and South Asia, (Khan, 2006). Family Coloubridae is the most abundant snake family with 249 genera, distributed all around the world except Antarctica. It has non venomous snakes. Colubroids were difficult to classify because of their specific characteristics which made them similar to elapids than to each other. In the recent past, due to development in specie recognition methods such as molecular characterization, they have been placed in a separate family.

For the taxon to which the specie is considered to belong, the DNA sample and its sequence will serve as an essential part of the type specie and as a sort of marking,

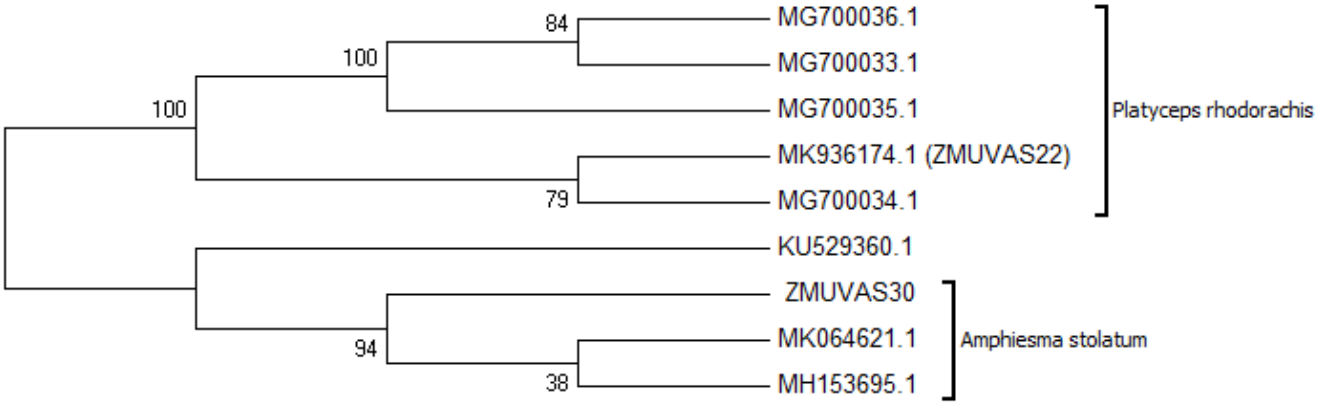

Fig. 2: Neighbor-joining tree of specimens based of COI sequences based p- distances. Bootstrap values are given above the nodes.

Table 2: The interspecific genetic identities of family Colubridae.

Species	MK064621.1	MH153695.1	MH220698.1	KU529360.1	MK936174.1	MG700034.1	MG700036.1	MG700033.1	MG700035.1
MK064621.1	ID								
MH153695.1	1	ID							
MH220698.1	1	1	ID						
KU529360.1	0.996	0.996	0.996	ID					
MK936174.1	0.811	0.811	0.811	0.813	ID				
MG700034.1	0.824	0.824	0.824	0.822	0.885	ID			
MG700036.1	0.822	0.822	0.822	0.824	0.869	0.841	ID		
MG700033.1	0.822	0.822	0.822	0.824	0.869	0.841	1	ID	
MG700035.1	0.82	0.82	0.82	0.822	0.871	0.843	0.998	0.998	ID

or 'barcode.' There are significant benefits to this type of method, especially in groups where morphology is hard to observe or in very disparate people where many taxa still aren't defined. In these circumstances specimens may be allocated easily to known organisms which are most genetically related. These methods can easily distinguish organisms from treated tissues that are often difficult to recognize using morphological methods at different stages of their life cycle.

Since it would be unreasonable to depend solely on morphological features for the classification of species, it is best not to concentrate solely on gene sequences but to use data from several different genes. Results from phylogenetic analysis on data collected from a particular genetic site or multiple linked sites can retrieve the evolutionary history of the gene but do not represent the history of the organism. However, once species boundaries are defined, sequences from an 'ordinary' set of unrelated genes can be used to position individuals within those boundaries

At the sequence variation point, DNA barcoding specie recognition is used where interspecific divergences are substantially greater than intraspecific divergences using a standard DNA fragment (Hebert *et al*., 2003). In snake studies, the COI and the Cytb genes have given distinguishing barcode snipped-off scores. This suggests that COI in snakes is more realistic for barcoding DNA than Cytb. (Rubinoff *et al*., 2006). In comparison, the COI data set showed several unique nucleotide mutations that separated each species but not the Cytb. This indicates that the Cytb gene replacement rate was higher than the COI gene's (Laopichienpong, 2016;Folmer *et al*. 1994).

The DNA sequences showed accurate and precise identification of organisms. Newly generated *Platyceps rhodorachis* sequences have been submitted to GenBank and accession numbers have been obtained. In the Neighbor-joining tree were clustered together *Platyceps rhodorachis* and closely related sequences. N-J tree based on COI sequences of *Platyceps rhodorachis* clearly differentiated from other members of the family colubridae as an out group. *Platyceps rhodorachis* (Cliff Racer) and *Amphiesma stolatum* (buff striped keelback) mean intraspecific identities were 0.11 and p-distance dependent, respectively.

In the forensics Mitochondrial DNA is now a valuable resource, since the increased amount of copies in each cell enhances susceptibility and inadequacy of hybridization which facilitates the lack or formation of polymorphs of mtDNA. The success of identification using Neighbor joining tree was initially assessed by Herbert *et al*., (2003). The sequence identification is considered effective when

sequences formed are species-specific clusters. As mentioned above, identification of species through barcodes allow, a wide resolution, of the geographical origin of the samples. This makes barcoding of DNA an excellent method for tracking these animals' natural and human-mediated migration activities. The COI gene provides a viable alternative for species identification, but appropriate databases and further work will need to be backed up. For practical reasons, the collection of data through DNA sequencing using recognized markers should advance quickly, with growing demand for identification of biodiversity in bio resource restoration and management.

The molecular identification of the family Colubridae presents a significant advancement in understanding the evolutionary relationships, genetic diversity, and species delimitation within this large and diverse group of snakes. Colubridae, one of the largest snake families, comprises a vast range of species with diverse ecological roles, morphological characteristics, and geographic distributions. Traditional taxonomic classifications based on morphology often face limitations due to the phenotypic plasticity and convergence found within the family. This research, utilizing molecular techniques, provides a more robust framework for identifying and resolving the taxonomic complexities of the family Colubridae.

Molecular methods, particularly DNA barcoding and sequencing of mitochondrial genes like cytochrome c oxidase I (COI) and 16S rRNA, have proven to be invaluable in distinguishing cryptic species and identifying evolutionary lineages. In the present study, molecular markers successfully differentiated species within Colubridae, confirming that molecular identification is a more reliable tool than traditional morphology-based taxonomy, especially when dealing with cryptic species. The use of multiple molecular markers strengthens the accuracy of identification by cross-referencing between genes, thereby reducing the risk of misidentification that may occur due to gene-specific evolutionary constraints.

One of the key findings in the study was the revelation of significant genetic divergence between species that were previously thought to be closely related based on morphological characteristics. This underscores the importance of molecular data in resolving taxonomic uncertainties. The mitochondrial COI gene, widely used in DNA barcoding, provided high-resolution species-level identification, while 16S rRNA, being more conserved, offered deeper insights into the phylogenetic relationships between different genera within the family. The integration of both markers not only enhanced the resolution of species identification but also allowed for the detection of potential new species or subspecies that had not been recognized before due to morphological similarities.

Furthermore, this molecular approach has ecological and conservation implications. Accurate species identification is crucial for developing conservation strategies, particularly in regions where Colubrid snakes are under threat due to habitat loss, climate change, or human-wildlife conflict. Misidentification of species can lead to ineffective or misguided conservation efforts. By applying molecular tools, this study provides a foundation for more precise ecological assessments and habitat management plans for Colubrid snakes, which play key roles in controlling pest populations and maintaining ecosystem balance.

The study also highlights the role of molecular identification in understanding biogeographic patterns and evolutionary history. Phylogenetic analysis based on molecular data revealed the presence of several distinct lineages within Colubridae that correspond to geographic isolation and adaptation to different environmental conditions. These findings support the hypothesis that historical biogeographic events, such as continental drift and climatic shifts, have played a critical role in shaping the current diversity and distribution of Colubrid snakes. Such insights into the evolutionary dynamics of the family Colubridae could inform further research on the adaptive strategies and speciation processes in reptiles.

Despite the clear advantages of molecular identification, there are still challenges that need to be addressed. The reliance on mitochondrial DNA (mtDNA) markers, although useful for species identification, may not always capture the full genetic diversity present in nuclear genomes. Therefore, future studies should aim to incorporate nuclear markers, such as microsatellites or single nucleotide polymorphisms (SNPs), to provide a more comprehensive view of genetic diversity and evolutionary relationships within Colubridae. Additionally, the limited availability of reference sequences in public databases remains a constraint for identifying less-studied species, emphasizing the need for continued efforts to expand the molecular database for Colubridae and other snake families.

Conclusion and Recommendations

It can be concluded that, identification of reptiles on morphological basis is still considered authentic. However, large scale molecular analysis of these taxa is required for exact species identification and to report any new species from area.