FORENSIC DNA MARKERS IN SOME THREATENED SPECIES OF SNAKES: ISOLATION AND CHARACTERISATION OF MICROSATELLITE FROM RAT SNAKE (*Ptyas mucosa*)

Thesis submitted for the

DEGREE OF DOCTORATE OF PHILOSOPHY (SCIENCE)

JADAVPUR UNIVERSITY

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CERTIFICATE FROM THE SUPERVISOR(S)

This is to certify that the thesis entitled **"Forensic DNA Markers in Some Threatened Species of Snakes: Isolation and Characterisation of Microsatellite from Rat Snake (*Ptyas mucosa*)"** submitted by **Smt. Ishani Mitra** who got her name registered on **7th August 2014** with **Index No.: 150/14/Life.Sc./23** for the award of Ph. D. (Science) degree of Jadavpur University, is absolutely based upon her own work under the supervision of **Dr. Ikramul Haque**, Director, Central Forensic Science Laboratory, Chandigarh and co-supervision of **Dr. (Mrs) Soma Roy**, Assistant Director, Central Forensic Science Laboratory, Kolkata and that neither this thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.

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Certificate of Permission

Permission for the collection of biological samples had been obtained from competent authority: under Section 12 of Wildlife Protection Act, 1972, Ministry of Environment, Forests and Climate Change (Wildlife Divisions), Govt. of India, New Delhi, India and Principal Conservator & Chief Wildlife Warden, West Bengal, India.

(Ref: F. No. 1-28/20015 WL-I Dated: 21st May, 2015
Memo No. 3843/WL/4R-6/2015; dated 13th July, 2017)

Dedicated

to my Father

Late Dilip Kumar Mitra

&

my Son

Master Amitrajit Datta

"There is no one giant step that does it, it's a lot of little steps".

- **Peter A Cohen**

ACKNOWLEDGEMENTS

Our faith is made effectual when we acknowledge everything good in us.
- Joel Osteen

I take this opportunity to acknowledge and extend my sincere gratitude to all who made this Ph.D. thesis a possibility. The work described in this thesis would not have been possible without the support and necessary facilities from ***Central Forensic Science Laboratory, Kolkata*** *where I continued my research work and* ***Jadavpur University, Kolkata*** *where I have registered my name as research fellow. I would like to express my sincere gratefulness to* ***Mr. Priyankar Ghosh (Ex-Director of Central Forensic Science Laboratory, Kolkata)****,* ***Dr. Anil Kumar Sharma (Director of Central Forensic Science Laboratory, Kamrup) and Mr. B. Badoniya (Director of Central Forensic Science Laboratory, Kolkata)*** *for letting me pursue my dream by giving me all kind of opportunities and providing me with all the facilities at their esteemed laboratory.*

At this point, I take immense pleasure in expressing my heart-felt gratitude and deep sense of thanks for my Ph.D. supervisor respected ***Dr. Ikramul Haque (Director of Central Forensic Science Laboratory, Chandigarh)*** *for his infinite patience, constant encouragements to look forward and the inspiration to develop a deep knowledge to excel in scientific world which made me the researcher I am today. I greatly appreciate the guidance and advice you offered whenever needed sir. I thank you from the bottom of my heart for nurturing me with your guidance and care****.***

My heart-felt appreciation to my Ph.D. co-supervisor ***Dr. (Mrs) Soma Roy (Assistant Director (Biology) of Central Forensic Science Laboratory, Kolkata)*** *for giving me training on practical knowledge and her constant motivation, inspiration and support during the period of my work without which I could not have made this thesis possible.*

Thanks are also due to all my respected and concerned faculties ***Prof. Parimal Karmakar, Prof. Ratan Gachhui, Prof. Biswadip Das*** *and* ***Dr. Paltu Kumar Dhal****, from the department of Life Science and Biotechnology, Jadavpur University for their lectures, notes and supports in clearing doubts during the period of Course work for Ph.D. there as well as all to the Scientific officers* ***Dr. Kshitij Chandel****,* ***Dr. Devendra Singh Yadav****,* ***Mr. A. K. Mandi****,* ***Mrs. Sumita Bhattacharya****, Senior-scientific assistant* ***Mrs. J. P. Purti*** *from Biology division of CFSL Kolkata for their teaching, training and guidance during this tenure of work.*

*I will be forever grateful to my seniors **Dr. P. R. Meganathan, Dr. Bhawna Dubey** and **Dr. K. Naga Joagayya** who rendered their helping hand whenever needed and kept me motivated with their inspirational words and feedback.*

*A special thanks to my juniors **Mr. Dinesh D** and **Miss Shreya Saha** who are working as research fellow in the Biology Division, the librarian, the office staff and all others who have helped me directly or indirectly in the successful completion of my thesis at **Central Forensic Science Laboratory, Kolkata, West Bengal, India.***

*I am thankful to the **Ministry of Environment and Forest (Wildlife Divisions), Government of India, New Delhi, India** and **Principal Conservator & Chief Wildlife Warden, West Bengal, India** for providing the permission to collect the research samples from the **Zoological Garden, Alipore, Kolkata, West Bengal, India; Wild Animal Rescue & Transit Facility Centre, Salt Lake, West Bengal, India** and **Junglemahal Zoological Park, Jhargram, West Bengal, India**. I would like to sincerely thank **Shri Asis Kumar Samanta, IFS; Dr. Basavraj S Holeyachi** for kindly providing me with research samples from their respective zoological gardens. Also I am thankful to **Anthropological Survey of India, Kolkata, West Bengal, India** for providing me with technical help during laboratory experiments.*

*My deepest sense of gratitude, respect and love to my **Late Father, my elder sister, my younger brother** and the dearest friend of mine who eventually at present my **better half,** for their whole-hearted inspiration, sacrifices and wishes. A special heart-felt gratitude and deepest appreciation to my **Maa (my Mother)** who always stood beside me, sacrificed for me and take care of my family in my absence. **Maa** without your kind support and constant encouragements this endeavour of mine would never get its final shape. Please stay beside me like this in future too **Maa**.*

*Lastly, I would like to thank my little baby **Master Amitrajit Datta** who came to my life during this period of my research journey and whose presence always enlightened my gloomy mood, whose struggle to reach every growth milestone became my inspiration to keep trying on my work and who never failed to make me smile during the worst phase of my work which made the journey of my research endearing.*

*Above all I humbly thanks to **Almighty God**, whose sustaining grace and blessings have been sufficient for me to complete this endeavour.*

ISHANI MITRA

ABSTRACT

Destroying habitats and illegal trading of body parts can endanger the animals, plants, and other organisms that live at a biodiversity area. The impact of unprecedented declination due to illegal trafficking of species can be extremely detrimental to the endurability of exposed populations in the wild and is a serious worldwide concern for wildlife management to stop the illegal smuggling, hunting and poaching of wildlife, be it for their medicinal value or ornamental body parts.

Technological advancement in human forensic provides a backbone for Wildlife investigation. Use of molecular markers for identification of protected species offers a greater premise in the field of conservation biology. The information on genetic diversity of wildlife is necessary to ascertain the genetically deteriorated populations so that better management plans can be established for their conservation. Accurate classification of these threatened species allows understanding of the species biology and identification of distinct populations that should be managed with utmost care. Molecular markers are versatile tools for identification of populations with genetic crisis by comparing genetic diversities that in turn helps to resolve taxonomic uncertainties and to establish management units within species. The genetic marker analysis also provides sensitive and useful tools for prevention of illegal hunting and poaching and for more effective implementation of the laws for protection of the endangered species. This piece of work proposes to study tools of DNA markers technology for application in molecular diversity analysis with special emphasis on wildlife conservation.

Hence, for this study we have selected the Indian snake species which are listed in the Appendix II and III of CITES. According to 2009 Red list these species are lower risk and it's near to threatened. The information gather from the field of genetics/molecular biology could be of immense value for conservation efforts of these snake species in Indian flora and fauna. In view of the above, it is considered conservation and rebuilding goals are thus urgently required. There is always a need to use genetic data for better understanding of evolutionary relationships, the genome composition and variations in the endangered species.

Thus, this thesis is focused on finding new molecular identification techniques for the proper identification of snake exhibit specimens. It is divided into two Parts, Part I containing two chapters i.e. Introduction with Objective of this study and Literature Review and Part II containing three chapters which are formatted in a paper style consisting of a preface, introduction, materials and methods, results and discussion, conclusions, and references sections. The Part II describes newly designed primers for snake species identification, building up mitogenome database as well as phylogenetic inference from complete mitogenome study and development of novel microsatellite markers from Indian Rat snake (*Ptyas mucosa*) for conservation purpose. The DNA based study is expected to offer some prospective taxonomic characterization to some unknown or unstudied snake samples. Hence, a wise approach has been taken to develop new identification techniques of snake species through molecular genetics and developing microsatellite marker for conservation studies which will enable investigation of the population genetic characteristics of this species throughout its distribution.

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ABBREVIATIONS

AFLP	–	Amplified Fragment Length Polymorphism
$BLAST_n$	–	Basic Local Alignment Search Tool
C-mos	–	Cellular Murine Oocyte Sarcoma
CITES	–	Conservation on International Trade in Endangered Species of Wild Fauna and Flora
CMCF	–	C-mos Common Forward Primer
CMDRR	–	C-mos *Daboia russellii* Reverse
CMPMR	–	C-mos *Ptyas mucosa* Reverse
CMXPR	–	C-mos *Xenochrophis piscator*/ *Fowlea Piscator* Reverse
DNA	–	Deoxy-ribo Nucleic Acid
EST	–	Expressed Sequence Tag
IUCN	–	International Union for Conservation of Nature and Natural Resources
mtDNA	–	Mitochondrial DNA
NCBI	–	National Centre for Biotechnology Information
NGS	–	Next Generation Sequencing
PCR	–	Polymerase Chain Reaction
RFLP	–	Restriction Fragment Length Polymorphism
RAPD	–	Randomly Amplified Polymorphic DNA
SNP	–	Single Nucleotide Polymorphism
STR	–	Short Tamdem Repeats
WPA (1972)	–	Wildlife Protection Act, 1972
12S rRNA	–	12S ribosomal Ribo Nucleic Acid
16S rRNA	–	16S ribosomal Ribo Nucleic Acid
16MCF	–	16S ribosomal Ribo Nucleic Acid Common Forward
16MDRR	–	16S ribosomal Ribo Nucleic Acid *Daboia russellii* Reverse
16MPMR	–	16S ribosomal Ribo Nucleic Acid *Ptyas mucosa* Reverse
16MNNR	–	16S ribosomal Ribo Nucleic Acid *Naja naja* Reverse

CHAPTER I

INTRODUCTION

"We should preserve every scrap of biodiversity as priceless while we learn to use it and come to understand what it means to humanity."

~ E. O. Wilson

1.1 Biodiversity & Conservation

Ever since the origin of life on earth about 3.8 billion years ago, there had been enormous changes of life forms on earth. Be it a microorganism or a fungi or plants or complex animals, earth has witnessed all of them and their changes with time. The existence of different range of life also developed biological diversity among them. Biodiversity, the short form of biological diversity, includes all living organisms, species and populations and their interrelatedness with the environment. It is the term given to the diverse range of life on Earth to support the functions of ecosystem (Avise, 1995). It is said that diversity breeds diversity. Having a diverse range of living organisms allows other organisms to take advantage of the resources provided. For example, trees provide habitat and nutrients for birds, insects, other plants and animals, fungi, and microbes. It enriches the worth of our lives in ways that are not easy to enumerate. Biodiversity is the most precious gift of nature that mankind is blessed with. As all the organisms in an ecosystem are interlinked and interdependent, the value of biodiversity in the life of all the organisms including humans is enormous.

The biodiversity of an organism is measured at three levels - the Genetic diversity, the Species diversity and the Ecological diversity (Wilson, 1988). Genetic diversity is all the variety of traits contained in all individual plants, animals, and microorganisms. For example, the colour of human eye i.e. Brown and Blue. It occurs within a species as well as between species. Species diversity is the number of different species and population of those species that are found in a particular location. For example, India's Western Ghats is endemic for Nilgiri Tahr or *Hemitragus hylocrius*, Lion tailed macaque or *Macaca silenus*, along with other species of animals and plants like Asian elephant, Indian Gaur, Sloth bear, Wild Durian, Kindal etc. Ecological diversity refers to the variations in the ecosystem within a geographical region and their impact on humans as well as on the environment as a whole. It includes different habitats, different biological communities, and ecological processes, as well as variation within individual ecosystems. For examples, geographical regions like deserts ecosystem or forests ecosystem or oceans ecosystem. They all bears different types of species which are directly or indirectly associated with each and every species surviving on that ecosystem.

Thus, protecting biodiversity is in our self-interest. Some consider that it is an important human responsibility to be stewards for the rest of the world's living

organisms. It is the moral responsibility of a Wildlife Biologist to conserve wildlife species and their habitat, to preserve those diverse species which is necessary to balance the ecosystem, and pass it on to our next generation in good state (Frankham *et al.*, 2002). The International Union for Conservation of Nature and Natural Resources or IUCN is a membership Union composed of both civil society and government organisations to conserve nature and accelerate the evolution to sustainable advances. They work on the status ofthe nature,its resources and the measures neede to safeguard them. The organization is best known for compiling and publishing the IUCN Red List of Threatened Species**,** 1964 which assesses the conservation status of species worldwide and is the world's most comprehensive inventory of the global conservation status of biological species. According to the IUCN Red List (2021) more than 1.3 million types of species including all microorganisms, plants and animal species have been recorded in the world but it is assumed that there still might be nearly 6 million species which are waiting to be discovered and named. It documents the extinction of 784 species (including 87 plants species, 338 vertebrates and 359 invertebrates) with more than 38,500 species threatened with extinction in the last 500 years (including 41% of amphibians, 37% of fishes, 34% of conifers, 33% of reef building corals, 26% of mammals and 14% of birds)

1.1.1 Biodiversity of India

India is bestowed with rich and diverse floral, as well as faunal range due to its unique and wide distribution of bio-geographic areas. It is one of the world's most bio-diverse regions which covers an intensive range of ten eco-zones such as, the trans-Himalayan, the Himalayan, the North-East, the Gangetic plain, the Western ghats, the Deccan Plateau, the Semiarid, the Deserts, the Coastal and the Islands (Rodgers *et al.*, 1988, 2000). In India approximately 47, 000 types of plants species and twice their numbers of animal's species have been reported. Hence, all the organisms which are presents in such geographic locations needs to be conserve for India's ecological system as India is affluent of biodiversity and placed 12th among 17 mega diversity countries in the world (https://www.biodiversitya-z.org/content/megadiverse-countries). This immense diversity has resulted in the inclusion of India as biodiversity hotspots among twenty five other global Biodiversity hotspots (Myers *et al.*, 2000). They are the Western ghat, the Eastern Himalaya and the hilly range of India-Myanmar border, which encompassing the world's diverse ecosystem. These hotspot regions are abode for many wild species like Royal Bengal Tiger, One-horned

Rhinos, Asiatic Lion, Asiatic Elephant, Snow Leopard, Nilgiri Tahr, Lion tailed Macaque, Indian Rock Python, Indian Cobra, Great Indian Bustards, Indian Vulture etc. to name a few (Table 1.1 briefly describes about some endangered Indian Wild Species with reference of their body parts that are used illegally). But, due to over exploitation, illegal poaching and trading, the wildlife populations in India is being vehemently destroyed and many rare elements of Indian wild fauna are facing a threat to their survival, many are almost at the verge of extinction (Freeland, 2005).

Table 1.1: List of some endangered Indian Wild Species with reference of their body parts that are used illegally

Species	Type of body parts used
Tiger	Skin, Claw, Head, Teeth, Bones
Elephant	Tusk
Rhinoceros	Horn, Blood
Crocodile	Skin, Meat
Indian Rock Python	Skin, Meat
Gharial	Tissue, Blood
Cobra	Tissue, Skin
Scarlet Macaw	Feathers
Tibetan Antelope	Skin, Horn, Shehtoosh shawls
Turtle	Skin, meat

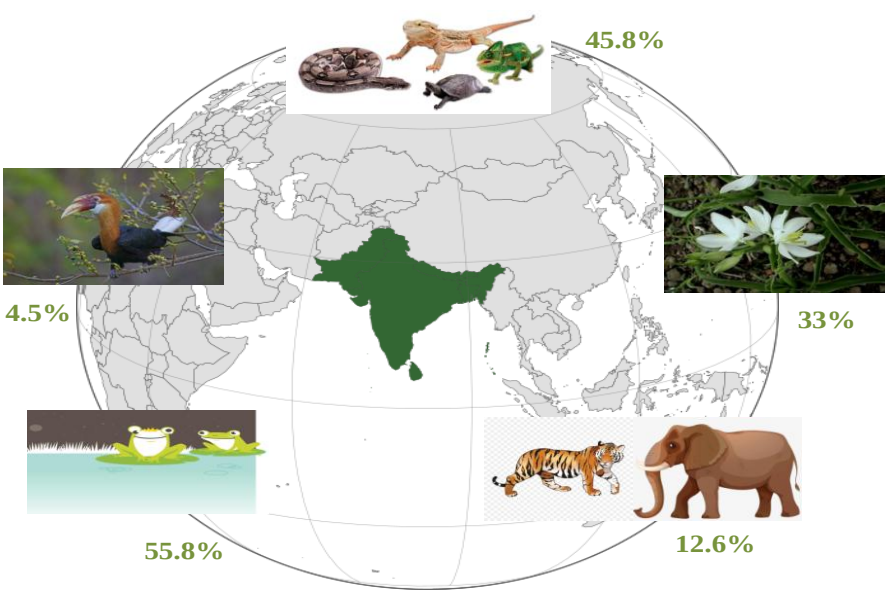

Figure 1.1: A species wise endemic content of the Indian Biodiversity

1.2 Illegal Wildlife Trading and Role of Legislative Agencies in Conservation

The illegal wildlife hunting and trading is one of the the major money making trades which attracts approximately an annual value of US$23 billion globally. (Alacs and Georges 2008, Wyler and Sheikh 2008). The South-Eastern Asia is considered as a central hub for the illegal wildlife trading and poaching due to apparently lower risk of trafficking of wild endangered species across this region (Rosen and Smith, 2010) and it's worth 25% of the total illegal trading of wildlife. (Lin, 2005; Elliott, 2007). This illegal trading is fuelled by constant demand for rare, endangered, and lucrative wild species across the continents. The body parts of different endangered animals are mostly traded to East Asian countries like China, Korea, Japan and to South-East Asian Countries like Indonesia, Malaysia, Philippines, Singapore, etc. from India for earning lucrative value. They are often killed due to fear, or wrong belief that all snakes are venomous, or due to the medicinal value of their blood, bones and gallbladder, or for their nutritious meat and lastly for entertainment purposes. They are used as traditional Chinese medicines, exotic cuisines, symbols of good luck and wealth, manufacturing of accessory items like leather shoes, bags, belts, wallets etc. using their attractive and charismatic skin or as interior decorations (Roberts *et al.* 2002). Some of the extremely highly prized animals include mammals like tigers, rhinoceros, elephants, pangolin, bears etc.; reptiles like pythons, vipers, cobras etc.; birds like parrots, macaw, cockatoos etc. and fishes. (Thomas *et al.*, 2006; Alacs and Georges, 2008; Linacre and Tobe, 2008; Zaw *et al.*, 2011) and plants species includes sandalwood, timber woods and rare species of orchids (Beek *et al.*, 2010; Lee *et al.*, 2010; Parveen *et al.*, 2012).

The Convention on International Trade of Endangered Species of Wild Fauna and Flora (CITES) was created in an effort to fight the illegal trade of endangered species at international level. CITES was first initiated in July 1975. There are three Appendices of CITES. According to the conservation status of species Appendix I list those species which are in direct threat of extinction and species listed in Appendix II include those where illegal trading may impact. Lastly, Appendix III are those that have been selected for trade by another parties in controlling their international trade (CITES 2013).

India has been a member party of CITES since 20 July 1976. The international and national trade of wildlife in India as it pertains to CITES listed species is governed under several laws including The Indian Forest Act (1927) and Forest Acts of State

Governments, Wildlife Protection Act (1972), The Forest Conservation Act (1980), The Environment (Protection) Act (1986), Foreign Trade Act (1992) and ForeignTrade Policy, National Forest Policy (1998), The Biological Diversity Act (2002) and National Wildlife Action Plan (2002-2016). The main provisions of CITES are enforced through Customs Act 1962. The principle law governing wildlife protection on a national bais is the Wild Life Protection Act 1972, which has been amended several times, i.e., in 1991, 2002, 2003 and latest on 2006, to include new species, hard protection and higher penalties (Heinrich *et al.,* 2021).

However, illegal trade in endangered species remains abundant. In India as per Wildlife Protection Act, (1972) the maximum punishment is 5 years imprisonment with fine which may extend to fifty thousand rupees.

1.3 Conservation through Genomics

The ultimate foundation of biodiversity is genetic diversity and for conservation of animal species genetic technology has many applications (Ogden *et al.,* 2009; Iyengar, 2014). The science of Genetics looks at inherited characteristics and the genes that underlie them. Conservation genetics uses a combination of ecology, molecular biology, population genetics, mathematical modelling and evolutionary taxonomy (the study of family relationships). It is both a basic and an applied science. A population with high genetic diversity will have a genetic makeup that allows them to survive local environmental conditions (Kumar *et al.*, 2000). These individuals will reproduce, and the population will survive. Conserving these adaptations increases the ability/chances for species to survive long (Saccone *et al.*, 2005). In case of threatened and endangered species to increase the genetic diversity of their population they can be ‘forced’ migrate or set up captive breeding programs. Conservation genetics or the application of genetics to the preservation of species has received increasing attention in recent years (Allendorf *et al.*, 2007; Frankham, 2003). The loss of genetic diversity results in lower individual fitness and poor adaptability (Lenton *et al.,* 2006). Genetic studies can reduce the extinction risk by helping population management programs.

1.3.1 Role of Wildlife Forensic in Conservation

Forensic science is any scientific field that is applied to the field of law and Wildlife forensic science is science that is applied to legal questions involving wildlife. It applies a range of scientific disciplines to legal cases involving non-human biological

evidence, to solve crimes such as poaching, animal abuse, and trade in endangered species (Butler, 2005). Wildlife Forensic Science deals with activities including illegal trafficking of protected wildlife, unfettered poaching and smuggling of their body parts. It is essentially concerned with the identification of evidence items in order to determine the species, population, relationship or individual identity of a sample (Ogden *et al.*, 2009). Most often, the evidence items are morphologically altered and go through many wearing and tearing as well as turn into confiscated items. Identification of a specimen becomes challenging when such evidentiary materials are seized.

To implement the WPA (1972), it becomes necessary to properly identify each crime exhibits upto species level. The ability to enforce CITES legislation along with WLPA in countries has been limited due to the lack of accurate and validated analysis techniques that can unambiguously identify the presence of endangered or trade restricted species in seized wildlife materials which results in difficulties in building a case against alleged criminals and leads to an inability to fully prosecute those suspected of committing wildlife crime. The variety of evidence in Wildlife Forensic cases is infinite, potentially encircling the entire biodiversity of the planet. It can range from a trunk full of animal fur to a van full of leather garments and boots made from skin of snakes or crocodiles, to shipments of elephant tusks, pangolin scales and meat, shark fins, trophy elk, sun bear bile, oil-soaked birds to wild ginseng and coral jewellery (Cassidy *et al.*, 2005; Gupta, 2012). Hence, Methods for species identification testing need to be fast and have a high degree of accuracy for submission as evidence in a court of legal proceedings. The field of wildlife forensics is in much need of validated and standardised tools, as in human forensics, for successful prosecution of wildlife trafficking.

1.3.1.1 Forensic Identification using Morphological Analysis Techniques

The preliminary examination of an evidence sample is performed by morphological analysis. This process includes identification using microscope for hair, feather or pollen identification, or other techniques that are used in anthropology (Singh *et al.*, 2006). When the samples are in their ideal condition they can be easily identified by their morphological features. This requires the examiner to be highly skilled and experienced to perform analysis on different types of samples (Bell, 2011). However when the samples are degraded or chemically treated or very small in quantity it becomes difficult to identify them. Therefore, advanced techniques, such as

chromatography, mass spectrometry, proteomics or DNA techniques are applied for species identification.

1.3.1.2 Forensic Identification using Chemical Techniques

To identify confiscated wildlife materials and for wildlife forensic applications many chemical examinations are performed in the forensic laboratory. Mainly chromatographic and mass spectrometry techniques are frequently practice to identify traces of plant species within traditional medicines (David *et al.,* 2010). Metabolomic is another technique that can also be performed to identify the presence of adulterants in herbal pharmaceutical drugs (Liu *et al.*, 2001, Buriani, Garcia-Bermejo *et al.*, 2012). Also, there is optical immunoassay method which was developed to identify the snake venom (Dong *et al.*, 2004). But, these methods also fail when the seized samples are heat treated as well as chemically altered.

1.3.1.3 Forensic Identification using Molecular Marker

The discovery that some DNA sequences are unique to an individual has revolutionized the field of Forensic science to the level where almost any species can be genetically characterized with a high degree of certainty (Budowle *et al*., 2005; Ibrahim *et al.*, 2011). With the development of national and international legislation to protect ever diminishing habitat and biodiversity, DNA forensics is now becoming a key investigative tool to combat wildlife crimes (Gupta *et al., 2011*). Recent advances in molecular genetics such as mitochondrial DNA and Microsatellite DNA can be undertaken non-invasively using single blood drop, shed skin fragments or faecal samples (Dubey, 2010). Thus, DNA fingerprinting from non-human DNA sample is a fast evolving area of research and professional practice and its applications in Wildlife Forensic Science is gaining increasing profile (Table 1.2 briefly shows name of some Endangered Indian species with their expensive body parts and types of Investigation in DNA Forensic).

A Molecular marker or a genetic marker is a gene or DNA sequence with a known location on a chromosome that can be used to identify individuals or species. Basically two types of markers are used for Wildlife DNA forensic analysis.

i. Mitochondrial Markers
ii. Nuclear Markers

Markers derived from nuclear genes are not available for a majority of wildlife, but that might change. Currently, mtDNA markers dominate the wildlife area for species identification.

Table 1.2: Name of some Endangered Indian species with their expensive body parts and Types of Investigation in DNA Forensic

Species	Type of body parts used	Types of Investigation in DNA Forensic
Tiger	Skin, Claw, Head, Teeth, Bones	Species identification, Individual identification. (Xu *et al.* 2005, Linacre and Tobe. 2008, Gupta *et al.* 2011)
Elephant	Tusk	Origin determination, Species identification. (Wasser *et al.* 2008, Lee *et al.* 2013)
Rhinoceros	Horn, Blood	Species identification, Individual identification. (Hsieh *et al.* 2003, Harper *et al.* 2013,)
Crocodile	Skin, Meat	Species identification, Individual identification. (Meganathan *et al.* 2011, Naga Jogayya *et al.* 2013)
Asiatic black bear	Hair, Leather	Species identification. (Peppin *et al.* 2008, Jun *et al*.2011)
Gharial	Tissue, Blood	Species identification. (Naga *et al.* 2013)
Cobra	Tissue, Skin	Species identification. (Gaur *et al.* 2012)
Scarlet Macaw	Feathers	Species identification. (Abe *et al.* 2012)
Tibetan Antelope	Skin, Horn, Shehtoosh shawls	Species identification. (Ahmed *et al.* 2016)
Turtle	Skin, meat	Species identification. (Hseih *et al.* 2006, Stirtzel *et al.* 2007, Lee et al. 2009)

1.3.2 Mitochondrial DNA and its Application in Wildlife Forensic

The cytoplasm of each cell contains several hundred mitochondria. Through the oxidative phosphorylation process (OXPHOS), these essential organelles produce ATP, the energy source used in cellular metabolism. Sometimes called the 47th chromosome, mitochondria have their own distinct DNA called mtDNA, which is comprised of a small double stranded circular molecule and 16-17 kb in length (Robin, 1988; Naga *et al.*, 2013b). The mtDNA molecule contains only 37 genes. These include 24 genes involved in transcription of DNA into proteins (tRNA and rRNA genes) and 13 protein coding genes (PCG's) that code for enzymes used in the mitochondria (the complex involved in OXPHOS). These PCG's evolve much faster than other mitochondrial gene (mtGene) and work as a powerful marker to study the

phylogenetic history inferring families, genera and species. Among 13 PCG's Cytb, COI, NDI & ND2 are regularly used for solving a non-human case as universal primers are available for them. Besides, the ribosomal subunits are considered to be highly conserved and therefore used for the study of phyla and genera of a specimen. As the mitochondrial control region or D-Loop is fastest evolving region of the genome they are broadly used for inter and intra specific phylogenetic analysis and can be used for the study of geographical distribution of a species. This also helps in replication of mitochondrion (Brown *et al.*, 1979. The mutation rate in mtDNA is ten times higher than in nuclear DNA because mtDNA are subject to damage from reactive oxygen molecules released as a by product during OXPHOS (Boore JL, 1999; Parson *et al.*, 2001). In addition, the mtDNA also lacks the DNA repair mechanisms found in the nucleus. This MtDNA is much more resistant to the factors causing nuclear DNA degradation (Gupta *et al.*, 2005).

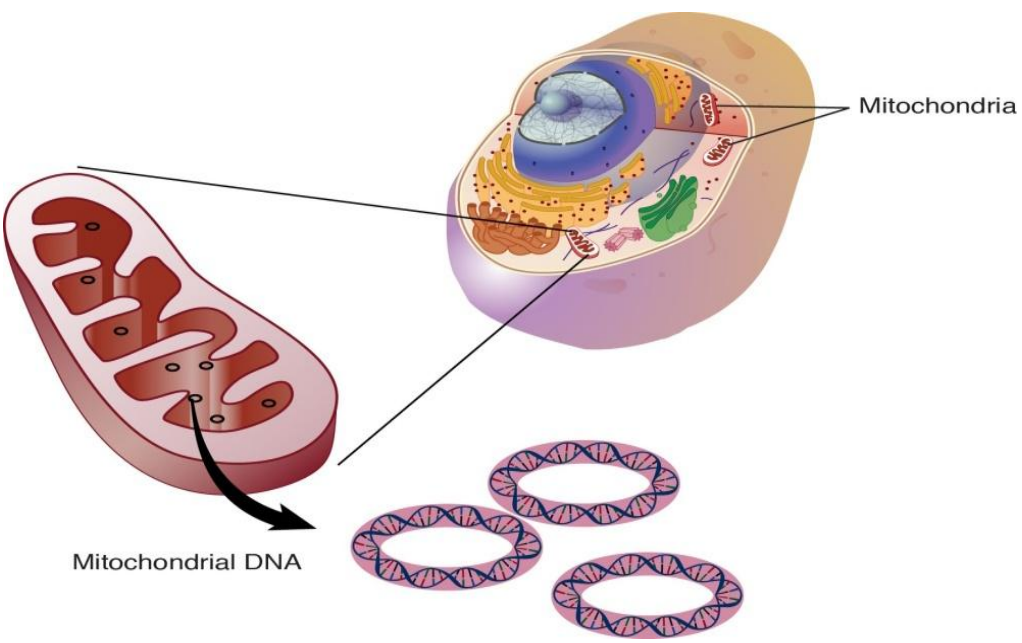

Figure 1.2: Animal mitochondrion diagram

1.3.2.1 Commonly used Mitochondrial Markers in Wildlife Forensics

In Wildlife Forensic the use of Mitochondrial DNA markers has many advantages over the nuclear markers (Parson *et al.*, 2001; Dubey *et al.*, 2009a):

i) The mtDNA genes code for essential life processes and hence remain conserved over many generations.
ii) Each cell has thousands of copies of mtDNA genes.

iii) The structure and location of mitochondrion in the cell makes mtDNA more stable, enabling to test very old samples and can be extracted from a very small sample.

iv) They show maternal inheritance and hence more secure markers for studying family trees.

v) mtDNA sequences evolve at a faster rate than the nuclear DNA sequences and genetic drift occurs more slowly among the alleles at nuclear locus.

The most commonly used mitochondrial markers are cytochrome b (Parson *et al.*, 2000; Varma *et al.*, 2003; Hsieh *et al.* 2006) and cytochrome oxidase subunit I (COI) (Herbert *et al.*, 2003a, b) because of their high mutation rates and availability of universal primers. Apart from these the ribosomal genes: 12S rRNA and 16S rRNA have been successfully utilized in forensic identification studies (Dubey *et al.*, 2009b; Meganathan *et al.*, 2011; Naga *et al.*, 2013b) and subunits of NADH dehydrogenase and control region or D-Loop are others markers used in forensic purpose. But works on all Indian endangered species are not reported till date.

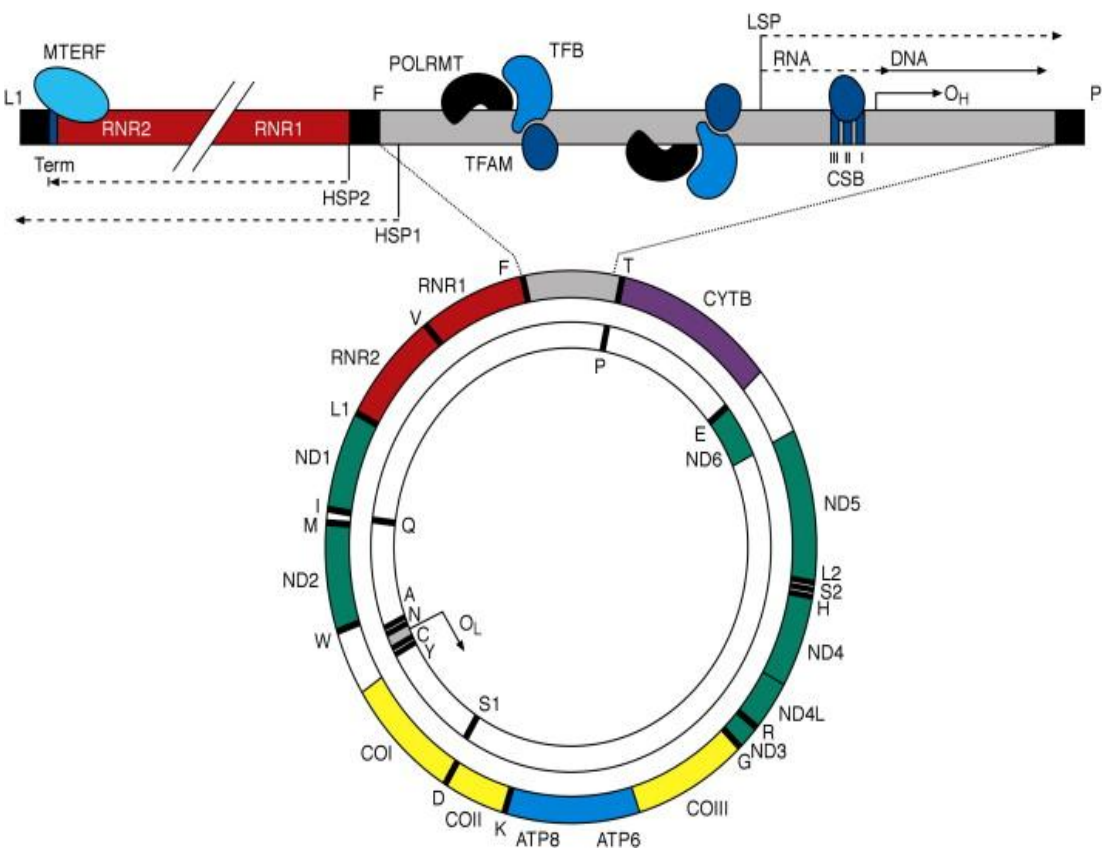

Figure 1.3: Typical vertebrate mitochondrial genes and their organization

Table 1.3: Forensic identification of non-human specimen using mitochondrial PCG's

Species	Marker used	Type of investigation
Red deer, buffalo, cattle, sheep, goat, domestic pig, horse, chicken & turkey	Cytochrome b (89 to 362 bp); 12S rRNA and 16S rRNA; D-loop region (370 bp)	Species identification in food items (Meyer *et al.* 1995; Matsunaga *et al.* 1998; Fajardo *et al.* 2006; Stirtzel *et al.* 2007; La Neve *et al.* 2008; Tobe *et al.* 2008; Haunshi *et al.* 2009; Murugaiah *et al.* 2009)
Rhinoceros	Cytochrome b (402 bp)	Species identification from horn (Hsieh *et al.* 2003)
Shark	Cytochrome b (180 bp)	Species identification from fins (Yan *et al.* 2005)
Camel	Cytochrome b (208 bp)	Species specific PCR amplification of camel (Chen *et al.* 2005)
Roe deer	Cytochrome b (900 bp)	Species identification from meat & hair (An *et al.* 2007)
Tiger	Cytochrome b gene	Species identification in traditional East Asian medicine (Linacre and Tobe, 2008)
Asiatic black bear	Cytochrome b (175 bp); Control region (279–744 bp)	Species identification in medicine(Peppin *et al.* 2008; Jun *et al.* 2011)
Seahorses	Control region (533 bp)	Species identification and determination of geographical origin. (Sanders *et al.* 2008)
Horse	Control region (662 bp & 209 bp)	Individualisation for forensic purposes, (Gurney *et al.* 2010)
Asian elephant	Control region (137 bp)	Species identification from ivory idol (Gupta *et al.* 2011)
Elephant	Cytochrome b (357 bp)	Species identification from Ivory seals (Lee *et al.* 2013).
Australian marsupials	Cytochrome c Oxidase subunit I, NADH 2	Species identification (Wilson-Wilde *et al.* 2011)
Indo-Chinese spitting cobra	Cytochrome b (472 bp)	Species identification from snake wine (Gaur *et al.* 2012)
Scarlet macaw	Cytochrome c Oxidase subunit I (648 bp)	Species identification from feathers (Abe *et al.* 2012)
Parrots and cockatoos	12S, rRNA (230 bp)	Species identification from embryonic tissue (Coghlan *et al.* 2011)

1.3.3 Nuclear DNA Markers and its Application in Wildlife Forensic

Beside mitochondrial DNA analysis nuclear DNA analysis also helps in inferring the species in questions as nuclear DNA markers are widely dispersed in non-coding areas of the whole genome. It becomes an advantage of using these markers for identification of species up to individual level (Dawnay *et al.* 2008). The second important advantage of using these markers is the elevated rate of sequence evolution related to organelle genes, which helps for phylogenetic studies (Satoh *et al.*, 1999).

1.3.3.1 Commonly used Nuclear Markers in Wildlife Forensics

The most commonly used nuclear markers for DNA fingerprinting are Amplified Fragment Length Polymorphism (AFLP), Randomly Amplified Polymorphic DNA (RAPD) and Microsatellites or Short Tandem Repeats (STRs) because of their large number of loci, which can be screened simultaneously (Fajardo *et al.*, 2006; Naga *et al.*, 2013a). Micro-satellites are multiple copies of short tandem repeats, generally 1-5 bp long, located in both coding and non coding regions and fairly evenly distributed throughout the genomes. They are co-dominant markers with bi-allelic or multi-allelic presentation in an individual or a population. Micro-satellites are highly polymorphic and can be easily amplified by PCR (Verma *et al.* 2003) (Table 1.6 shows some characteristics features of various nuclear DNA markers).

Table 1.4: Some characteristics features of various nuclear DNA markers

	RAPD	AFLP	Micro-satellites
Allelic information	Dominant	Dominant	Co-dominant
Locus presentation	Multi-locus	Multi-locus	Single locus
DNA required	0.02	0.50	0.05
PCR-based	Yes	Yes	Yes
Restriction digestion	No	Yes	No (Yes for development)
Reproducibility	Low	High	Low
Development cost	Low	Moderate	High
Cost per assay	Low	Moderate	Low

1.3.3.2. Individual Identification using Nuclear Markers

The most powerful DNA analysis we can perform is individual DNA profiling which allows a forensic scientist to link trace evidence from a suspect to a specific incident. Mitochondrial DNA analysis only gives the identification of species but cannot discern the individuality among the same species being identified. Individual identification of an animal or plant or insects is achieved using Microsatellites or STR profiling. Microsatellites are multiple copies of short tandem repeats, generally 1-5 bp long, located in both coding and non-coding regions and fairly evenly distributed throughout the eukaryotic genomes. These are co-dominant markers with bi-allelic or multi-allelic presentation in an individual or population. These markers are highly polymorphic and can be amplified easily through PCR, making them highly versatile markers for molecular fingerprinting. However, not much work has been carried out in the field of non-human STR typing. This technique in general is used for domestic pet's identification which is killed as a result of animal cruelty (Table 1.5 shows some example of individual identification of non-human specimen using STR loci). As there are still scarcity of allele frequency databases of wild animals, wildlife scientists are extensively working on achieving their aim to fulfil the database.

Table 1.5: Individual identification of non-human specimen using STR loci

Species	Marker used	Type of investigation
Tiger	STRs	Individual identification (Xu *et al.* 2005)
African elephants	STRs	Geographical origin determination (Wasser *et al.* 2008)
Red deer	STRs	Individual identification (Socratous *et al.* 2009)
Wolf	STRs	Individual identification (Caniglia *et al.* 2010)
Chimpanzees	STRs	Individual identification (Ghobrial *et al.* 2010)
Fox	STRs	Individual identification (Wesselink and Kuiper, 2011)
Northern European brown bear	STRs	Individual identification (Andreassen *et al.* 2012)
White tailed black cockatoos	STRs	Individual identification (White *et al.* 2012)
Rhinoceros	STRs	Individual identification (Harper *et al.* 2013)
Gharial	STRs	Population assignments (Naga *et al.* 2013)

1.4 Technologies used in Forensic Laboratories for Proper Applications of the Molecular Markers

1.4.1 Gel Electrophoresis

Electrophoresis is essentially a method of separating molecules by their size through the application of an electric field. The same principle is applied during DNA analysis. The individual fragments of DNA can be separated using electrophoresis to produce the distinct 'DNA fingerprint'. Samples to be analysed are placed in small wells at the top of the gel using pipettes. A control sample and a standard/marker sample is often run simultaneously. As the electric current is applied, the negatively charged DNA fragments begin moving through the gel towards the positively charged anode. Following electrophoresis, it is necessary to visualise these bands using radioactive or fluorescent probes or dyes. This not only separates DNA but also allows for the fragments to be measured.

1.4.2 PCR Amplification

During the investigation of a crime the amount of DNA evidence obtained is often very small, thus for successful DNA profiling, amplification of the isolated DNA is mandatory. In the year 1985 Kary B. Mullis invented the techniques which allows for the exponential amplification of DNA fragments to lengths of approximately 10,000 base pairs. This is known as Polymerase Chain Reaction (PCR). Here a single copy of a DNA fragment could be amplified to millions of copies easily. This process is beneficial in the amplification of minute or degraded samples. A successful PCR reaction requires primers which are complementary to the DNA target and mark the target to be amplified, with two primers being used.

Various alterations have been made to improve the PCR method. Combined with RFLP (PCR-RFLP) method this process helped in proper species identifications of wildlife animals without any requirement of further sequencing. Apart from that, multiplexing PCR Reaction involves the amplification of numerous DNA sequences in a single reaction through the use of primers that produce non-overlapping allele sizes, allowing numerous regions of a sample to be tested simultaneously.

1.4.3 Real time PCR (Rt PCR)

A Real-time Polymerase Chain Reaction (Rt PCR) is a technique based on the polymerase chain reaction (PCR). It is used for the detection of a specific DNA sequence in a sample by measuring the accumulation of amplified products during PCR using fluorescent technology which monitors the amplification of a targeted DNA molecule during the PCR, i.e. in real-time, and not at its end, as in conventional PCR. Real-time PCR can be used quantitatively (Quantitative real-time PCR), semi-quantitatively, i.e. above/below a certain amount of DNA molecules (Semi quantitative real-time PCR) or qualitatively (Qualitative real-time PCR).

1.4.4 Sequencing of PCR Products

The most used method for wildlife species identification on molecular basis is DNA sequencing. Identification of exhibits is achieved by comparing the sequence of their genomic region with a reference database after the completion of PCR amplification and post PCR clean up. There are so many software and technologies are available in the forensic laboratories to perform the sequencing of exhibits.

1.4.4.1 Data Interpretation of Sequenced Products

After performing the DNA sequencing, to interpret the identity of questioned species data interpretation of the result is done by matching the sequences with established database of victim species. This is done by performing BLAST search from GenBank of the sequenced material. If there is100% homology between the reference and questioned sample it can be concluded that the questioned sample was from the same species as of reference one. If the homology is 99.50% then it may be concluded that either the questioned sample comes from the species and the differences are due to intraspecific variations or it comes from an unknown but closely related species with 99.5% sequence match that has not been documented in the database.

1.4.5 Next Generation Sequencing

Since the advent of Next Generation Sequencing or high throughput sequencing, the whole genome sequencing of any organism has become fast, less laborious technique than previous Sanger sequencing. The course of parallel sequencing gives the chances to identify new STRs or SNP from a pool of repetitive DNA sequences (Liu *et al.*,

2012). These newly identified loci can be used to characterize the number of alleles, heterozygosity, linkage equilibrium, polymorphic content, deviations from Hardy-Weinberg equilibrium etc. which in turn will help in the study of population of an organism (Glenn, 2011; Quail, Smith *et al.*, 2012.).

Recently, by using NGS it has been successfully possible to differentiate the content of traditional medicine which is often mixture of different wild specimens. Somehow this needed to get validated for regular implementation in forensic usage. There are various NGS instruments available at present that function by the use of different sequencing techniques. They are applicable from smaller to larger scale functions. For example, Illumina (CA, USA) manufactures the HiSeq 2000 for larger scale applications and for smaller scale applications the MiSeq (Shokralla *et al.*, 2012). The table 1.6 describes and compare briefly about different types of available NGS instruments.

Table 1.6 Brief comparisons of different Next Generation Sequencing instruments.

Sequencing method	Sequencing platform	Maximum read length (base pairs)	Maximum sequence output	Advantages	Disadvantages
Pyrosequencing	Roche 454	700	700Mb	long read length	prone to homopolymer error
Sequencing by ligation	SOLiD	75	250Gb	High accuracy	Short read length
Sequencing by synthesis	Illumina (HiSeq, MiSeq)	300	600Gb	High throughput	Short read length
Single nucleotide real-time sequencing	PacBio	1100	100Mb	Longest read length	Lower throughput
Ion semiconductor	Ion Torrent	400	1Gb	Fast sequencing	Prone to homopolymer error

1.5 Threat to Indian Snakes

India is abode of about 275 species of snakes and among them Python, King cobra, Rat snakes, Viper's, Sand Boa, Water snakes etc. are declared as endangered and threatened species by CITES, IUCN and WLPA. They are often killed due to fear, or wrong belief that all snakes are venomous, or due to their attractive and charismatic skin for making leather shoes, bags, belts, wallets etc., or due to the medicinal value of their venom, blood and bones, for their nutritious meat and lastly for entertainment purposes. Despite of all the conservation laws in place, rampant poaching and covert trade continues to pose a threat of losing these precious species from their habitat. India has been one of the world's biggest suppliers of snake skins as indicated by the statistics of the past, for instance, in the year 1968, no less than 10 million snakes were slaughtered only for trade (Inskipp, 1981). According to one estimate, the number of snakes that were caught for snake skin trade was so large that once a tannery in South India handled 9000 skins per day (Daniel, 1970). An emerging illegal market is snake venom trading (TRAFFIC International, 2007). Table 1.7 shows list of major Indian snake species

Table 1.7: List of Major Indian Snakes Species in WLPA

Snake Species	Scientific Name	WLPA	CITES
1) Indian Rock Python	*Python molurus*	Schedule I	Appendix I
2) Indian Cobra	All subspecies of *Naja*	Schedule II	Appendix II
3) King Cobra	*Ophiophagus hannah*	Schedule II	Appendix II
4) Checkered Keelback	*Fowlea piscator*	Schedule II	Appendix III
5) Olivaceous Keelback	*Atretium schistosum*	Schedule II	Appendix III
6) Rat Snake	*Ptyas mucosa*	Schedule II	Appendix II
7) Russel's Viper	*Daboia russellii*	Schedule II	Appendix III
8) Dog Faced water Snake	*Cerberus rynchops*	Schedule II	Appendix III

Figure 1.4: Skin of Rat snake ready to be traded.

Figure 1.5: Items made from snake skin specimen.

In most of the illegally traded wild life cases including different snake exhibits, the seized materials are highly processed, their body parts goes through severe wear and tear and treated with heavy chemical treatment before illegal trading and routine morphological identification does not yield conclusive results. It becomes very difficult to find out or identify the proper species of confiscated snake material and for implementing right Wildlife (Protection) Act, (1972) it is important to identify the confiscated and camouflaged snake exhibits at molecular level to ascertain their sources. Thus, implementing Wildlife (Protection) Act, 1972 for the protection of endangered snake species, it is necessary to properly identify those seized materials on species level

So, far work on identification of different snake species on molecular level is limited. Therefore, the proposed study aims to develop some molecular techniques which can be implied on highly degraded samples for proper identification and also cost effective as well as easy to perform. As from degraded snake samples it is difficult to recover the complete mt-DNA and isolation of nuclear DNA becomes hard at times, it is required to identify the samples through smaller gene sequences so the species specific identification of samples can be accomplish. Thus it is necessary to build a database of complete mt-DNA sequencing of each snake species so that minute amount of recovered DNA can act as DNA detective of any unknown sample. It will further help in establishing the species relatedness and thereby fixing identity of species. Also, establishing quick and simple identification technique can fasten the species analysis method and such goal can be achieved through developing species specific molecular markers. Hence, this study will characterize the complete mitochondrial genome of some snake species for development of new forensic

markers as well as focus on isolation and characterization of microsatellite markers from snake specimen.

1.5.1 The Aim and Objectives of the Present Study

(i) To study and validate the species–specific mtDNA markers and to develop primers which are specific for some endangered Indian snakes.
(ii) To develop and validate molecular techniques for forensic identification of some endangered Indian snake species.
(iii) To characterize complete mitochondrial genome of Asiatic water snake or *Fowlea piscator* and study of their phylogeny.
(iv) To isolate and characterize novel microsatellite markers of Rat snake or *Ptyas mucosa*.
(v) To standardized the usage of the isolated STR markers for purposeful genetic studies for future usages.
(vi) To publish the outcome of the above research for their immediate uses.

1.5.2 Methodologies Adopted in the Present Study

(i) For Isolation of DNA from Shed skin of snakes, Fetzner (1999) DNA isolation protocol is used.
(ii) Isolation of genomic DNA from tissue and saliva samples performed using commercial kit.
(iii) Primers were designed using MEGA 7.
(iv) Will use Multiplex PCR technique for species identification.
(v) GeneAmp PCR system 9700 will be used for performing PCR.
(vi) Sequencing of isolated DNA will be done using 3100 Avant Genetic analyzer.
(vii) Next-generation sequencing will be performed to study the complete mitogenome of the snake species.
(viii) Microsatellite markers will be isolated using Glenn and Schable 2005 protocol.

1.6 Accreditation in Court of Law

The purpose of accreditation is to ensure that the result of any test performed is reliable, repeatable and robust. Hence, the international standard ISO 17025 is

globally working to accredit such laboratories for maintaining all of them. Besides identifying the exhibits up to species or individual level punishing the perpetrator of such heinous crime is what forensic scientists are working for and to establish the same, quality of all laboratory techniques, performance of tools and staffs are look out by the court of law. Unlike human identification techniques, non-human forensic practices are yet to be globally accepted in the court of law because of their variability, existence of numerous populations within a species, their inbreeding nature and most of the time encountered samples are degraded, camouflaged or altered though some identification techniques are legible in the court of law. Researchers are laboriously working on this to develop some gold standard identification techniques for non-human specimens.

1.7 Conclusion

The major part of biodiversity is forest and the wildlife present there. As human we get benefitted directly by using and depending on our hugely diverse ecosystem through food, fibre, wood, medicine, etc. There are many indirect benefits also that we receive through ecosystem services such as pollination, pest control, climate moderation and flood control. It gives resilience to the biosphere but if it degrades then different communities as well as human also become vulnerable. It is observed that population pressure, natural habitat destruction for urbanization, and excessive animal hunting for illegal trading are affecting the existence of forests and wildlife in the ecosystem (Charlesworth, 1999; Crandall *et al.*, 2000). The loss of populations, species, or groups of species from an ecosystem can upset its normal function and disrupt these ecological services.

In present day there is an urgent need to develop exact forensic procedure to fight against wildlife crime. With passing time, crime against wildlife species including endangered one is increasing, leading to improper implications of WPA, 1972. Due to the advent of DNA technologies in forensic field and their power to discriminate between two or more individuals, it's become necessary to manage the wildlife crime scene with more consideration to avoid contamination. A forensic investigation can only succeed if the crime scene investigation and laboratory techniques are performed right. It is very much necessary to handle the crime scene exhibits with utmost care, starting from proper recognition, collection and preservation to transferring them in a forensic laboratory where they will be analysed. Also, by enacting different Wildlife (Protection) Act, (1972) the animals can be protected from illegal poaching and

trafficking of their body parts. But, as the matter of fact the punishment prescribed in the wild life act is according to the crime involving endangered wildlife species. Thus the perpetrator of the crime remains escaped.

It is to mention that species identification involving endangered or protected animals or plants mostly relies on mitochondrial DNA analysis as punishment for illegal killing or poaching or trafficking will be implies if only species has been determined. On the other hand individualization of animals is required for captive or pet animals. Hence, their genomic database is required to establish the same. With the anticipation of developing new identification markers and by generating useful genomic database for Indian endangered snake species, the next chapters of this thesis progresses.

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CHAPTER II

REVIEW of LITERATURE

"If we can teach people about wildlife, they will be touched. Share my wildlife with me. Because humans want to save things that they love."

~ Steve Irwin

2.1 Introduction

Snakes are limbless, long, elongated, colourful reptiles belonging to a single order, Squamata, and form a suborder Serpentes, whose huge variations throughout the continents have always mesmerized human mind. There are about 3900 species of modern snakes arranged in some 20 families and 520 genera (Reptile data base, 2021) (Table 2.1 shows a list of Snake families with their genera and species number). Living snakes are found on every continent except Antarctica. According to IUCN RED LIST 12% of total world population of snakes are Endangered and 4% are near threatened. On the whole, they make up a significant proportion of the middle-order predators that keep our natural ecosystems working. Without them the numbers of prey species would increase to unnatural levels and the predators that eat snakes struggle to find food (Whitekar *et al.*, 2004; Dubey *et al.*, 2009). Not only are they part of a healthy ecosystem but they have a place in this world, a place in the wild to control rodent populations and provide food for other animals. Throughout history, humans have regarded snakes with both fascination and horror. They are not evil creatures that are out to hunt down and ruthlessly kill people. They are peaceful and attractive creatures of our land and prefer to be left alone. But, often they are killed due to fear or wrong belief that all snakes are venomous or due to their attractive and charismatic skin for making leather shoes, bags, belts, wallets etc. or due to the medicinal value of their venom and blood, for meat and entertainment purposes. Although snakes have many unique features such us, shading their skin, keeping their eyes always open, lack of external earlobe, forked tongue etc. but it is their attractive hides which are demanding items for illegal skin trading. According to CITES data base for the period 2015-2020, worldwide the total amount of traded skin items from different snake species is 25,55,890 or nearly 2.6 million. Although snakes are a poorly studied group as there is inadequate information available regarding their population, distribution and threats (Vidal *et al.*, 2009).

The wildlife scientist has adopted many strategies for the conservation of endangered Indian snake species. Among them protection of habitat of the endangered and threatened animals and captive breeding are on for frontiers. But, without their identification the perpetrator of wildlife crime will not be punished. Hence, here in this study we have selected the following Indian snake species as they are being targeted by the wildlife goons and randomly deploying from their natural habitat so that they can be conserved at their natural habitat without further depletion.

Table 2.1: List of Snake families with their genera and species number

Snake Families	Approx. Number of Genera	Approx. Number of Species
1) Acrochordidae (Wart Snakes)	2	3
2) Aniliidae (Cylinder Snakes)	3	9
3) Anomochilidae (Dwarf pipe snakes)	1	3
4) Boidae (Boas)	14	61
5) Bolyeriidae (Split jaw snakes)	2	2
6) Colubridae (Typical Snakes)	258	2500
7) Cylindrophiidae (Asian pipe snakes)	1	14
8) Elapidae (Cobras, Mambas etc.)	55	359
9) Homalopsidae (Homalopsids)	28	53
10) Lamprophiidae (Atractaspididae and 6 subfamilies from colubrids)	60	314
11) Leptotyphlopidae (Thread snakes)	2	50
12) Loxocemidae (Mexican burrowing snakes)	1	1
13) Pareidae (Snail eating snakes)	3	20
14) Pythonidae (Pythons)	8	40
15) Tropidophidae (Dwarf Boas)	2	34
16) Typhlopidae (Blind Snakes)	5	200
17) Uropeltidae (Shield-tails snakes)	8	55
18) Viperidae (Vipers and Pit Vipers)	35	340
19) Xenopeltidae (Sunbeam Snake)	1	2
20) Xenodermidae	6	18

2.2. Snakes Species chosen in this Study

The Indian snake species chosen and collected for this study are described next.

2.2.1 Indian Cobra or *Naja naja* (Linnaeus, 1758):

Listed in appendix II of CITES, these are highly venomous species of snakes from family Elapidae. They are distributed throughout India, Pakistan, Bangladesh and most of the countries. An adult can grow on average upto 2 mt long. They can be

easily identified by the presence of spectacle or hood like mark on the back of their hood. Their body colour greatly depends on their geographic origin starting from shades of brown to yellow to greyish black. Their venom which is neurotoxic in nature is believed to have medicinal values mainly in manufacturing Chinese medicines, hence, illegally killed and trafficked for its venom. Also, heavily used by snake charmers and trafficked for illegal skin trading from India to other South East Asian countries (https://indiabiodiversity.org/species/show/238905).

Kingdom - Animaliia
Phylum - Chordata
Class - Reptalia
Order - Squamata
Family - Elapidae
Genus - Naja
Species - *Naja naja*

Figure 2.1: *Naja naja*
(Giri, 2005; https://indiabiodiversity.org/observation/show/15196491)

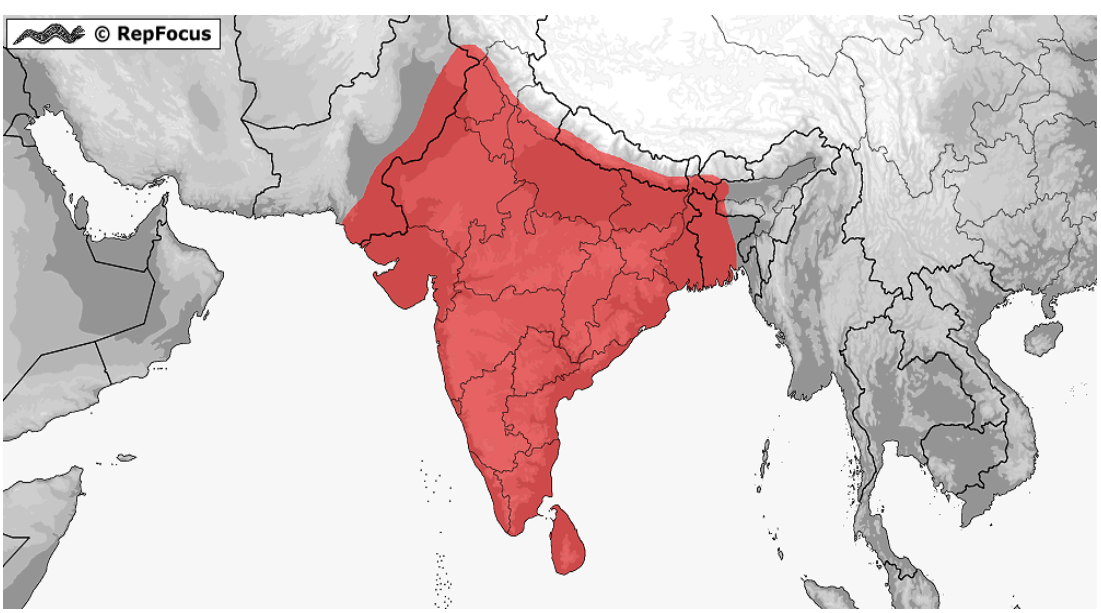

Figure 2.2: Geographic distribution of Indian Cobra (*Naja naja*)

2.2.2 Rat Snake *or Ptyas mucosa* (Linnaeus, 1758):

Listed in appendix II of CITES, these are non-venomous snake from family Colubridae. They are famous for their fast crawling and quite larger size than other species which are found in India. They can achieve height of 2.5 mt on average after maturation. This is an essential species to check the population of rodents, toads and frogs around human habitat and agricultural lands by preventing loss of a agricultural productivity and over populations. They were present in the past, but recently extinct from Singapore (Baker & Lim, 2008). Currently distributed throughout the other Asian countries they are illegally trafficked and killed for their fleshy meat, lucrative skin and also due to fear as it has similar nature with Indian Cobra or common Cobra which are highly poisonous. Snake charmers also use them as they are large and non harmful to handle (https://indiabiodiversity.org/species/show/257422).

Kingdom - Animaliia
Phylum - Chordata
Class - Reptalia
Order - Squamata
Family - Colubridae
Genus - Ptyas
Species - *Ptyas mucosa*

Figure 2.3: *Ptyas mucosa*
(Manoj, 2021; https://www.inaturalist.org/photos/167885506?size=medium)

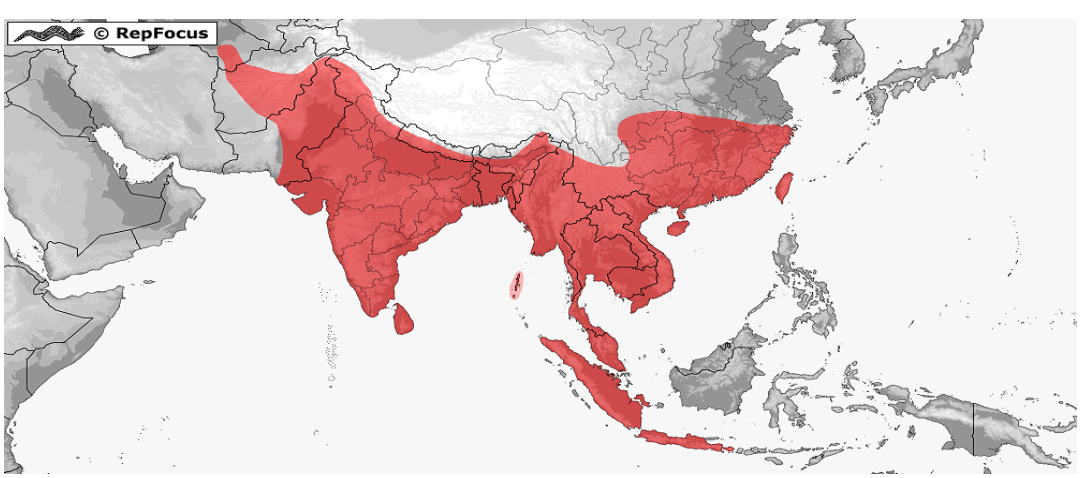

Figure 2.4: Geographic distribution of Rat Snake (*Ptyas mucosa*)

2.2.3 Russell's Viper or *Daboia russelii* (Shaw & Nodder, 1797)

This is one of the most bite and death causing highly venomous snake in India which belongs to the family Viperadae. Listed in appendix III of CITES they are distributed throughout the Pakistan, India, Nepal, Sri Lanka, Bangladesh, Bhutan, Myanmar, Thailand etc. South -East Asian countries. They can grow to a maximum total length of 166 cm (5.5 ft) and averages about 120 cm (4 ft). They are famous for their large spitting range and hugely trafficked from India to core South-East Asian countries for medicinal purpose of its venom, skin and meat (https://indiabiodiversity.org/species/show/238674).

Kingdom - Animaliia

Phylum - Chordata

Class - Reptalia

Order - Squamata

Family - Viperidae

Genus - Daboia

Species - *Daboia russellii*

Figure 2.5: *Daboia russellii*
*(*Sharma *et al.* 2013; https://www.researchgate.net/figure/Russells-Viper*)*

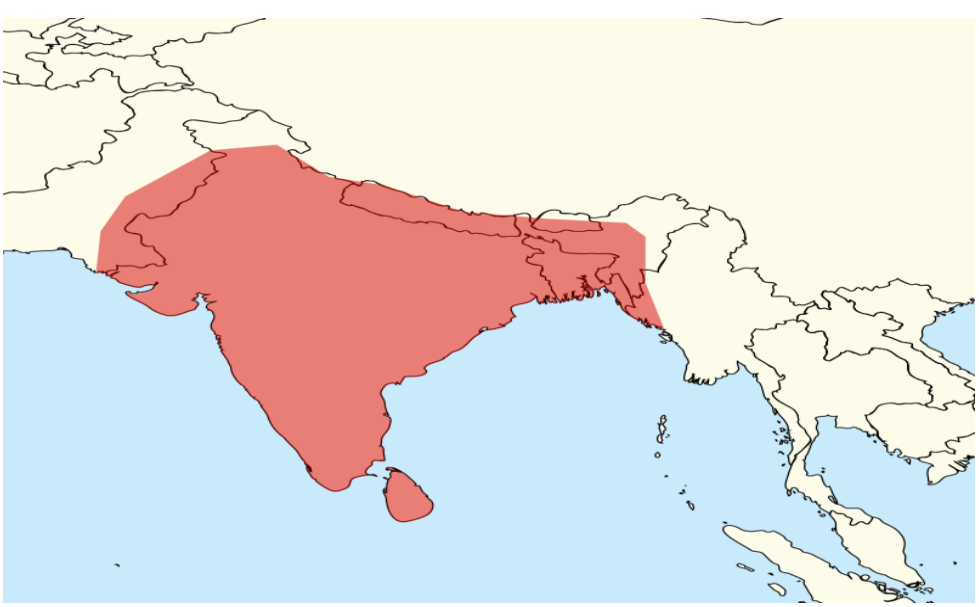

Figure 2.6: Geographic distribution of Russell's viper (*Daboia russellii*)

2.2.4 Checkered Keelback or *Fowlea piscator* (Schneider, 1799)

A non-venomous snake species from family Colubridae and subfamily Natricinae these snakes are listed in appendix III of CITES. They are found in fresh water bodies and an adult can be of 1.5 mt of length. Its wide range of colours and patterns makes its identification tough sometimes. Due to its aggressive behaviour it can be confused with Cobra. As it is well settled in urban areas, habitat destruction does not seem to be a serious threat for this species. In many parts of its range it is consumed by few communities. Major threats to this species are due to road accidents and intentional killing by people for their attractively textured skin and nutritious protein rich meat (https://indiabiodiversity.org/species/show/238773).

Kingdom	- Animaliia
Phylum	- Chordata
Class	- Reptalia
Order	- Squamata
Family	- Colubridae
Sub family	- Natricinae
Genus	- Fowlea
Species	-*F. Piscator*

Figure 2.7: *Fowlea piscator*
(Kasambe, 2016;ttps://commons.wikimedia.org/wiki/File:Checkered_Keelback)

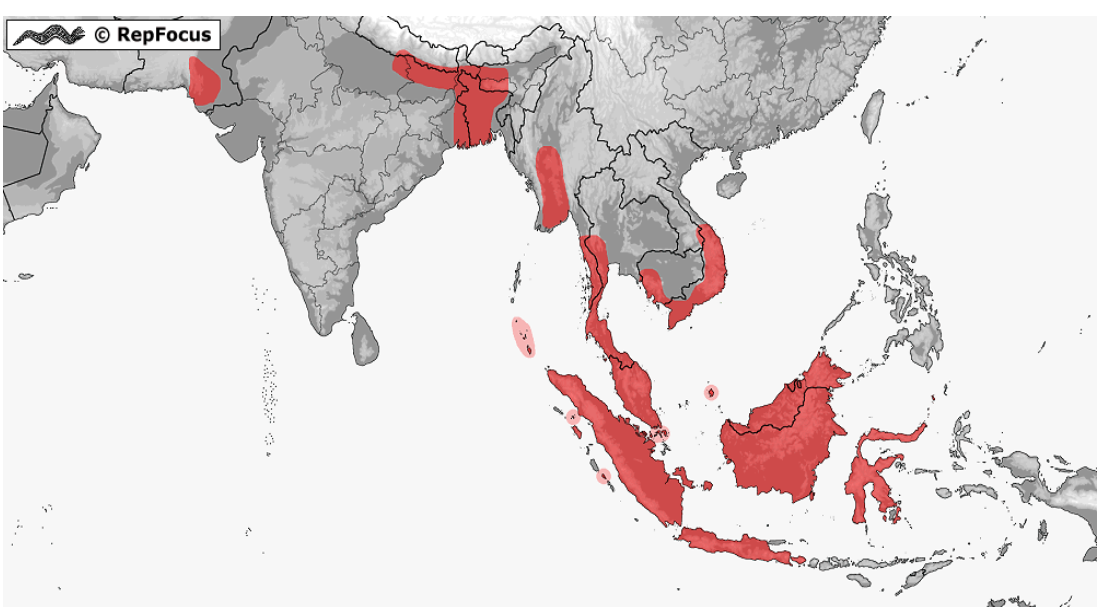

Figure 2.8: Geographic distribution of Checkered keelback (*Fowlea piscator*)

2.3 Review of Literature

As a whole, two types of analysis are mainly used for forensic identification of crime exhibits involving endangered snake species and they are,

i) Identification using forensically informative mitochondrial markers, and by
ii) Development and characterization of microsatellites or STR markers.

This section presents a brief review on the literature studied on the application of mitochondrial as well as nuclear markers for snake species identification especially for legal aspect.

2.3.1 Identification using Forensically Informative Mitochondrial Markers

The sequencing of mtDNA from non-human source has only recently gained attention, with applications in the profiling of 'silent witnesses' of crimes, traceability of food products and control of illegal trade of endangered species or drugs, among others. It is imperative to merge a solid knowledge of mitochondrial genetics with rigorous methods of error prevention in order to permit the full approval of non-human mtDNA profiling in routine forensic work (Pereira *et al.*, 2010). Most of the time the crime exhibits are chemically treated, morphologically altered and highly degraded. In those cases nuclear DNA isolation turns difficult whereas a minute amount of mtDNA can be recovered from those confiscated samples. Hence, creation of a database with small size amplicons could help in identification of exhibit specimens. Although many works has been reported till date for such applications but it required many more databases for easy identification. Table 2.2 shows list of some snake species identified using mitochondrial markers.

The higher-level of snake phylogeny can be inferred from sequence analyses of one nuclear gene (c-mos) and three mitochondrial genes (12S rRNA, 16S rRNA and cytochrome *b*) has been shown by **Vidal *et al.* (2002)**. In his paper he has shown that present snakes species belong to two lineages. Firstly the fossorial Scolecophidia, and secondly, the diverse group of typical snakes or Alethinophidia. With the help of mitochondrial gene sequencing it was possible to clearly distinguish between the clades Henophidia and Caenophidia, that belongs to Alethinophidia.

Table 2.2: Forensic identification of snake specimen using mitochondrial markers

Species	Marker used	Type of investigation
Indian snakes	Cytochrome c Oxidase subunit I (175/245 bp)	Species identification from shed skin (Dubey *et al*. 2011)
Indo-Chinese spitting cobra	Cytochrome b (472 bp)	Species identification from snake wine (Gaur *et al.* 2012)
Snake	16S rRNA (130, 265 and 380 bp)	Identification of snake species (Dubey *et al*. 2009)
Sea snake	Cytochrome b, 12S rRNA and 16S rRNA	Species Identification from adulterated meat (Suntrarachun *et al*. 2018).
Garter snake	Cytochrome b and NADH subunit 2	Species identification and origin determination (Neuman-Lee *et al.* 2011)
King Cobra and Banded Krait	Cytochrome b gene	Population discrimination (Kundu *et al.* 2020)
Chinese snake species from four families	COI	Species identification of medically important snakes (Chen *et al*. 2018)

It reveals the closest relatives within Alethinophidia are Aniliidae or genus *Anilius* and Tropidophiidae or genera *Trachyboa* and *Tropidophis*. Although the data were insufficient to robustly infer the ancestral mode of life of snakes, evidence of plasticity in the basic ecological and trophic modes of snakes was found.

Pook *et al.* (2005) demonstrate the species identification method by extraction of whole mitochondrial DNA followed by sequencing of 12S rRNA gene from dried snake venom. It can be used to validate the species in questions through comparison against existing sequences in the NCBI GenBank database and using phylogenetic analyses with other sequences. Pooled venom can also be screened for the presence of mixed species. This new method of DNA barcoding of snake venoms ensures the identification of specific species even after future taxonomic changes.

For the identification of three endangered Indian snake species namingly, *Python molurus molurus, Naja naja* and *Xenochrophis piscator* **Dubey *et al.* (2009a)** developed a simple and cost-effective molecular technique with PCR-RFLP. They amplified a 431-bp amplicons from Cytochrome b gene using novel primers followed by restriction fragmentation Mbo II and Fok I enzymes. It enabled successful identification of highly degraded shed skin samples from three Indian snake species.

Later, **Dubey *et al.* (2009b)** also developed a novel multiplex PCR assay for the identification of three Indian snake species *Python molurus*, *Ptyas mucosa* and *Naja naja* using 16S rRNA gene. They designed three reverse primers and a common forward primer to generate three different size species-specific PCR fragments. Absence of any PCR amplification in non-target species proves the specificity of the newly designed primers. These four primers were combined in a multiplex assay to enable identification of three snake species in a single reaction. It is expected that this effort will help strengthening conservation efforts for these species.

Neuman-Lee *et al.* (2011) found a Garter snake (*Thamnophis*) outside of Haines, Alaska, in August 2005. The poor condition of the specimen was preventing a positive identification based on morphological analysis alone. Furthermore, Alaska is not a native place for snakes. Therefore they undertook a molecular approach to determine the species and geographic origin of the individual. They partially sequenced cytochrome b and NADH subunit-2, mito genes from the Alaskan specimen and seven specimens from localities in the lower North-Western United States. Phylogenetic reconstruction using the sequences and additional GenBank

samples revealed that the Alaskan specimen is *Thamnophis ordinoides*. In light of these analyses, the likelihood that the specimen represents a relict population, a recent natural colonization, or a fresh introduction was assessed.

Gaur *et al.* (2012) reported a novel set of universal primers to amplify and sequence a specific fragment of mitochondrial cyt b gene from a vast range of animal species and demonstrated that it can identify animals to the species level. An interesting case related to confiscation of a snake-wine bottle from a local bar in Bangalore city of Karnataka, India was solved by using the primers. The authorities suspected that the snake in the wine bottle to be an Indian cobra (*Naja naja*), which is a scheduled animal under the Indian wildlife protection act (1972). The morphological examination, from competent authorities like the Zoological Survey of India (ZSI), Southern Regional Station, Chennai and Bombay Natural History Society (BNHS), Mumbai, certified that the specimen was indeed of an Indian cobra i.e. *Naja naja*. However, to confirm the species, a molecular testing approach was taken. PCR product of 472 bp was obtained and after editing a consensus sequence of 421 bp was analyzed using BLAST program of NCBI. The cyt b sequence obtained from confiscated specimen (tail piece and scales) had maximum similarity (99.14%) with *Naja siamensis (*accession no. AF155214) i.e. an Indo-Chinese spitting cobra, whereas, it had 88.47% sequence similarity with *N. naja* (accession no. AF540932) i.e., an Indian cobra. Hence, confirming the sample to be *Naja siamensis.*

A simple method for identification of six snake species using the gel filtration chromatographic profiles from snake venoms was presented by **Asmari *et al.* (2014)**. The venoms of *Echis coloratus, Echis pyramidum*, *Cerastes gasperettii*, *Bitis arietans, Naja arabica,* and *Walterinnesia aegyptia* were milked, lyophilized, diluted and centrifuged to separate the mucus from the venom. The clear supernatants were filtered and chromatographed on fast protein liquid chromatography (FPLC). The obtained 16S rRNA gene sequences of the above species and chromatograms from different snake species showed peculiar patterns based on the number and location of peaks. The dendrograms generated from similarity matrix based on the presence/absence of particular chromatographic peaks clearly differentiated Elapids from Viperoids. Molecular cladistics using 16S rRNA gene sequences resulted in jumping clades while separating the members of these two families. These findings suggest that chromatographic profiles of snake venoms provide a simple and reproducible chemical fingerprinting method for quick identification of snake species.

However, the validation of this methodology requires further studies on large number of specimens from within and across species.

Suntrarachun *et al.* (2014) developed a method for species identification of venomous snakes on the basis of amplification of a specific part of the mitochondrial cytochrome b gene using PCR. To distinguish among venomous species, they obtained 1,144 bp PCR products and cut them with different restriction endonuclease, Alu I and Hinf I, resulting in species-specific restriction fragment length polymorphism (RFLP). Each enzyme generated different-sized fragments which specific to the six neurotoxic and six hematotoxic snake species in Thailand. However, identical patterns were found between *Daboia siamensis* and *Trimeresurus popeorum* among hematotoxic snakes when Alu I has been used. These results could be resolved by using additional enzyme such as Hinf I due to no cross reaction between the species was detected in their restriction patterns. The PCR-RFLP developed here is simple, rapid, reliable and reproducible; hence it can be routinely applied for snake species identification, essential for conservation and management of endangered snake species in Thailand.

Sharma *et al.* (2016) applied the forensic genetics techniques for identification of snake species involving snake bite cases to choose appropriate treatment and anticipate complications. This is particularly important for neurotoxic envenoming which, depending on the snake species involved, may not respond to available antivenoms. Adequate species identification tools are lacking. This study used a combination of morphological and molecular approaches (PCR-aided DNA sequencing from swabs of bite sites) to determine the contribution of venomous and non-venomous species to the snakebite burden in southern Nepal. Out of 749 patients admitted with a history of snakebite to one of three study centres, the biting species could be identified in 194 (25.9%). Out of these, 87 had been bitten by a venomous snake, most commonly the Indian spectacled cobra (*Naja naja;* n = 42) and the common krait (*Bungarus caeruleus*; n = 22). When both morphological identification and PCR/sequencing results were available, a 100% agreement was noted. The probability of a positive PCR result was significantly lower among patients who had used inadequate "first aid" measures (e.g. tourniquets or local application of remedies). This study is the first to report the use of forensic genetics methods for snake species identification in a prospective clinical study. If high diagnostic accuracy is confirmed in larger cohorts, this method will be a very useful reference diagnostic tool for epidemiological investigations and clinical studies.

Later, **Suntararachun *et al.* (2018)** developed a species-specific PCR-RFLP marker to distinguish between sea snake species. The PCR products of cytochrome *b* (Cyt b), 12S and 16S rRNA were sequenced and cut with different restriction endonuclease, Alu I and Hinf I. Each enzyme generated different - sized fragments which specific to Cyt b of eight sea snake species. However, the identical pattern was found among *Hydrophis* group. This result could be resolved by using these enzymes 12S rRNA digestion. This technique was successfully applied to blood, shed skin, raw meat, cooked meat, sea snake-fish binary admixture, and sea snake-pork binary admixture. Hence, it could be applied for identification of meat adulteration in meat products which are sold as meatballs to reduce production costs. Hopefully, this technique would improve sea snake species identification when morphological examination is no longer possible because the animals are already processed. This is very important to track when sea snake species are being hunted and also used to assess the conservation and management of the sea snakes in Thai waters, especially the Gulf of Thailand.

Kundu *et al.* (2020) generated the mitochondrial Cytochrome b gene of two morphologically identified deadly elapid species from Mizoram. Both, the King Cobra (*Ophiophagus hannah*) and Banded Krait (*Bungarus fasciatus*) showed monophyletic clades in the BA topology and cohesively clustered with the database sequences generated from distant geographical locations. The studied *O. hannah* depicted 2.7–7.6% K2P genetic distances with the specimens collected from China, Vietnam, and Thailand. Further, the northeast Indian *B. fasciatus* revealed 3.3–4% K2P genetic distance from Chinese, Vietnamese, Thailand, Indonesian, and Australian specimens. The TCS network showed distinct haplotypes for both the species collected from northeast India. The genetic information of these venomous snakes would be helpful for further rapid identification from the museum as well as from road-killed specimens, curbing the venom poaching and medical avenues.

2.3.1.1 Complete Mitochondrial Genome: The Ultimate Tool for Phylogeny Study

Guo *et al.* (2008) sequenced the entire mitochondrial DNA (mitogenome) of Russell's snapper (*Lutjanus russellii*) using long PCR and primer-walking methodology (GenBank Accession No. EF514208). The mitogenome was similar in gene composition and order to those of other vertebrates, having 37 structural genes, i.e., two ribosomal RNAs, 22 transfer RNAs, and 13 protein-coding genes. Phylogenetic

analyses based on the mtDNA sequence of Russell's snapper supported a close relationship between *Lutjaninae* and *Caesioninae,* consistent with taxonomic hypotheses based on morphology. More studies utilizing mitogenomes are needed to resolve high-level relationships among snappers.

Dubey *et al.* (2012) reported the complete mitochondrial genome sequence of an endangered Indian snake species *Python molurus molurus.* A typical snake mitochondrial (mt) genome of 17,258 bp length comprising of 37 genes including the 13 protein coding genes, 22 tRNA genes, and 2 ribosomal RNA genes along with duplicate control regions was described. The *P. molurus molurus* mt. genome is relatively similar to other snake mt. Genomes with respect to gene arrangement, composition, tRNA structures and skews of AT/GC bases. The phylogenetic analyses involving the concatenated 13 protein coding genes of P. *molurus molurus* confirmed to the previously established snake phylogeny.

Liu *et al.* (2015) studied the whole mitochondrial genome of a colubridae snake *Elaphe schrenckii.* It is a circular molecule of 17,165 bp in size and consists of 13 protein-coding genes, 22 transfer RNA (tRNA) genes, 2 ribosomal RNA (rRNA) genes and 2 control regions (CRI and CRII). Except for eight tRNAs and ND6 gene, all other mitochondrial genes were encoded on the heavy strand (H strand). The gene order and orientation of *E. Schrenckii* mitogenome are basically identical to that of other alethinophidian snakes. Mitochondrial genome analyses based on MP, ML and NJ yielded identical phylogenetic trees, indicating a close phylogenetic affinity of 12 species of Colubridae snakes. This study will facilitate the further research of the population genetics of this species and systematic analyses of the genus Elaphe.

Zhou B *et al.* (2016) studied the complete mitochondrial genome sequence of the *Ptyas mucosus*. The total length is 17,151 bp and sequence analysis showed its structure is in accordance with other snakes. The complete mitochondrial genome contains 2 rRNA genes, 21 tRNA genes, 13 protein-coding genes (PCGs), 2 control regions and 1 putative origin of L-strand replication. The gene order and nucleotide composition of *P. mucosus* are very similar with *E. bimaculata, E. anomala* and *E. schrenckii*. A phylogenetic tree of mitochondrial genomes indicated *P. mucosus* had the most closely relationship with *E. bimaculata,* and formed a monophyletic group with *E. bimaculata, E. anomala* and *E. Schrenckii*.

Zhou Dan *et al.* (2016) also studied the complete mitochondrial genome of *Rhabdophis tigrinus* (Reptilia: Squamata). It is a circular molecule of 17 415 bp in length (GenBank accession no. KU641019), consisting of 13 protein-coding genes, 22 transfer RNA genes, two ribosomal RNA genes (12S and 16S rRNA) and two control regions (D-loop), with the typical gene order and direction of transcription in Serpentes. The overall base composition is 33.65% A, 26.70% C, 13.16% G and 26.49% T. Mitochondrial genomes analyses based on NJ method yield phylogenetic trees, including 14 reported snakes belonging to four families (Colubridae, Elapidae, Viperidae and Typhlopidae). These molecular data presented here provide a useful tool for systematic analyses of genus Rhabdophis and family Colubridae.

Sun *et al.* (**2017)** investigated the complete mitochondrial genome of the greater green snake *Cyclophiops major*, a protected and colubrid species. The genome is 17,217bp in size, including 13 protein coding genes, 2 rRNA genes, 22 tRNA genes, 2 control regions, and an origin of light-strand replication. All genes are distributed on the heavy strand, except for ND6 gene and 8 tRNA genes. The AT content of the overall base composition of light strand is 59.83%, showing AT bias. Phylogenetic tree was built based on the genome of *C. major* and other related snakes to analyze their phylogenic relationship.

Wu *et al.* (2017) sequenced the complete mitochondrial genome of *Thermophis shangrila* by using the next-generation sequencing technique. The total length of the mitogenome was 17,407 bp, which was composed of 13 protein coding genes, two rRNA genes (12s and 16s rRNA), 22 tRNA genes, and two control regions (CRI and CRII). The base composition was 32.6% for A, 23.9% for T, 30.0% for C, and 13.5% for G. They added a fragment about 150 bp in length at Control region I, which Peng *et al*. failed to obtain using Sanger di-deoxy sequencing.

2.3.2 Development and Characterization of Microsatellites or STR Markers

Hoffman *et al.* (2010) isolated nine microsatellite loci from the Tibetan hot spring snake, *Thermophis baileyi*, using a shotgun sequencing approach. Loci were amplified in 49 individuals from three different populations. The number of alleles per locus ranged from 3-24 and the observed heterozygosity was between 0.162 and 0.844. No evidence for linkage disequilibrium between pairs of loci was found and almost all loci conformed to Hardy-Weinberg expectations with the exception of marker TbA12, which showed evidence of null alleles. Six of the microsatellites showed cross-

amplification in the sister species *Thermophis zhaoermii* and might be applicable to investigate the population genetic structure in this species.

Kwiatkowski *et al*. (2010) isolated and characterized 16 microsatellite loci from the Louisiana pine snake, *Pituophis ruthveni*. Loci were screened in 24 individuals from locations throughout its distribution in Louisiana and Texas. The number of alleles per locus ranged from 4 to 12, observed heterozygosity ranged from 0.200 to 0.875, and the probability of identity ranged from 0.043 to 0.298. They examined cross-species amplification at these loci in *P. catenifer* (bullsnakes and gopher snakes) and *P. melanoleucus* (pine snakes). These new markers provide tools for examining the conservation genetics of this species complex. Louisiana pine snakes face numerous threats: population densities are extremely low and their natural habitat has been severely altered and fragmented. In southern Canada, *P. catenifer* is at the northern extreme of its range and limited by the availability of suitable over-wintering sites. Hence, for these two species reduction of heterozygosity, potential for inbreeding, and increased effects of genetic drift are all of considerable conservation concern.

Lukoschek *et al.* (2011) developed ten microsatellite loci for the elegant sea snake, *Hydrophis elegans*, from partial genomic DNA libraries using a repeat enrichment protocol. Eight loci had nine or more alleles per locus (maximum 20), while the other two had three and seven. All ten loci amplified successfully in 11 of the 15 additional hydrophiine sea snake species screened. Nine loci amplified successfully for three species and eight amplified successfully for the remaining species. Based on this highly successful cross-amplification we expect these ten loci to be useful markers for investigating population genetic structure, gene flow and parentage for all sea snake species from the Hydrophis group.

Zheng *et al.* (2014) characterize ten polymorphic microsatellite loci isolated from *Ptyas korros*, a colubridae snake. Fifty-seven individuals were collected from Quanzhou population in Guangxi, China. These markers revealed a high degree of genetic diversity (4–14 alleles per locus) and heterozygosity (HO ranged from 0.296 to 1.000, and HE ranged from 0.419 to 0.868). No locus exhibited significant deviations from Hardy–Weinberg equilibrium. There was no evidence of linkage disequilibrium among pairs of loci. These microsatellite markers will be useful for the study of gene flow, population structure and evolutionary history of *P. korros*.

Frankham *et al.* (2015) developed 16 novel microsatellite loci using 454 sequencing for one of Australia's most threatened elapid snake species, the broad-headed snake (*Hoplocephalus bungaroides*). To curb the illegal collection of individuals, the species recovery program recommended genetic typing of all individuals in captivity to validate alleged pedigrees. All loci were polymorphic with a mean of 8 (±0.79) alleles per loci. These new forensically informative markers improve the power of available molecular markers to identify the illegal movement of this species, and provide a useful tool for conservation management of this species, not only in captivity but also the wild.

Unger *et al.* (2015) isolated and characterized 33 microsatellite loci for the brown tree snake, *B. irregularis*. The loci were screened across 32 individuals from Guam. The number of alleles per locus ranged from three to ten, with an average of 4.62. The expected (He) and observed heterozygosity (Ho) ranged from 0.294 to 0.856 and from 0.031 to 0.813, with an average of 0.648 and 0.524, respectively. Significant deviations from Hardy–Weinberg equilibrium were detected at seven loci after Bonferroni correction. Probability of identity values ranged from 0.043 to 0.539.

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CHAPTER III

IDENTIFICATION THROUGH FORENSICALLY INFORMATIVE DNA MARKERS

"The industry is becoming very ready for animal identification."

~ Mike Johanns

Due to the constant pressure of ever increasing human population, wildlife habitat loss, global urbanization, illegal wildlife trafficking and poaching, the Indian wild life flora and fauna is depleting, day by day, resulting in ecological imbalance. India enacted its "Wildlife Protection Act in 1972" with wide ranging provisions for punishment of offenders. Among different endangered animal species, snakes are most neglected creature looked at with apathy and therefore, are ruthlessly killed, illegally trafficked and poached for their venom, lucrative skin, meat and bones for manufacturing of medicines, accessories and food items to South East Asian countries. Establishing the identity of the endangered snake species is important for punishing the offenders but morphological characters fail to establish identity as they are often altered. The technique of identification of snake species at molecular level holds very effective conclusion in punishing offenders. The development of DNA-based markers has revolutionised wildlife forensic examination there by conservation of species. Molecular markers in forensic examination are used for detection of variations in nucleotide sequences using PCR technique (Avise *et al.*, 1994; Linda *et al.*, 1995). These markers could also determine genetic diversity, leading to their application in management of the natural resources and genetic improvement programs (Hillis *et al.*, 1996; O'Brien *et al.*, 1991). There are many reports that state use of these molecular markers for identification of plants, reptiles, birds or mammals, in terms of conservation and wildlife forensic identification purposes (Meyer *et al.*, 1994; Cocolin *et al.*, 2000; Arslan *et al.*, 2006; Magnussen *et al.*, 2007). Among all types of molecular markers, in recent past, DNA typing using mitochondrial DNA has become a powerful tool for the identification of non-human species (Pereira *et al.* 2010). The unique features of mtDNA can be applied for wildlife forensic testing in different taxonomic groups and by using appropriate laboratorial and analytical procedures, the erroneous interpretations can be avoided. The mitochodrial genes like Cytochrome b, COI, 12S rRNA, 16S rRNA, NADH 2, NADH 4, NADH 6 shows species specific conserved areas and on the basis of their unique base sequences many mitochondrial markers for the identification of different endangered snake species has been developed till date. They are used in combination of PCR-RFLP or gene sequencing technologies for species identification purpose (Dubey *et al*., 2009; Gaur *et al.*, 2012; Suntarachun *et al.*, 2018). Also, higher-level of snake relationships can also be constructed from sequence analyses of three (3) mitochondrial genes (COI, Cytochrome *b* and 16S rRNA) and one nuclear gene (C-mos) (Bornstein *et al.* 2018). These techniques are reasonably sensitive, specific and cost effective, compared to other DNA based assays (Mane *et al.*, 2006).

Identification of Three Indian Snake Species using Single Tube Multiplex PCR Techniques on Mitochondrial 16S rRNA Gene

3.1.1 Introduction

In India snakes are not treated as important creatures but their body parts are illegally trafficked from India to all South-East Asian countries because of lucrative value of skin, bones and meat resulting in depletion of their numbers in Indian bio-reserves. This requires serious attention in the context of conservation and protection of them (Cox *et al.*, 2012). "Wildlife Protection Act (1972)" has the sufficient provisions for preserving these precious creatures. In addition, CITES also regulates the illegal trafficking of snakes worldwide. Thus, it can be said that there are enough provisions in the law to curb the illicit trade, killing and poaching of these animals (Avise, 1994). However, punishment of animal poachers and traffickers depends on identification of species of animal. In the absence of scientific proof, it is difficult to implement the "WPA (1972)" law. Furthermore, snake skins are treated with chemicals and physically altered, which render morphological and serological examination of species difficult leading to misidentification resulting in lesser punishment and continuous depletion of endangered species (Cox *et al.*, 2012). Therefore, in order to impose appropriate wildlife laws, it is time to invent reliable and fast molecular identification techniques to identify the exact species of traded animals. Hence, it's essential to develop modern identification techniques in order to authenticate seized animals up to species level rather than depending upon basic morphology based identification techniques which are all time-consuming and difficult in cases of physically altered specimens. Many modern techniques based on molecular approaches, are needed in addition to traditional taxonomic methods in order to authenticate identification of animal up to species level and already several methods have been developed for easy identification of the snake exhibit upto species level for proper application of "WPA (1972)". Therefore, the species-specific PCR based assay has been considered imperative for identification of three snake species in this study (Suntarachun *et al.*, 2014). However, fast and cost effective methods are always preferred for examination.

The 16S rRNA is a mitochondrial DNA gene which encodes for large subunit of mitochondria or mt LSU. Approximately 1500 base pairs in length and can be found in both prokaryotic and eukaryotic cells. They are conservative in nature, shows lack of introns and relative abundance in cells. Contain hypervariable regions that can

provide species-specific sequences useful for identification of species. Hence, it has been extensively used for phylogenetic study of a species (Flook et al., 1995). It can be a valuable tool for species identification of extremely degraded exhibit samples. Therefore, in this study, we have generated one set of "species-specific multiplex PCR" (Polymerase Chain Reaction) markers using mitochondrial "16S rRNA" gene for identification of three (3) commonly found, poached and trafficked Indian snake species, namely "*Ptyas mucosa*", "*Daboia russellii*" and "*Naja naja*" which are listed in "Appendix-'II' & 'III'" of "CITES" or "Convention on International Trade in Endangered Species of Wild Fauna and Flora" and "Schedule 'II' Part 'II'" of Indian "WPA, 1972 or Wildlife Protection Act (1972)". Amplified products are then identified by using the simple 2% Agarose Gel electrophoresis techniques. It may consider as a functional tool for wildlife Forensic Identification of morphologically altered exhibits samples from these Indian snake species and promising to be useful for their conservation.

3.1.2 Materials and Methods

This section describes about materials that we used in this study along with methodologies of DNA extractions, PCR amplifications and multiplexing strategies.

3.1.2.1 Sample Collection

The biological samples of "Indian Rat snake" or "*Ptyas mucosa"* (25n), "Russell 's viper" or "*Daboia russellii"* (25n) and "Indian Cobra" (25n) or "*Naja naja"* (25n) were collected from Alipore Zoological Garden, Kolkata, West Bengal, India; Wildlife Rescue Centre and Transit Facility, Salt lake, Kolkata, West Bengal, India and Snake Transit House, Jabalpur, Madhya Pradesh, India. The other non-target species like *Python molurus molurus*, *Chrysopelea ornata*, *Eryx johnii* and distant species like *Crocodylus porosus*, *Varanus bengalensis*, *Katla katla* for specificity testing were obtained from repository of Central Forensic Science Laboratory, Kolkata, India which were collected from Madras Crocodile Bank Trust, Mamallapuram, Chennai, India and Alipore Zoological Garden, Kolkata, West Bengal, India.

3.1.2.2 Extraction of Genomic DNA and Quality Check

From shed skin samples DNA was extracted using "Fetzner protocol" (Fetzner, 1999). From tissue samples whole DNA was extracted using "Qia tissue DNA extraction kit" (Qiagen, Valencia) as per the manufacturer's procedure.

DNA quality and quantity were checked by using 1% agarose gel electrophoresis (Figure-3.1.1). For this we used one blank and one control sample of 100ng/µl along with isolated DNA in the gel block and run the electrophoresis. The more brighter the fluorescence, the higher the concentration i.e.>100ng/µl and vice-versa.

Figure 3.1.1: 1% Agarose gel electrophoresis; **A-** Control DNA of 100ng/µl; **B**– *Ptyas.mucosa*; **C**– *Daboia russellii*; **D**– *Naja naja*; **E**– *Crocodylus porosus*; F-Water.

3.1.2.3 Polymerase Chain Reaction Amplification and Sequencing:

At first, PCR amplification of the targeted 16s rRNA gene was performed using established forward and reverse primers (Palumbi *et al.*, 1998). Standard PCR procedure was performed using 10× PCR Buffer, 5mM MgCl2, 200µM dNTPs, 1U/µl Taq polymerase (Applied Biosystem), 1.25µM forward and reverse primers of both the gene markers and 20ng/µl extracted genomic DNA. The final reaction volume was set as 20 µl. PCR cycle were set as, "initial denaturation" at 94°C for 4minute, followed by 38 cycles of "denaturation" at 94°C for 30seconds, "primer annealing" at 56°C for 1minute 30seconds, "primer extension" at 72°C for 7minutes, then "post cycling extension" at 72°C for 10 min followed by hold at 4°C. The amplified PCR products were detected by 2% agarose gel electrophoresis methods, using Ethidium Bromide stain (0.5µg/ml) (Figure-3.1.2). The 16S rRNA gene showed amplifications around 450bp. All the amplified products were then cycle sequenced using BigDye Terminator Cycle sequencing kit v 3.1 (Applied Bio-systems, Foster City, CA). DNA sequencing was performed on ABI Prism 3100 Genetic Analyzer.

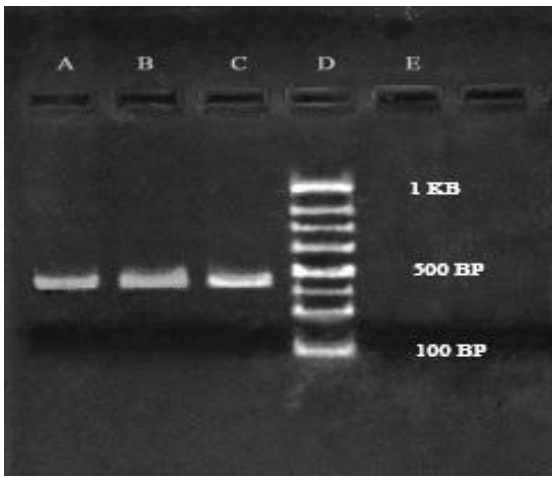

Figure 3.1.2: 2% Agarose gel electrophoresis using 16S rRNA universal primer which shows fluorescence at 450 bp. **A**- *P. mucosa*; **B**- *D. russellii*; **C**- *N. naja;* **D** - 1kb *Ladder;* **E** –Water.

3.1.2.4 Species Specific Multiplex PCR Primer Design

We used mitochondrial 16S rRNA gene to design species specific Multiplex-PCR primers. All the sequences for this gene from snake species were obtained from available public database NCBI (National Centre for Biotechnology Information) and the accession numbers are given in the table no. 3.1.3. To identify species-specific positions we aligned those sequences of snake species along with the partially sequenced gene in our laboratory and aligned them using MEGA 7 software. To design species-specific reverse primers, interspecific nucleotide sequence differences were observed (Henegairu *et al.*, 1997; Dubey *et al.*, 2009; Meganathan *et al.*, 2011). These reverse primers were paired with the newly designed forward primer for 16S rRNA gene and amplified three different snake species via PCR set. Table 3.1.1 shows the designed common primer and species-specific reverse primers. The multiplex-PCR assays were developed to differentiate all four snake species with two sequential panels. Strategies for designing both the multiplexing primers are shown in Figure 3.1.3.

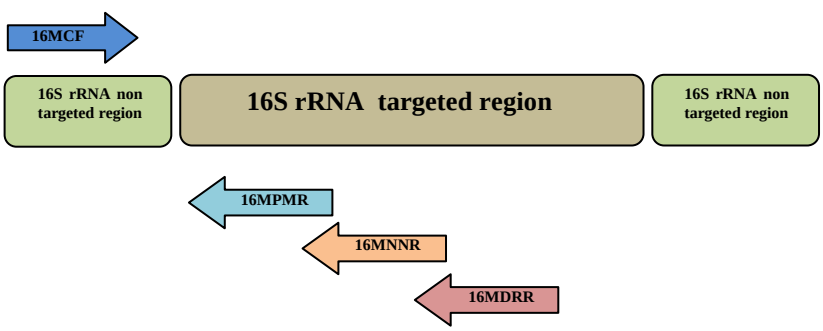

Figure 3.1.3: Strategy for designing 16S rRNA Multiplex PCR primer set.

Table-3.1.1: Newly constructed 16S rRNA gene primers along with their amplicon size.

S. No.	Primer	Sequence	Amplicon size	Specific to
1	16MCF	AAGACCAGAAGACCCTGTGA		
2	16MPMR	GTCATTATGGGGTTTAATGG	50bp	*Ptyas mucosa*
4	16MNNR	TGGCCTAGAAGTGGATGACT	180bp	*Naja naja*
3	16MDRR	AAATGTGGCTTGTTTGCCTT	200bp	*Daboia russellii*

The multiplex-PCR assays were developed to differentiate all three snake species with one sequential panel. The designed primers were subject to BLAST (Basic Local Alignment Search Tool) search using $BLAST_x$ and $BLAST_n$ program.(The result of BLAST search of the designed primers with specificity % is given in table no. 3.1.2) The specificity of each primer for each species was tested in separate reactions. The validation studies were carried out using targeted and non targeted species DNA with sufficient numbers of individuals from a single species.

Table 3.1.2: The result of BLAST search of the designed primers with specificity %

Primers	Max. Score	Total Score	Query cover	E-value	Percent Identity	Accession Length	AccessionNo.
16MPMR	40.1	40.1	100%	1.2	100.00%	485	MK209321.1
16MNNR	40.1	40.1	100%	1.2	100.00%	530	MW282747.1
16MDRR	40.1	40.1	100%	1.2	100.00%	407	KT313023.1

3.1.2.5 Multiplex PCR Amplification

Multiplex PCR amplification involves simultaneous running of multiple PCR primers or target markers in a single tube PCR reaction (Henegairu *et al.*, 1997; Dubey *et al.*, 2009). It was designed for speedy identification of species without former sequencing (Unajak *et al.*, 2011; Datukishvili et al., 2015). A single tube multiplex PCR amplifications was carried out to detect three different snake species groups simultaneously. The Multiplex PCR reaction was carried out using 10x PCR buffer, 5 mM MgCl2, 0.2 mM dNTPs, 1.50µm forward and 1.50µm species- specific reverse primers, 1.0U/µl of Taq polymerase (Applied Biosystem), and 20 ng of extracted DNA. PCR cycle was set as follows; "initial denaturation" at 94°C for 4 minute,

followed by 38 cycles of “denaturation” at 94°C for 30 seconds, species-specific “primer annealing” temperature at 56°C, for 1 minute 30 seconds, “primer extension” at 72°C for 1 minute 15 seconds, then post cycling or “final extension” at 72°C for 7 minutes followed by final hold at 4°C. The final amplified PCR products were detected by 2.5% agarose gel electrophoresis methods, using Ethidium Bromide stain (0.5µg/ml) (Figure-3.1.4).

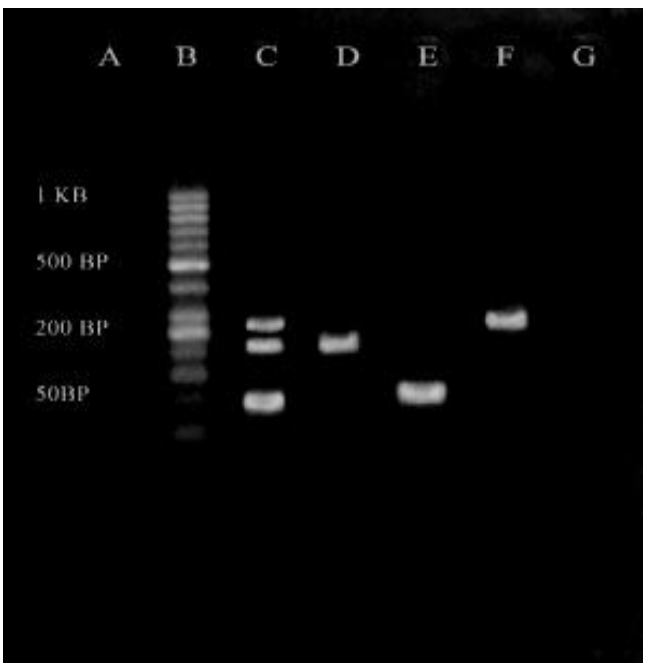

Figure 3.1.4:- 2.5% agarose gel electrophoresis results using 16MCF, 16MPMR, 16MNNR and 16MDRR primers designed in this study.**A-** Water; **B-** 1Kb Ladder; **C-** Single tube Multiplex; **D-** *N.naja (180 bp)***; E-***P.mucosa (50 bp)*; **F-** *D.russellii (200 bp).*

3.1.3 Results and Discussions

Multiplex PCR is a variant of PCR process where two or more loci are simultaneously amplified in the same run. Here, a common forward primer and three (3) species-specific reverse primers have been constructed for both the genes, separately, to amplify three different size species- specific amplicons. Both the multiplex-PCR panel shows the rapid identification of individual species assigned to each of them. Firstly, single tube multiplex PCR reaction (panel A for Figure 3.1.4) was performed using species specific three reverse primers 16MPMR, 16MNNR, 16MDRR in combination with common forward primer 16MCF designed in this study allowing the following amplifications, visible under 2.5% agarose gel electrophoresis run for 30 min:

For Multiplex PCR using 16S rRNA marker (Figure 3.1.4):

(i) A single band of 180 bp for *Naja naja* (panel D)

(ii) A single band of 50 bp for *Ptyas mucosa* (panel E),

(iii)A single band of 200 bp for *Daboia russellii* (panel F),

Secondly, parallel specificity run was also performed in a single PCR amplification (Panel D, E & F) using individual species -specific primers and forward primers for each sample. The results show the DNA bands occurred at expected length from all the studied species (Figure 3.1.4).

Species-specific multiplex PCR markers were used to analyze the three snake species samples that we studied. The results showed expected amplification at the designed band. These sets of primers are the first DNA markers to exactly identify the three snake species examined. Our multiplex PCR assay clearly identified three snake species (*Ptyas mucosa, Daboia russellii and Naja naja*) in two simultaneous runs followed by 2.5% Agarose gel electrophoresis check. Therefore these markers can be effectively used for rapid and accurate identification of species in wildlife forensic case work, conservation studies and medicinal research.

3.1.4. Conclusion

Forensic DNA testing is a key investigative tool for combating wildlife crimes. Conservation biologists also focus on the progress of novel DNA based technologies for wildlife protection strategies. Since the inception of the Multiplex PCR techniques it has been successfully applied in many arena of Forensic DNA testing. Here we describe Multiplex- PCR reaction kits based on two separate multiplex PCR marker sets which can be used for the rapid identification of all the four Indian snake species. Species identification can be established by performing PCR followed by 2.5% Agarose gel electrophoresis which is definitely economic, fast and accurate. This method of species identification using Multiplex–PCR techniques described in this paper is cost effective and less time consuming compared to other techniques based on DNA sequencing.

Therefore, this Multiplex-PCR assay technique can be used in wildlife forensic cases involving indigenous snake samples. The newly developed multiplex PCR kits are expected to help the forensic scientist for providing proper identification of the exhibits from three Indian snake species.

Table 3.1.3: List of snake species along with their accession numbers that we have used while designing 16S rRNA gene multiplex primers

Serial No.	Gene	Species Name	Accession No.
1	16S rRNA	*Vipera raddei*	AJ275784.1
2	16S rRNA	*Vipera berus*	AJ275781.1
3	16S rRNA	*Daboia russellii*	AJ275776.1 KT313023.1 DQ305436.1 GQ398147.1
4	16S rRNA	*Echis coloratus*	AJ275760.1
5	16S rRNA	*Bitis arietans*	AJ275743.1
6	16S rRNA	*Naja atra*	EU729433.1
7	16S rRNA	*Deinagkistrodon acutus*	EU729428.1
8	16S rRNA	*Ophiophagus Hannah*	Z46480.1
9	16S rRNA	*Python reticulates*	Z46478.1
10	16S rRNA	*Bangarus fasciatus*	Z4650.1
11	16S rRNA	*Eryx jayakari*	HQ267804.1
12	16S rRNA	*Oxyrhopus melanogenys*	AF158489.1
13	16S rRNA	*Ahaetulla fasciolata*	KX660204.1
14	16S rRNA	*Hydrophis fasciatus*	KT966164.1
15	16S rRNA	*Ptyas fusca*	KX660202.1
16	16S rRNA	*Typhlops pusillus*	AF36683.1
17	16S rRNA	*Ptyas mucosa*	MH423745.1 KC589121.1 AY611815.1
18	16S rRNA	*Xenochrophis piscator*	KX27727.1
19	16S rRNA	*Xenochrophis vittatus*	EF395846.1
20	16S rRNA	*Naja naja*	GQ359756.1 EU624270.1 EU547137.1
21	16S rRNA	*Crotalus pricei pricei*	JN022947.1
22	16S rRNA	*Pelamis platurus*	DQ234050.1
23	16S rRNA	*Trimeresurus macrops*	KR021133.1
24	16S rRNA	*Demansia papuensis*	KF736327.1
25	16S rRNA	*Pantherophis guttatus*	AM236349.1
26	16S rRNA	*Boa constrictor*	AM236348.1
27	16S rRNA	*Lycodryas maculates*	HE798447.1
28	16S rRNA	*Pseudechis papuanus*	AJ749374.1
29	16S rRNA	*Vipera raddei*	AJ275784.1
30	16S rRNA	*Atheris squamigera*	AJ275737.1

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Identification of Three Indian Snake Species using Single Tube Multiplex PCR Techniques on Nuclear C-mos Gene

3.2.1 Introduction

India is a country famous for its rich wild flora and fauna. In order to conserve wildlife species in their natural habitat and to facilitate the sustainable management of natural resources there is a requirement to regulate the illegal trading, killing and poaching of wild flora and fauna. The implementation of various agreements designed to achieve this, such as the Convention on the International Trade in Endangered Species (CITES), requires identification of animals and plants or their products and derivatives; however this is often difficult where morphological characters have been lost or where the issue concerns the geographic or familial origin of a sample. Wildlife DNA forensics involves the application of molecular genetics identification techniques to present evidence in wildlife crime investigation cases and require robust molecular markers, informative at the level of the species, population and individual, in a wide range of taxa (Butler, 2005). Over the past few years many molecular identification techniques have been proved to be effective tool for species identification (Fajardo *et al*., 2006) and have already undergone forensic validation, such as the mitochondrial cytochrome b (Budowle *et al.*, 2005) and cytochrome oxidase I (Dawnay *et al.* 2008) gene regions. Thus, DNA sequencing has proved to be most robust and reliable technique for species identification. Mitochondrial DNA sequence analysis is also enormously useful in studies of phylogenetic relationship and allowed researchers to analyze evolutionary relationships among species (Watson *et al.*, 1982; Jerome *et al.*, 2003). Although the mitochondrial DNA sequences have shown their forensic utility in identification of species, constructing phylogenetic tree but some of the nuclear genes have also played very important role in forensic discrimination of wildlife products (Hillis *et al.*, 1992; Friedlander *et al.*, 1994; Jerome *et al.*, 2003).

Nuclear primers based species identification in forensic studies has the advantage as conclusions can be verified by examining a number of independent primers. This is very useful in cases, where PCR contamination is a possibility, or when hybridization between species is known to occur. Here, conserved genes are targeted for amplification with broad-ranged primers which are divergent enough to resolve closely related species. Therefore, the nuclear gene data can be best assessed to rebuild nodes at deepest levels of the reptilian tree for their discrimination (Palumbi *et*

al., 1998). In view of their utility, we have selected nuclear gene i.e. oocyte maturation factor (C-mos) for species identification purpose. The C-*mos* is a candidate nuclear gene (Graybeal *et al.*, 1994), which is a proto-oncogene that encodes a serine/threonine kinase expressed at high levels in germ cells. It is single-copy, without introns, and is just over 1000 base pairs (bp) in size. It has been found in the genomes of vertebrates like amphibians, birds and mammals. There are no repetitive bases in the sequences and few insertions or deletions would results as complicated sequence alignment among vertebrates (Saint *et al.*, 1998; Jogayya *et al.*, 2018). These characteristics make this gene very amenable to PCR amplification from genomic DNA and direct sequencing of PCR products.

Therefore, in this study, we have designed one set of "species-specific multiplex PCR" (Polymerase Chain Reaction) markers using nuclear "C-mos" gene for identification of three (3) commonly found, poached and trafficked Indian snake species, namely "*Ptyas mucosa", "Daboia russellii" and "Fowlea piscator"* which are listed in "Appendix-'II' & 'III'" of "CITES" or "Convention on International Trade in Endangered Species of Wild Fauna and Flora" and "Schedule 'II' Part 'II'" of Indian "WPA, 1972 or Wildlife Protection Act (1972)". The species-specific PCR based assay has been considered imperative for identification of three snake species in this study. Amplified products are then identified by using the simple 2% Agarose Gel electrophoresis techniques. It may consider as a functional tool for wildlife Forensic Identification of morphologically altered exhibits samples from these Indian snake species and promising to be useful for their conservation as, fast and cost- effective methods are always preferred for examination.

3.2.2 Materials and Methods

This section describes about materials that we used in this study along with methodologies of DNA extractions, PCR amplifications and multiplexing strategies.

3.1.2.1 Sample Collection

The biological samples of "Indian Rat snake" or "*Ptyas mucosa"* (25n), "Russell 's viper" or "*Daboia russellii"* (25n) and "Checkered keelback" (25n) or "*Fowlea piscator"* (25n) were collected from Alipore Zoological Garden, Kolkata, West Bengal, India; Wildlife Rescue Centre and Transit Facility, Salt lake, Kolkata, West Bengal, India and Snake transit house, Jabalpur, Madhya Pradesh, India. The other

non-target species like *Python molurus molurus, Chrysopelea ornata, Eryx johnii* and distant species like *Crocodylus porosus, Varanus bengalensis, Katla katla* for specificity testing were obtained from repository of Central Forensic Science Laboratory, Kolkata, India which were collected from Madras Crocodile Bank Trust, Mamallapuram, Chennai, India and Alipore Zoological Garden, Kolkata, West Bengal, India.

3.2.2.2 Extraction of Genomic DNA and Quality Check

From shed skin samples DNA was extracted using "Fetzner protocol" (Fetzner, 1999). From tissue samples whole DNA was extracted using "Qia tissue DNA extraction kit" (Qiagen, Valencia) as per the manufacturer's procedure.

DNA quality and quantity were checked by using 1% agarose gel electrophoresis (Figure-3.2.1). For this we used one blank and one control sample of 100ng/µl along with isolated DNA in the gel block and run the electrophoresis. The more bright the fluorescence, the higher the concentration i.e.>100ng/µl and vice-versa.

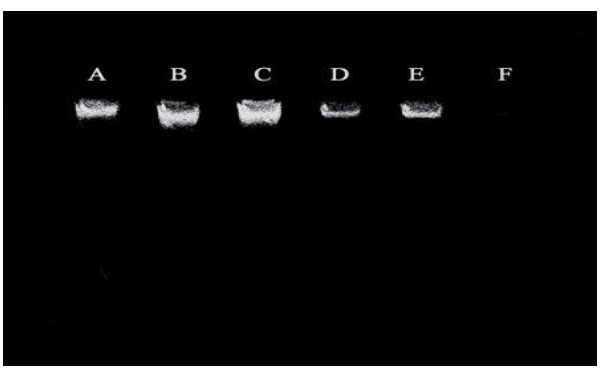

Figure 3.2.1: 1% Agarose gel electrophoresis.; **A**- Control DNA of 100ng/µl; **B**– *Ptyas.mucosa*; **C**– *Daboia russellii*; **D**– *Fowlea piscator*; **E**- *Crocodylus porosus* **F**-Water.

3.2.2.3 Polymerase Chain Reaction Amplification and Sequencing

At first, PCR amplification of the targeted C-mos gene was performed using established forward and reverse primers (Saint *et al.*, 1998). Standard PCR procedure was performed using 10× PCR Buffer, 5mM MgCl2, 200µM dNTPs, 1U/µl Taq polymerase (Applied Biosystem), 1.25µM forward and reverse primers of both the gene markers and 20ng/µl extracted genomic DNA. The final reaction volume was set as 20 µl. PCR cycle were set as, "initial denaturation" at 94°C for 4minute, followed by 38 cycles of "denaturation" at 94°C for 30seconds, "primer annealing" at 60.5°C

for 1minute 30seconds, “primer extension” at 72°C for 7minutes, then “post cycling extension” at 72°C for 10 min followed by hold at 4°C. The amplified PCR products were detected by 2% agarose gel electrophoresis methods, using Ethidium Bromide stain (0.5µg/ml) (Figure-3.2.2). The C-mos gene showed amplifications around 700bp. All the amplified products were then cycle sequenced using BigDye Terminator Cycle sequencing kit v 3.1 (Applied Bio-systems, Foster City, CA). DNA sequencing was performed on ABI Prism 3100 Genetic Analyzer.

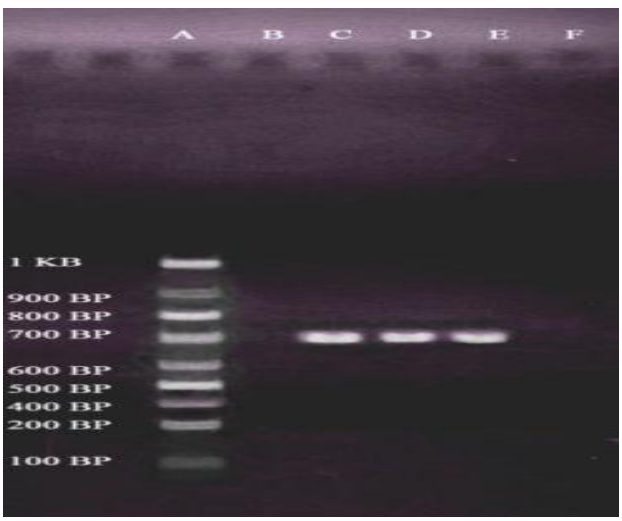

Figure 3.2.2: 2% Agarose gel electrophoresis using C-mos universal primer primer which shows fluorescence at 700 bp. **A**- 1kb *Ladder*; **B**-Water; **C**- *P. mucosa*; **D**- *D. russellii*; **E**- *F. piscator*.

3.2.2.4 Species Specific Multiplex PCR Primer Design

We used nuclear C-mos gene to design species specific Multiplex-PCR primers. All the sequences for this gene from snake species were obtained from available public database NCBI (National Centre for Biotechnology Information) and the accession numbers are given in the table no. 3.2.3. To identify species-specific positions we aligned those sequences of snake species along with the partially sequenced gene in our laboratory and aligned them using MEGA 7 software. To design species-specific reverse primers, interspecific nucleotide sequence differences were observed (Henegairu *et al.*, 1997; Dubey *et al*., 2009). These reverse primers were paired with the newly designed forward primer for C-mos gene and amplified three different snake species via PCR set. Table 3.2.1 shows the designed common primer and species-specific reverse primers. The multiplex-PCR assays were developed to differentiate all three (3) snake species within a single sequential panel. Strategies for designing both the multiplexing primers are shown in Figure 3.2.2.

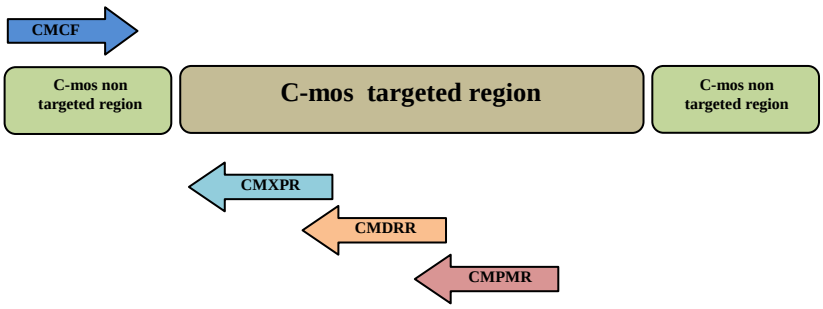

Figure 3.2.3: Strategy for designing C-mos Multiplex PCR primer set.

Table 3.2.1: Newly constructed C-mos multiplex-PCR primer set along with their amplicon size**.**

S. No.	Primer	Sequence	Amplicon size	Specific to
1	CMCF	CATAGTAGCTGCTAGCACATGTA		
2	CMPMR	GAGTTTTAAGCCCGTGCGCACAG	250bp	*Ptyas mucosa*
3	CMDRR	GGTCACATTTATGGCTATCATTG	150 bp	*Daboia russellii*
4	CMXPR	CCTGTTAAATCACCAGTTCCATAG	120bp	*Fowlea piscator*

The multiplex-PCR assays were developed to differentiate all three snake species with one sequential panel. The designed primers were subject to BLAST (Basic Local Alignment Search Tool) search using $BLAST_x$ and $BLAST_n$ program (The result of BLAST search of the designed primers with specificity % is given in the Table 3.2.3). The specificity of each primer for each species was tested in separate reactions. The validation studies were carried out using targeted and non targeted species DNA with sufficient numbers of individuals from a single species.

Table 3.2.2: The result of BLAST search of the designed primers with specificity %

Primers	Max. Score	Total Score	Query cover	E-value	Percent Identity	Accession Length	AccessionNo.
CMPMR	46.1	46.1	100%	0.019	100.00%	578	GQ225670.1
CMDRR	46.1	46.1	100%	0.019	100.00%	548	GQ225671.1
CMFPR	48.1	48.1	100%	0.007	100.00%	601	LC325792.1

3.2.2.5 Multiplex PCR Amplification

Multiplex PCR amplification involves simultaneous running of multiple PCR primers or target markers in a single tube PCR reaction (Henegairu *et al.*, 1997; Dubey *et al.*, 2009). It was designed for speedy identification of species without former sequencing (Datukishvili *et al.*, 2015). A single tube multiplex PCR amplification was carried out to detect three different snake species groups simultaneously. The Multiplex PCR reaction was carried out using 10x PCR buffer, 5 mM MgCl2, 0.2 mM dNTPs, 1.50µm forward and 1.50µm species- specific reverse primers, 1.0U/µl of Taq polymerase (Applied Biosystem), and 20 ng of extracted DNA. PCR cycle was set as follows; "initial denaturation" at 94°C for 4 minute, followed by 38 cycles of "denaturation" at 94°C for 30 seconds, species-specific "primer annealing" temperature at 60°C, for 1 minute 30 seconds, "primer extension" at 72°C for 1 minute 15 seconds, then post cycling or "final extension" at 72°C for 7 minutes followed by final hold at 4°C. The final amplified PCR products were detected by 2.5% agarose gel electrophoresis methods, using Ethidium Bromide stain (0.5µg/ml) (Figure-3.2.4).

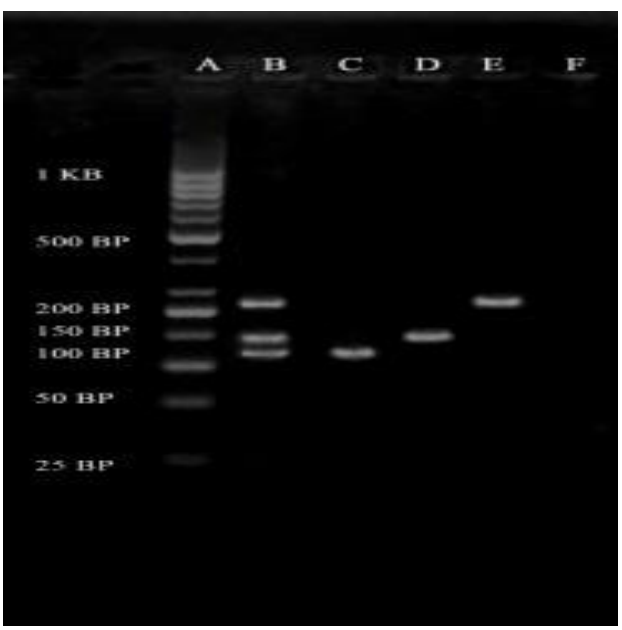

Figure 3.2.4: 2.5% agarose gel electrophoresis results using CMCF, CMPMR, CMXPR and CMDRR primers designed in this study. **A-** 1Kb Ladder; **B-** Single tube Multiplex; **C-** *F.piscator (120 bp)***; D**-*D.russellii (150 bp);* **E**- *P.mucosa (250 bp);* **F**- Water.

3.2.3 Results and Discussions

Multiplex PCR is a variant of PCR process where two or more loci are simultaneously amplified in the same run. Here, a common forward primer and three (3) species-specific reverse primers has been constructed for both the genes, separately, to amplify three different size species- specific amplicons. Both the multiplex-PCR panel shows the rapid identification of individual species assigned to each of them. Firstly, single tube multiplex PCR reaction (panel B for Figure 3.2.4) was performed using species specific three reverse primers CMPMR, CMDRR and CMXPR in combination with common forward primer CMCF designed in this study allowing the following amplifications, visible under 2.5% agarose gel electrophoresis run for 30 min:

For Multiplex PCR using C-mos marker (Figure 3.2.4):

(i) A single band of 120 bp for *Fowlea piscator* (panel C),
(ii) A single band of 150 bp for *Daboia russellii* (panel D) and
(iii)A single band of 250 bp for *Ptyas mucosa* (panel E).

Secondly, parallel specificity run was also performed in a single PCR amplification (Panel D, E & F) using individual species -specific primers and forward primers for each sample. The results show the DNA bands occurred at expected length from all the studied species (Figure 3.2.4).

Species-specific multiplex PCR markers were used to analyze the three Indian snake species samples that we studied. The result shows expected amplification at the desired band. These sets of primers are the first DNA markers to exactly identify the three snake species examined. Our multiplex PCR assay clearly identified three snake species (*Ptyas mucosa, Daboia russellii, and Fowlea piscator*) using one multiplex PCR run followed by 2.5% Agarose gel electrophoresis check. Therefore these markers can be effectively used for rapid and accurate identification of species in wildlife forensic case work, conservation studies and medicinal research.

3.2.4 Conclusion

Wildlife DNA forensic is a key investigative tool for combating wildlife crimes. Conservation biologists also focus on the progress of novel DNA based technologies for wildlife protection strategies. Since the inception of the Multiplex- PCR

techniques it has been successfully applied in many arena of wildlife DNA testing. At the same time, the way DNA evidences are generated and presented in the court, the system is also coming under renewed scrutiny. Here we described a Multiplex- PCR reaction kits which can be used for the rapid identification of three Indian snake species. Species identification can be established by performing PCR followed by 2.5% Agarose gel electrophoresis which is definitely economic, fast and accurate. This method of species identification using Multiplex–PCR techniques described in this paper is cost effective and less time consuming compared to other techniques based on DNA sequencing.

Therefore, this Multiplex-PCR assay technique can be used in wildlife forensic cases involving indigenous snake samples. The newly developed multiplex PCR kits are expected to help the forensic scientist for providing proper identification of the exhibits from three Indian snake species. Hence, we recommend the use of these novel primers in the forensic as well as populations of endangered crocodile species, which will help in the effective implementation of wildlife convention to conservation of these species.

Table 3.2.3: List of snake species along with their accession numbers that we have used while designing C-mos gene multiplex primers.

Serial No.	Gene	Species Name	Accession No.
1	C-mos	*Ptyas mucosa*	GQ225670.1 AF471151.1 KR814679.1
2	C-mos	*Ptyas korros*	AY486953.1
3	C-mos	*Ptyas luzonensis*	KX660392.1
4	C-mos	*Ptyas fusca*	KX660342.1
5	C-mos	*Naja naja*	AF435020.1
6	C-mos	*Naja nivea*	AY058939.1
7	C-mos	*Naja kaouthia*	AY058938.1
8	C-mos	*Naja atra*	KX694797.1
9	C-mos	*Daboia russellii*	GQ225671.1 AF471156.1 MH122714.1
10	C-mos	*Xenochrophis piscator*	GQ225669.1 LC325763.1 LC325764.1 LC325791.1
11	C-mos	*Ophiophagus hannah*	AY058940.1
12	C-mos	*Boa constrictor*	KC329979.1
13	C-mos	*Lichanura trivirgata*	AF544687.1
14	C-mos	*Eryx conicus*	DQ469787.1
15	C-mos	*Corallus hortulanus*	JX244317.1
16	C-mos	*Corallus annulatus*	JX244314.1
17	C-mos	*Crotalus atrox*	JN090135.1
18	C-mos	*Crotalus oreganus*	JN620895.1
19	C-mos	*Enhydris polylepis*	EF473655.1
20	C-mos	*Pythonodipsas carinata*	FJ404290.1
21	C-mos	*Python molurus bivittatus*	AF435016.1
22	C-mos	*Bungarus multicinctus*	AF435021.1
23	C-mos	*Bungarus fasciatus*	AF544732.1
24	C-mos	*Vipera ursinii*	AF433658.1
25	C-mos	*Oxyrhopus rhombifer*	GQ457876.1
26	C-mos	*Leptodeira annulata*	GQ457866.1
27	C-mos	*Boiga irregularis*	FJ710793.1
28	C-mos	*Micrurus surinamensis*	EF 137422.1
29	C-mos	*Natrix natrix*	AF544697.1
30	C-mos	*Cerastes cerastes*	AF544679.1

3.2.5 References

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CHAPTER IV

COMPLETE MITOCHONDRIAL GENOME: THE ULTIMATE TOOL FOR PHYLOGENY STUDY

"The work to protect one species benefits us all."

~ Jennifer Skiff

The study of the complete mitochondrial DNA (mtDNA, mitochondrial genome or mitogenome) of an organism leads to a better insight in to the history of their evolution and biological make up (Avice, 1994). It is one of the fundamental genetic marker for biodiversity studies, forensic identification, molecular ecology, population genetics and phylogenetics because of its conservative gene order, high copy number, lack of recombination, maternal inheritance, absence of introns and presence of protein coding genes that gives overall scenario of the genome (Wolstenholme, 1992; Boore, 1999). It can be vividly use for population structure analysis, conservation genetics and wildlife conservation purposes as well as to identify species from hybrid or cryptic individual in a population (Parham *et al.*, 2006). This features added the applications of the mtDNA in forensic identification of many unknown species which are reported earlier. Another application of complete mitogenome analysis is construction of phylogenetic tree to find the evolutionary relationships among species (Dong *et al.*, 2005; Zhang *et al.*, 2011). Further, the development of high throughput sequencing technologies or Next-generation sequencing aided much value to the study of different species by reducing time, labour and cost (Hahn *et al.*, 2013; Wu *et al.*, 2017). The recovery of entire mitochondrial genome (mtGenome) data and the additional discriminatory power those data offer is arguably the greatest benefit that NGS brings to forensic mtDNA analysis. MtGenome data development via NGS is technically feasible and may be more cost-effective than current Sanger-based control region sequencing. As a result, many laboratories, including the FBI Laboratory, are evaluating mtGenome assays for casework implementation.

The Complete Mitochondrial genome Study of Asiatic Water Snake (*Fowlea piscator*) using Next-Generation Sequencing

4.1. Introduction

Asiatic water snake or Checkered keelback or *Fowlea piscator or Xenochrophis piscator* (Schneider, 1799) are non-venomous, almost 3-5 ft long, fleshy snakes found near freshwater ponds, lakes or rivers and belong from the family Colubridae. They are listed as least concerned species by IUCN Red list and in schedule III of CITES, which plays an important role for controlling ecological balance of the environment (Hossain, 2016). This species comes from the family Colubridae and sub-family Natricinae but till date their classification or phylogenetic position is not confirmed genetically (Vogel *et al.*, 2012). Despite their wide distribution over Asian countries, they are still deploying in numbers and the main reasons for their declination are habitat loss for rapid urbanization, illegal trafficking to East Asian countries for their protein rich meat, lucrative chequered board pattern skin & killing due to fear of threat to life (Das, 2000, 2012; Whitekar, 1986, 2006). Hence, species *Fowlea piscator* and their body parts are now-a day's becoming products of significant commercial importance in the East Asian countries markets and may leads to turn into an endangered species in near future (Hossain, 2016).

Therefore from now conservation strategies must be start for them. In purpose to fulfil that, it is required to utilize large amount of data for better consideration of phylogenetics or evolutionary relationships among species. The information garnered from the field of molecular genetics could be of enormous help for conservation purpose of this species. However, in public database NCBI, there are 240 nos of complete mtDNA sequences from snake species are available, which may have been contributed to the study of snake phylogenetics. But, the complete mtDNA of none species from the genus Fowlea has been described yet in spite of having 12 species of snakes in the genus Fowlea presently (Schneider, 1792; Vogel and David, 2012).

In view of the above, it is important to generate the complete mitochondrial DNA sequences of the Asiatic water snake, *Fowlea piscator,* which can be used in the study of phylogenetics, gene variability and species identification to aid the perceptive of genetic relationships among snakes. Here, we sequenced and analysed the complete mitochondrial genome of *Fowlea piscator* from Eastern India using Next-generation

sequencing or High throughput sequencing, in relation to achieve the genetic information about this species, its genome resource, and phylogenetic position.

4.2. Materials and Methodology

The following materials and methodologies have been applied in this study.

4.2.1. Sample Collections

F. piscator's biological samples (saliva and shed skin) were collected from the Zoological garden, Alipore, Kolkata, West Bengal, India and Wildlife Rescue Centre, Kolkata, India.

4.2.2. Isolations, Qualitative and Quantitative Analysis of Mitochondrial DNA

Genomic DNA was extracted from the samples using the DNeasy Blood & Tissue Kit (Qiagen, Germany) as per manufacturer's protocol. Isolated DNA was run at 110 V for 30 minutes and checked on 1% agarose gel (loaded 5 µl) for the intact band (Figure 4.1). For determining concentration of DNA, 1 µl of sample was analyzed using Qubit Fluorometer 2.0 (Invitrogen, USA) which results in the range of 0.3 ng/µl with A260/280 ratios in the range of 1.89 (Table 4.1).

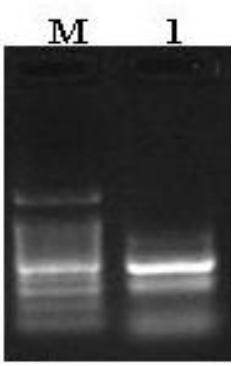


Figure 4.1: 1% gel image; Lane M: 100bp Ladder & Lane 1: CFSLK_IM_XP512567

Table 4.1: Results of quantification by Qubit Fluorometer 2.0

Sample ID	Concentration(ng/µl)	A260/280
CFSLK_IM_XP512567	0.3	1.89

4.2.3. Preparation of Library

The paired-end sequencing library was prepared from mitochondrial DNA samples of *Fowlea piscator* or coded as CFSLK_IM_XP512567, by using QIAseq FX DNA Library kit (Qiagen, Germany). It was designed based on the complete mitochondrial genome sequence of a closely related snake species *Rhabdophis tigrinus* (Accession No: KU641019.1) which was available on public database NCBI (Corodinator, 2018). The library preparation process was initiated with 100ng DNA. At first, the DNA was enzymatically sheared into smaller fragments. Then fragmented DNA made ready for adapter ligations by continuous step of end-repair. Following this step, the generated adapters were ligated to both ends of the DNA fragments. These adapters hold sequences, essential for binding to a flow cell for further sequencing. It allows the adapter-ligated fragments for binding with standard Illumina sequencing primers and PCR amplification. To ensure maximum yields, a high-fidelity amplification step was performed using HiFi PCR Master Mix (Takara Bio, Japan).). The average size of libraries was 324bp. The libraries were then sequenced on Illumina NextSeq 500 (Illumiina, San Diego, USA) platform (2x150bp chemistry) to generate ~1.5-2 GB data from the sample.

4.2.4. Quantity and quality check (QC) of library on Bioanalyzer

The amplified library was then analyzed in Bioanalyzer 2100 (Agilent Technologies) using High Sensitivity (HS) DNA chip (Figure 4.2). It gave concentration of 21,514.03 pg/µl in between 200 to 522 bp range. The average size of library was 324bp.

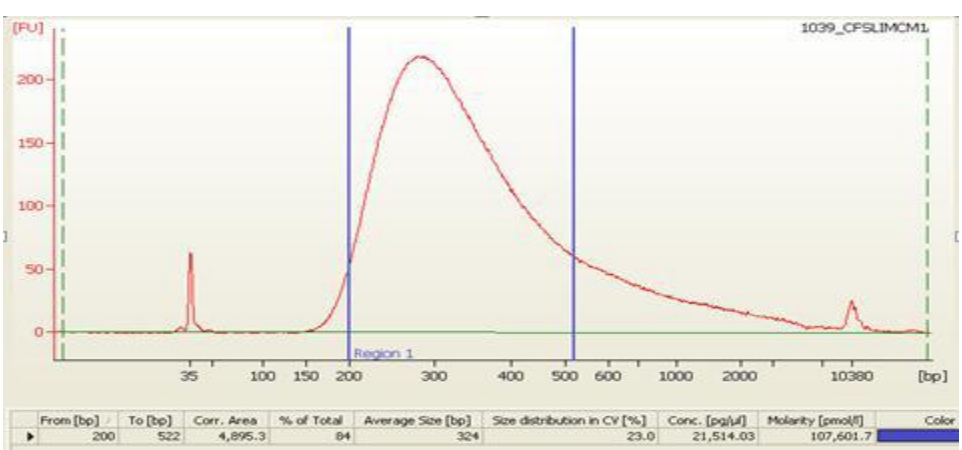


Figure 4.2: Bioanalyzer profiles of library loaded in Agilent DNA HS chip

4.2.5. Cluster Generation and Sequencing

After obtaining the DNA concentration the library was loaded on to Illumina platform for cluster generation and paired-end sequencing. It allows the template fragments to be sequenced in both directions. The generated library molecule gets binded with complementary adapter oligos on paired-end flow cell. The adapters were designed such as to allow selective cleavage of both forward and reverse strands during sequencing. The copied reverse strand was used to sequence from the opposite end of the fragment.

4.2.6. Sequencing and Assembling

After removing unpaired, short, poor-quality reads only top quality paired end reads were used for reference based sequence assembly using MITObim version1.9 (Hahn *et al.*, 2013). It gave Reads (R1 & R2) of 4200774. The total reads (R1+R2) were 8401548 and the total bases were 1,26,86,33,748 resulting in production of 1.2GB data (Table 4.2). Annotations were generated using MITOS WebServer 2. (Bernt *et. al.*, 2013), by comparison method with close species *Rhabdopis tigrinus* (GenBank Accession No. KU641019.1).

Table 4.2: Data statistics for the samples CFSLK_IM_XP512567

Samples	Reads(R1)	Reads(R2)	Total_Reads (R1+R2)	Total_Bases (R1+R2)	Total Data (GB)
CFSLK_IM_XP 512567	4200774	4200774	8401548	1268633748	1.2 GB

4.2.8. Gene Annotations

The genome was annotated by using MITOS WebServer 2 (Bernt *et. al.*, 2013). Next, the mitogenome was integrally inspected first using tRNA-Scan SE 2.0 (Lowe *et al.*, 2016) for detecting 22 tRNAs and their cloverleaf structure by using ARWEN program (Laslett, 2008). The annotated genes were then cross-checked to estimate the boundaries of protein coding genes, ribosomal RNAs and control region or D-Loop. Next, to assess the sizes of PCGs, the position of start and stop codon were used. For checking the annotations the proteins were then translated to amino acids and compared with subsequent genes. By aligning the newly generated sequences with other available snake mtDNA sequences, the two rRNA genes were identified. The

nucleotide length and composition for different PCGs were calculated in MEGA 7 (Kumar *et al.*, 2016) and codon usage for all PCGs of *Fowlea piscator* was estimated using the sequence manipulation suit's codon usage calculator (Stothard, 2000). Also the A, T, G, C content and base skews were calculated using MEGA 7 (Kumar *et al.*, 2016) to study the mitogenome.

4.3. Results and Discussions

The complete mitochondrial genome of *F. piscator* generated through this study was was 16,999bp in length with base composition of 33% A, 28% T, 27% C and 12% G, with a GC content of 39% and submitted to NCBI with GenBank Accession no. OK110208.1. The 17kb complete mitochondrial genome was composed of 22 numbers of transfer RNA or tRNA genes, 13 numbers of protein-coding genes or PCGs, 2 numbers of ribosomal RNA or rRNA genes, 2 numbers of control regions (CRs), and a origin of light-strand replication (OL), which is similar to typical snake mtDNA (Xu *et al.*, 2015; Sun *et al.*, 2017) (Table 4.4) (Figure 4.3).

4.3.1. Transfer RNAs:

All the 22 transfer RNA (tRNA) genes commonly found in metazoan were also present in this *F. piscator* species. The 22 tRNA genes ranged from 58bp (tRNA-Ser) to 84 bp (tRNA-Asn). It possesses typical cloverleaf secondary structure. The DHU loop was absent for tRNA Ser and tRNA Asn. The TΨC stem was usually 4-5 nucleotides but in case of tRNA Gly and tRNA Met it shortened to 3 nucleotides and much less in case of tRNA Phe (Kumazawa. 1996). The OL was 39 bp long and was typically located between tRNA-Asn and tRNA-Cys genes. Table 4.5 describes the cloverleaf structure of 22tRNA.

Table 4.3: List of Software used for bioinformatics analysis

Software	Version	Application
CLC genomics Workbench	6	For denovo assembly of reads
MITObim	1.9	For denovo assembly of reads
MITOS WebServer	2	For gene annotation
BLASTN	2.2.28	For similarity search against NT database
tRNAscan-SE	2.0	For tRNA scan
ARWEN	1.2	For tRNA cloverleaf structure
EGA	7	For Phylogenetic study

Table 4.4: Characteristics of Complete Mitochondrial genome of *F. piscator* (https://www.biologicscorp.com/tools/GCContent/)

Gene	From	To	Size(bp)	A	T	G	C	Start	Stop	Strand	Anti-codon
tRNA-Phe	1	67	67	24	19	11	13			H	GAA
12S rRNA	67	994	927	334	183	161	249			H	
tRNA-Val	995	1060	66	27	17	7	15			H	TAC
16S rRNA	1061	2531	1471	567	318	240	346			H	
ND1	2532	3495	964	299	257	116	292	ACA	TAA	H	
tRNA-Iso	3496	3552	57	19	12	16	10			H	GAT
CRII	3553	4627	1075	300	341	132	302				
tRNA-Leu	4628	4700	73	27	15	13	18			H	TAA
tRNA-Gln	4701	4771	71	26	17	7	21			L	TAG
tRNA-Met	4772	4835	64	22	11	12	19			H	CAT
ND2	4836	5862	1027	396	233	82	315	ATT	CCA	H	
tRNA-Trp	5863	5927	65	24	18	10	13			H	TCA
tRNA-Ala	5931	5995	65	21	19	9	16			L	TGC
tRNA-Asn	5996	6068	73	24	15	13	21			L	GTT
OL	6069	6107	39								
tRNA-Cys	6108	6167	60	13	15	15	17			L	GCA
tRNA-Tyr	6167	6240	74	21	21	17	15			L	GTA
COX1	6241	7852	1611	461	446	263	441	ATA	ATC	H	
tRNA-Ser	7843	7909	67	18	23	10	16			L	TGA
tRNA-Asp	7910	7977	68	16	19	16	17			H	GTC
COX2	7978	8662	685	220	158	105	202	ATG	TAA	H	
tRNA-Lys	8663	8727	64	23	22	9	10			H	TTT
ATP8	8728	8893	165	68	51	8	38	ATG	TAA	H	
ATP6	8883	9563	681	236	186	67	192	ATG	TAA	H	
COX3	9563	10346	784	242	200	120	222	ATG	TCT	H	
tRNA-Gly	10347	10407	61	23	15	9	14			H	TCC
ND3	10408	10750	343	106	93	38	106	ATC	AGT	H	
tRNA-Arg	10751	10815	65	21	18	11	15			H	TCG
ND4L	10816	11106	291	108	81	30	72	ATG	TAA	H	
ND4	11107	12444	1338	458	362	130	388	ATG	TAA	H	
tRNA-His	12445	12508	64	24	16	11	13			H	GTG
tRNA-Ser	12505	12568	64	15	17	12	20			H	GCT
tRNA-Leu	12566	12636	71	23	19	15	14			H	TAG
ND5	12658	14418	1761	638	445	174	504	ATC	TAA	H	
ND6	14414	14932	519	242	90	38	149	ATG	AGA	L	
tRNA-Glu	14933	14994	62	23	14	12	13			L	TTC
Cytb	14995	16111	1117	334	319	125	335	ATG	TAA	H	
tRNA-Thr	16112	16175	64	19	16	12	17			H	TGT
tRNA-Pro	16176	16237	62	23	15	7	17			L	TGG
CRI	16238	16999	762	182	243	111	226				
Total	16999			5675	4386	2201	4736				

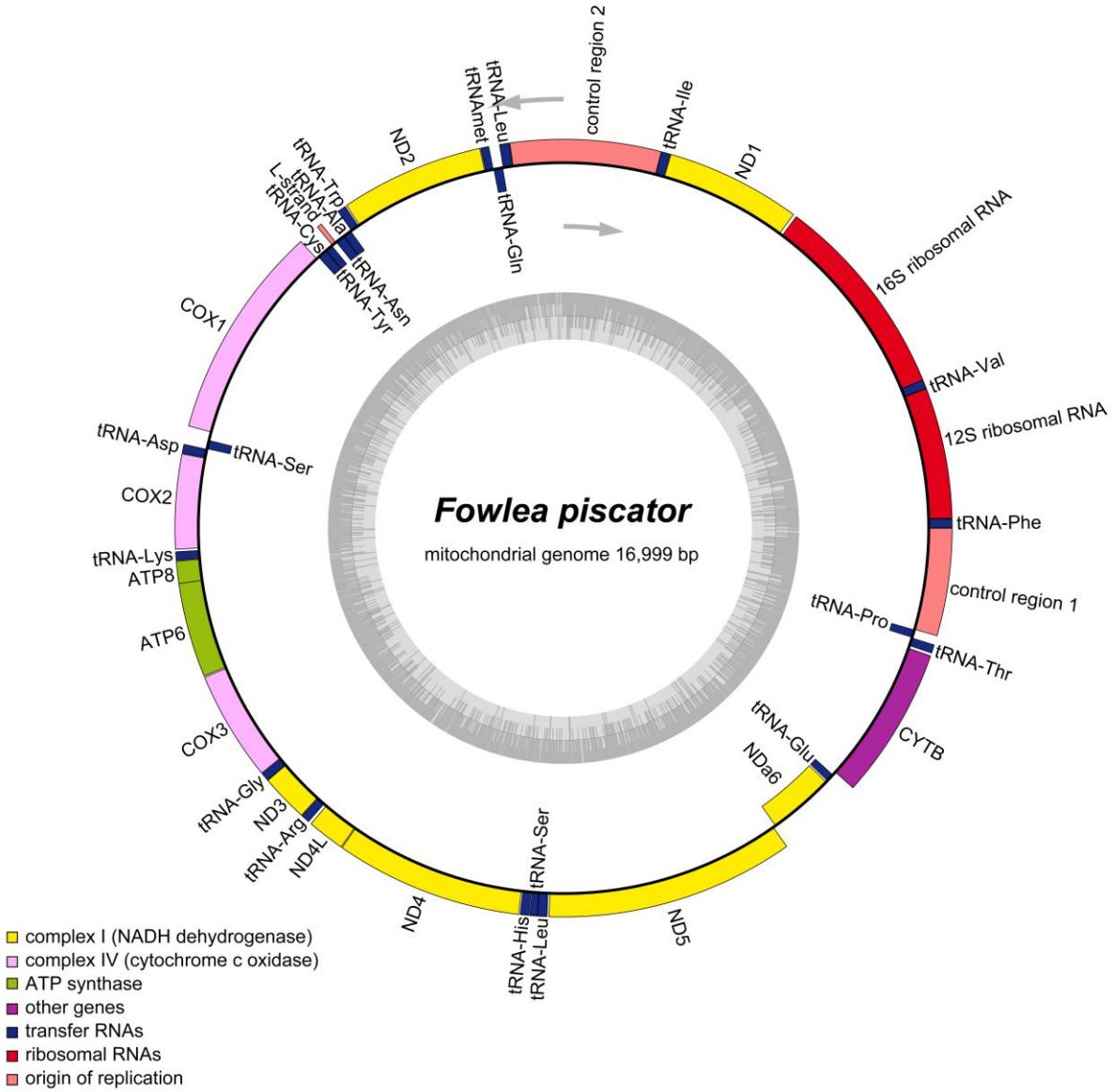
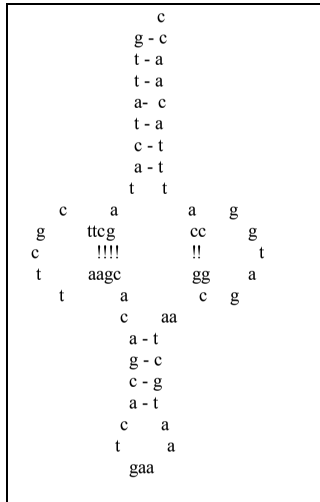
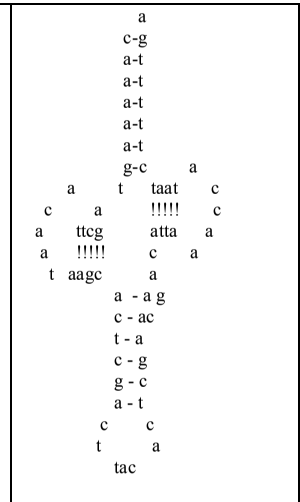


Figure 4.3: Circular maps of newly sequenced mitogenome from *Fowlea piscator* generated through OGDRAW. (Genes are represented by different coloured blocks, as presented in the legend below the maps. Coloured blocks outside of each ring indicate that the genes were on the forward strand, while coloured blocks in the rings indicate that the genes were located on the reverse strand).

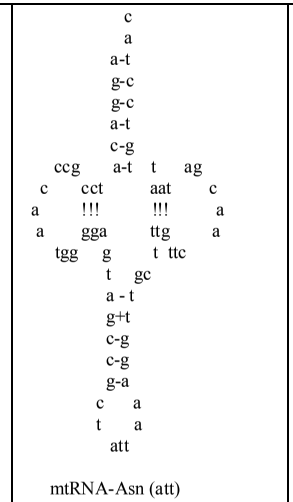
Table 4.5: 22tRNA cloverleaf structure



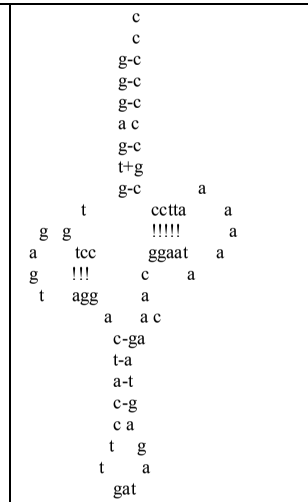
mtRNA-Phe (gaa)



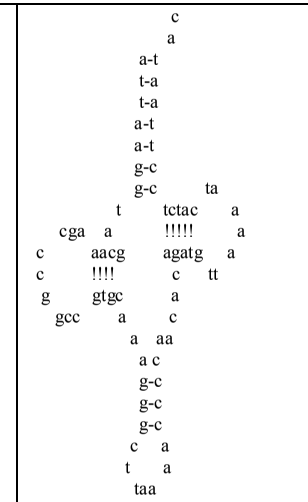
mtRNA-Val (tac)



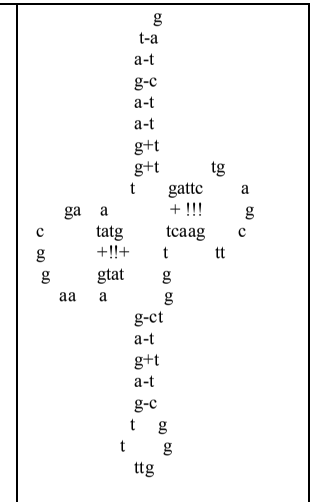
mtRNA-Asn (att)



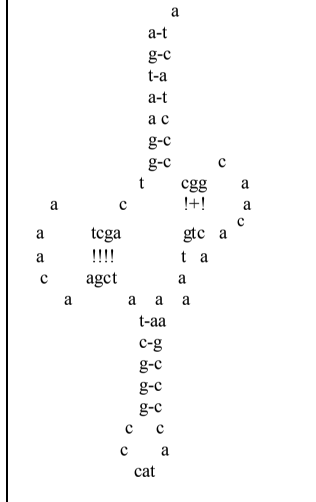
mtRNA-Ile (gat)



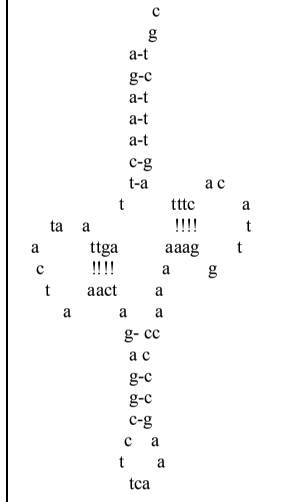
mtRNA-Leu (taa)



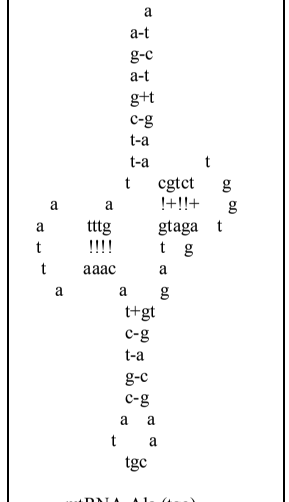
mtRNA-Gln (ttg)



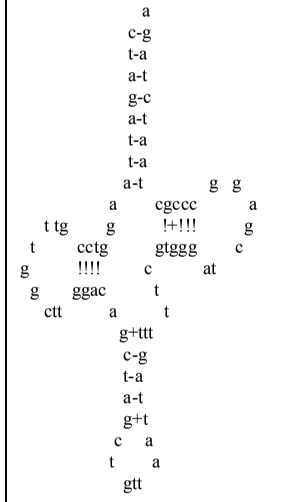
mtRNA-Met (cat)



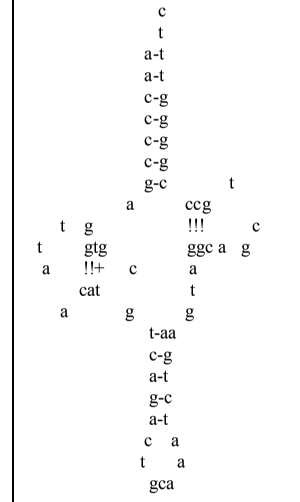
mtRNA-(Stop|Trp) (tca)



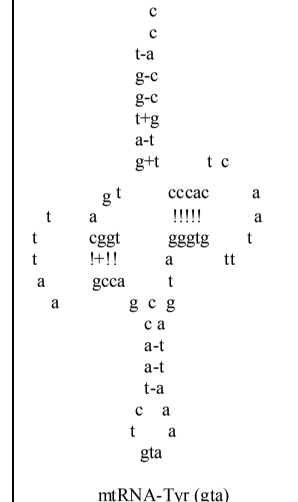
mtRNA-Ala (tgc)



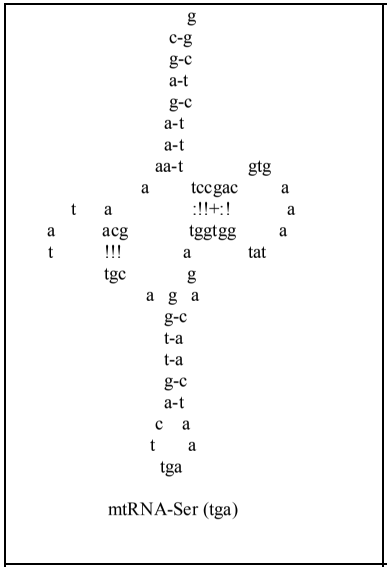
mtRNA-Asn (gtt)



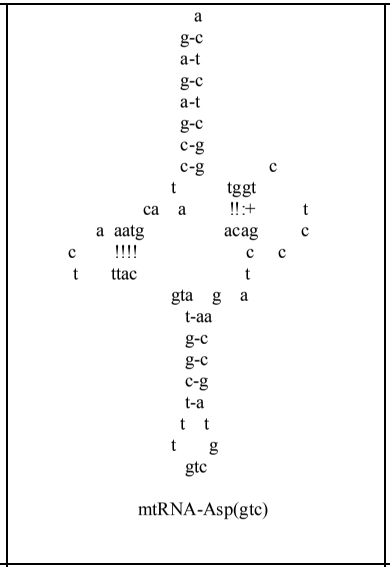
mtRNA-Cys (gca)



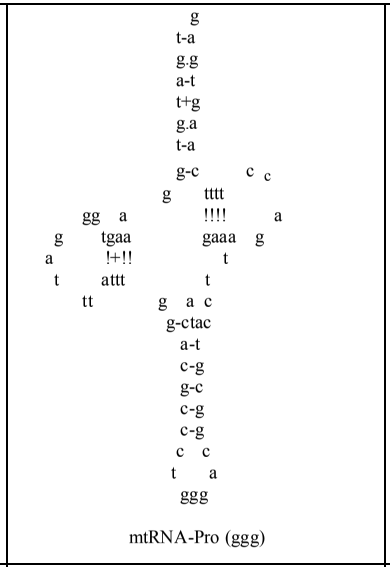
mtRNA-Tyr (gta)



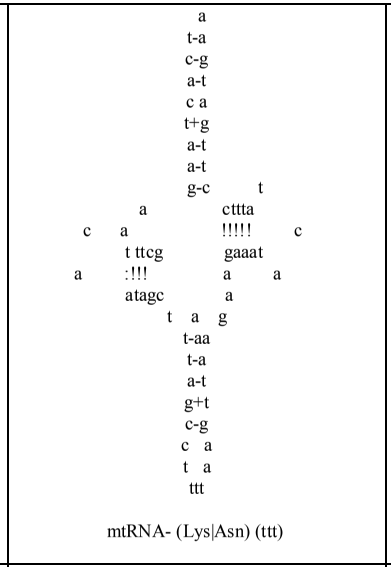
mtRNA-Ser (tga)



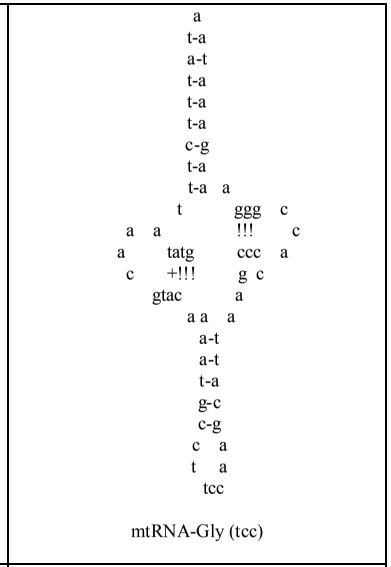
mtRNA-Asp(gtc)



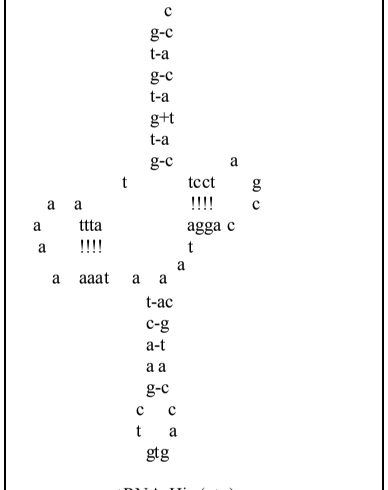
mtRNA-Pro (ggg)



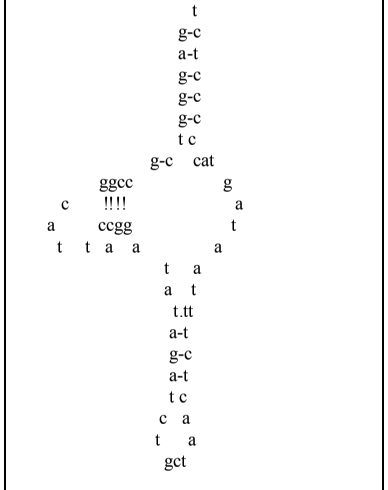
mtRNA- (Lys|Asn) (ttt)



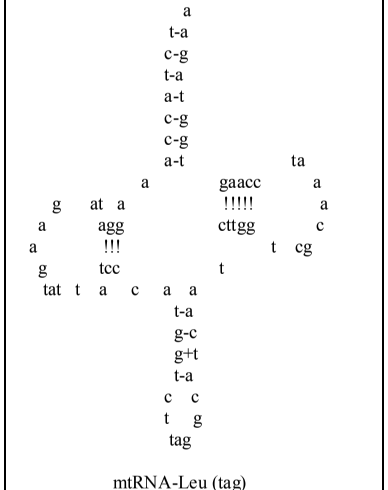
mtRNA-Gly (tcc)



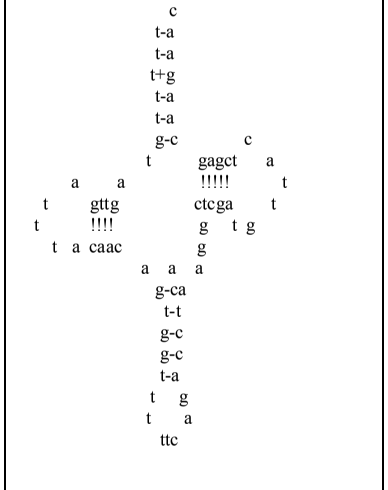
mtRNA-His (gtg)



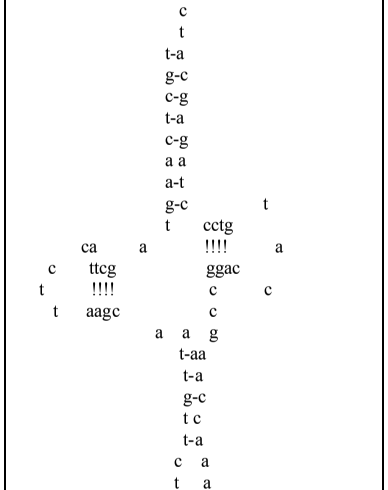
D-loop mtRNA-Ser (gct)



mtRNA-Leu (tag)



mtRNA-Glu (ttc)



mtRNA-Thr (tgt)

Contd.

4.3.2. Protein Coding genes and Nucleotide Compositions

Among all thirteen (13) PCGs, twelve (12) were encoded on '+ve' strand or heavy strand (H-strand) and one NADH 6 gene encoded on the '–ve' strand or light strand (L-strand). Like typical mitochodrial genes eight (8) protein-coding genes were initiated with 'ATG', NAD3 & NAD5 with 'ATC', ND2 with 'ATT', COX1 with 'ATA' and ND1 with 'ACA'. Similarly, eight (8) protein-coding genes were terminated with the typical stop codon 'TAA' i.e. Cytb, COX2, ND1, ND4L, ND4, ND5, ATP8 and ATP6, whereas, COX1 terminated with 'AGG' as a stop codon, and the rest showed an incomplete stop codon 'T' (Zhang *et al.,* 2011; Li *et al.,* 2009) which may turn into a functional codon (TAA) only after polyadenylation during transcription (Ojala *et al.,* 1981). An overlap of gene was found between ATP8/ATP6 and ND5/ND6 which is a common phenomenon in chordate mtDNA. The number of various codons along with their start and stop codons are given in the Table 4.6.

The start and stop codon of *F. piscator* and *R. tigrinus* were almost identical ("ATG/TAA"; "ATG/AGG"; "ATG/AGT"; "ATG/CTT"; "ATA/AAT"; "ATC/TAA") for most of the genes, the only different genes are NADH2, NADH3, NADH4L and NADH6, whereas for the outgroup species only two genes, ATP8 & ATP6 are similar with the Colubridae, Natricinae.

The nucleotide variations of 13 Protein coding gene was estimated by calculating gene-by-gene overall genetic distances in *F. piscator* along with *R. tigrinus*; another species of Colubridae, Natricinae and *Python molurus molurus*; a species from the closely related family, Pythonidae for which complete mitochondrial genome sequence data is available. The number of codons for each genes between *X. piscator* and *R. tigrinus* is almost same apart from NADH6 which is 173 with the former and 170 with the later (Dubey *et al.,* 2012; Velozo *et al*., 2017; Zhao *et al.,* 2021). The calculated overall genetic distance represents that the highest conserved gene among three of the sample is NADH1 (0.141), followed by COXIII (0.145), where as the least is NADH5 (2.058) (Table 4.7).

Table 4.6: Characteristics of protein-coding genes of *X. piscator* and comparison with *R. tigrinus* and *P. molurus* (XP- *Fowlea piscator*; RT-*Rhabdophis tigrinus*; PM- *Python molurus*)

Protein	No. of codons			Predicted start/stop codon			Overall genetic distances
	FP	***RT***	***PM***	***FP***	***RT***	***PM***	
ATP8	55	55	56	ATG/TAA	ATG/TAA	ATG/TAA	0.915
ATP6	227	227	227	ATG/TAA	ATG/TAA	ATG/TAA	0.420
COXI	537	537	534	ATA/AGG	ATA/AGG	GTG/AGA	2.003
COXII	228	228	229	ATG/AGT	ATG/AGT	GTG/TA	0.156
COXIII	261	261	261	ATG/CTT	ATG/CTT	ATG/T	0.145
CYT B	372	372	370	ATG/CCT	ATG/CCT	ATG/T	0.261
NADH1	321	321	321	ATA/AAT	ATA/AAT	ATA/T	0.141
NADH2	342	342	344	ATT/TTT	ATT/TAT	ATT/TAA	0.230
NADH3	114	114	114	ATC/AGT	ATT/AGT	ATA/T	0.398
NADH4	446	446	452	ATG/TAA	ATG/TAA	ATG/A	0.491
NADH4L	97	97	96	ATG/TAA	GTG/TAA	ATG/TA	0.362
NADH5	587	587	598	ATC/TAA	ATC/TAA	ATG/TAA	2.058
NADH6	173	170	171	TCT/CAT	ATA/AGA	ATG/TAG	1.519

4.3.3. Ribosomal RNAs

Two mitochondrial rRNA genes, 12S and 16S rRNA, generated in this study were 927bp and 1471bp long and were separated by the "tRNA-Val" gene. They were aligned with available 12S and 16S rRNA gene sequences of snakes' species in public database NCBI. These 12S rRNA and 16S rRNA gene sequences have been mostly used in the studies of higher level classification such as to explore phylogenetic relationships at familial level, genus level and below respectively (Jogayya *et al.*, 2013).

4.3.4. Non-coding Region

A duplicate control regions (CRI & CRII) or D-Loop, like typical snake mitochondrion pattern was also found in *F. piscator's* mitochondrial genome sequences. The CRI was typically situated between usual "tRNA Pro" and "tRNA Phe" and similarly the CRII was typically situated between usual "tRNA Ile" and "tRNA Leu-Gln-Met" cluster. Both the regions were nearly 90% identical to each other, as they have been proposed to develop in a highly constant manner (Kumazawa *et al.*, 1996). Apart from that, there were 39 nucleotides spread that were unassigned to any mito genes.

4.3.5. Relative Study

F. piscator's complete mitochondrial genome sequencing of 17Kbp shows nearly 91.28% similarities with its closest species *Rhabdophis tigrinus* (Acccession No. NC_030210.1) using BLASTn (NCBI) search engine. The difference in base composition and strand asymmetry was determined by AT and CG-skews. This was measured based on the formulas: "AT skew = [A-T]% / [A+T]%" whereas, "CG skew = [C-G]% / [C+G]%" (Perna *et al*. 1995). The A+T nucleotide content of 13 PCGs was 59.6% and overall A+T content was 61% using MEGA 7. The strand specific nucleotide frequency biasness is indicated by this CG skew and AT skew (Hassanin *et al.*, 2005) (Table 4.7). At all positive strand encoded genes the AT and CG skew were found to be positive, whereas for negative strand encoded genes (NADH6) it was found to be negative. The phenomenon of asymmetry in the nucleotide composition between H & L, i.e. more A and C in positive strand, is supported by the positive AT and CG skew values inferred from this study (Dubey *et al.*, 2012).

Table 4.7: Nucleotide composition and skews of *F. piscator* mitochondrial protein-coding genes

Gene	A	T	G	C	AT SKEW	CG SKEW
ATP8+	0.412	0.309	0.048	0.230	0.1428	0.6546
ATP6+	0.347	0.273	0.098	0.282	0.1193	0.4842
COXI+	0.286	0.277	0.163	0.274	0.0159	0.2540
COXII+	0.321	0.231	0.153	0.295	0.1630	0.3169
COXIII+	0.309	0.255	0.153	0.283	0.1010	0.2981
CYT B+	0.299	0.286	0.112	0.303	0.0222	0.4602
NADH1+	0.310	0.267	0.120	0.303	0.0745	0.4326
NADH2+	0.386	0.227	0.080	0.307	0.2593	0.5865
NADH3+	0.309	0.271	0.111	0.309	0.6551	0.4714
NADH4L+	0.371	0.278	0.103	0.247	0.1432	0.4114
NADH4+	0.342	0.271	0.097	0.290	0.1158	0.4987
NADH5+	0.362	0.253	0.099	0.286	0.1772	0.4857
NADH6-	0.173	0.467	0.288	0.073	-0.4593	-0.5955
Average	0.323	0.273	0.126	0.278	NA	NA

The maximum proportions of the *F. piscator* mitogenomes are protein-coding regions, taking up 66.5% of the whole mitogenomes. The rRNA segments accounted for 14.10%, the intergenic tRNA for 8.5% and two intronic regions, accounted for 10.80% (Figure 4.4) respectively. The total number of nucleotide sequences of all the 13 PCG here follow the pattern A>C>T>G (Graph 4.1).

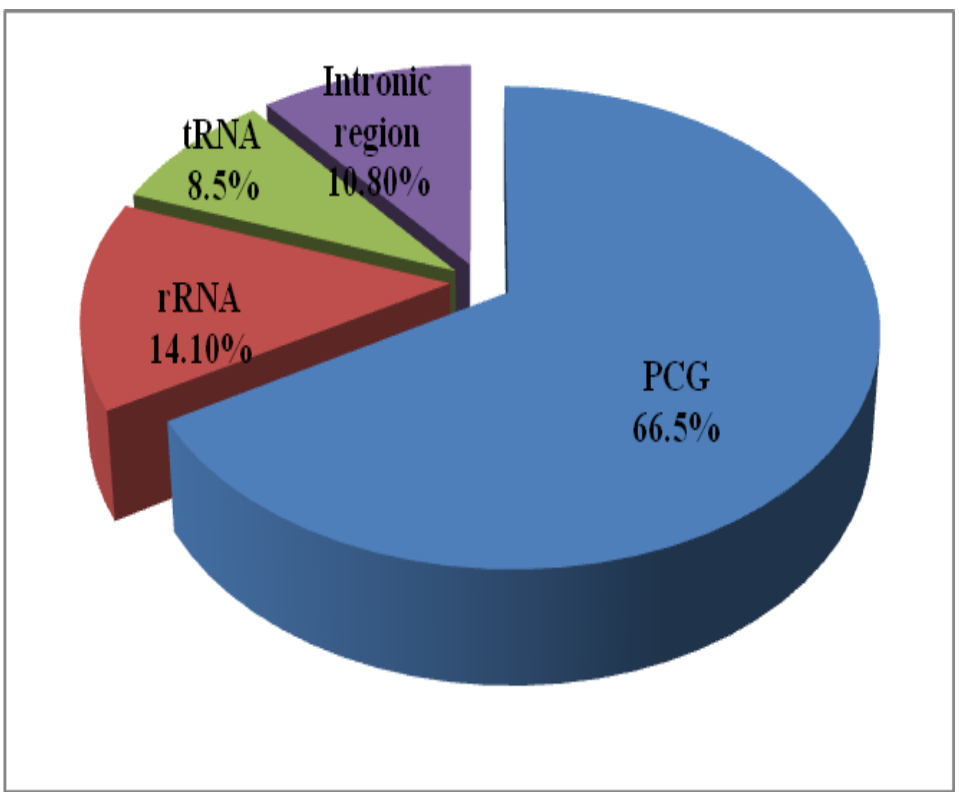
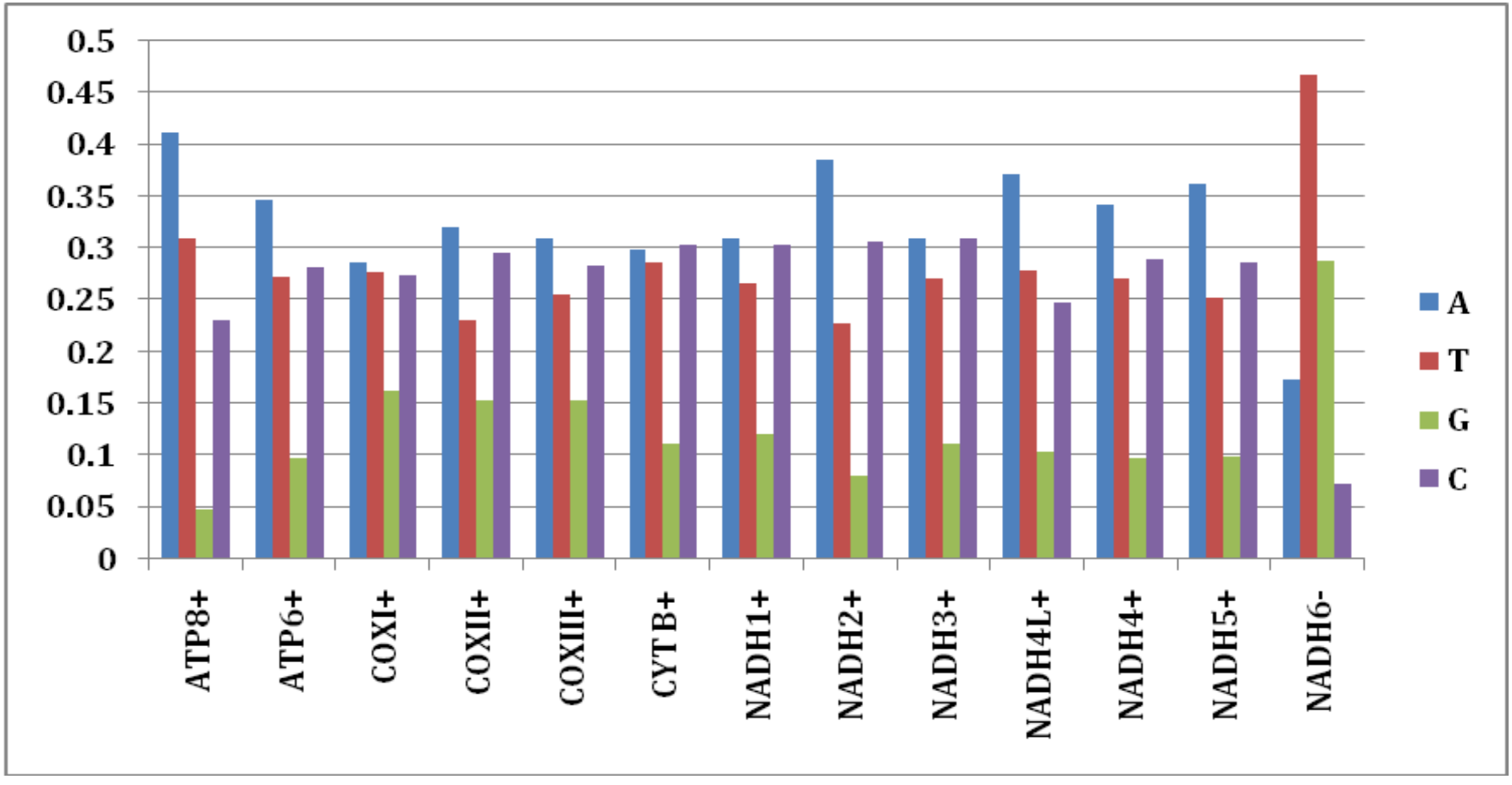


Figure 4.4: The proportion of the mitogenomes comprised by protein-coding, tRNA, rRNA and intronic regions for *X. piscator* gene.



Graph 4.1: Graphical representation of nucleotide composition and skews of *F. piscator* mitochondrial protein-coding genes

4.3.6. Phylogenetic Analysis

By using this newly generated complete mitochondrial genome sequences of *F. piscator*, the phylogenetic relationship within the family Colubridae and more precisely within the sub family Natricinae was validated. It was achieved by constructing; the Maximum Likelihood tree in MEGA 7.0 (Kumar *et al.,* 2016) which retrieved complete mitochondrial genome sequences of 24 species of which 18 species from the family Colubridae, and 6 from distant or outgroup family Pythonidae and Crocodilidae from NCBI genbank (Figure 4.5).

The Maximum Likelihood tree showed that *F. piscator* congregated with *Rhabdophis tigrinus* and *Pseudagkistrodon rudis*, highly supported by a bootstrap value of 100%, and then congregated with *Hebius optatum, Opisthotrophis latouchii, Opisthotrophis kuatunensis* which was in accordance with recent molecular works (Wang *et al.*, 2017; Zong *et al.*, 2020 and Wu *et al.*, 2021).

Apart from using complete mitogenome sequences another phylogenetic tree constructed using the Maximum Likelihood (ML) program based on the Tamura-Nei model analysis involving Cytochrome *b* gene sequences from 18 Colubridae snake species after eliminating all gaps and missing data in MEGA7 (Figure 4.6). It consist the accession numbers of retrieved sequences. The bootstrap values are shown for each nodes based on 1000 replicates. The tree with the highest log likelihood (-4310.54) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. This approach of constructing phylogenetic tree using Cytochrome b gene shows *Fowlea piscator* to be clustered with *Rhabdophis tigrinus* and *Hebius optatum*, and then congregated with *Pseudagkistrodon rudis, Opisthotrophis latouchii* and *Opisthotrophis kuatunensis* which was in close accordance with previous phylogenetic analysis using complete mitogenome.

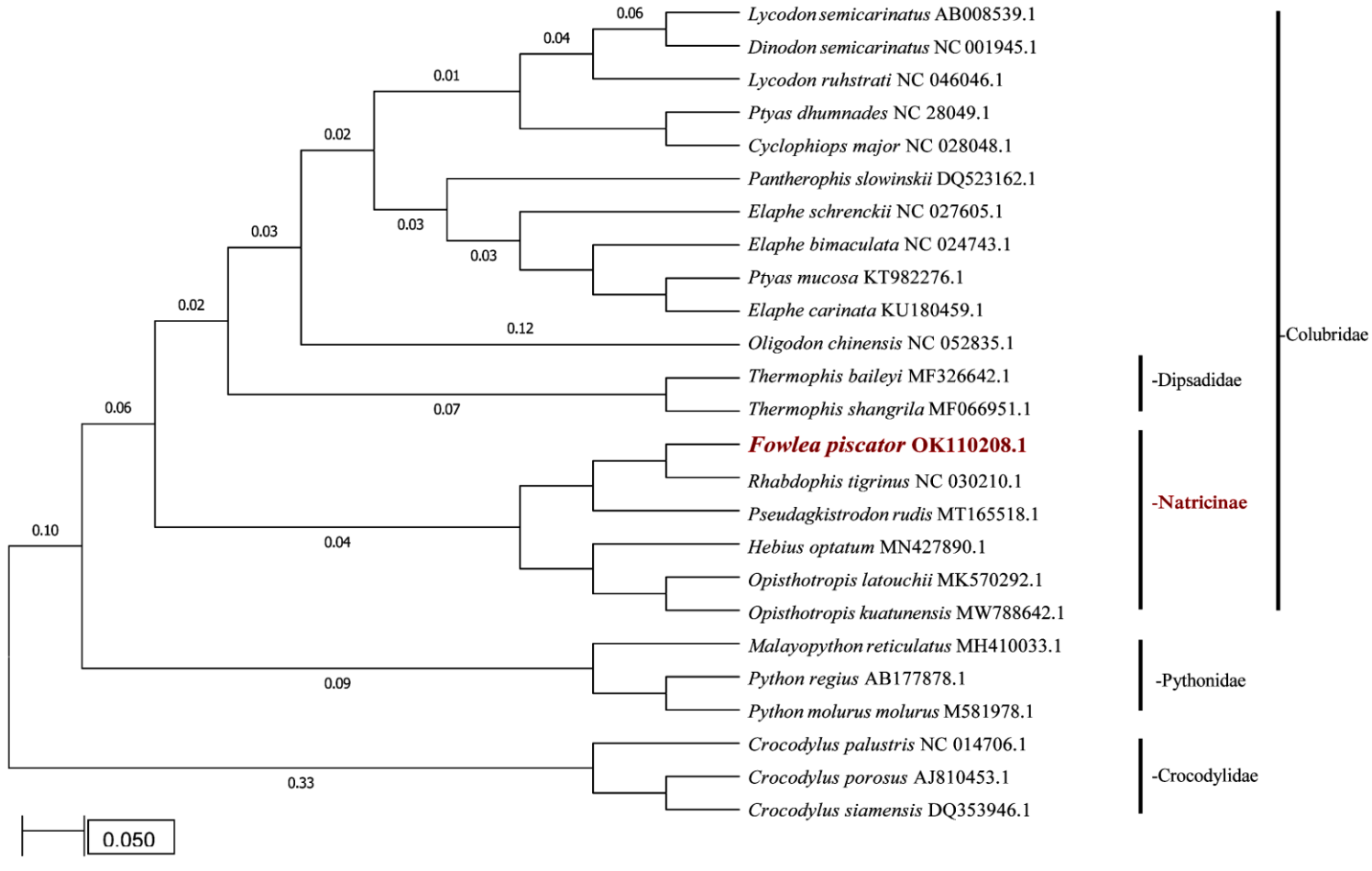


Figure 4.5: The Phylogenetic tree constructed using the Maximum Likelihood (ML) program based on the Tamura-Nei model analysis involving 25 complete mitochondrial genome nucleotide sequences after eliminating all gaps and missing data. It consist the accession numbers of retrieved sequences. The bootstrap values are shown for each nodes based on 1000 replicates.

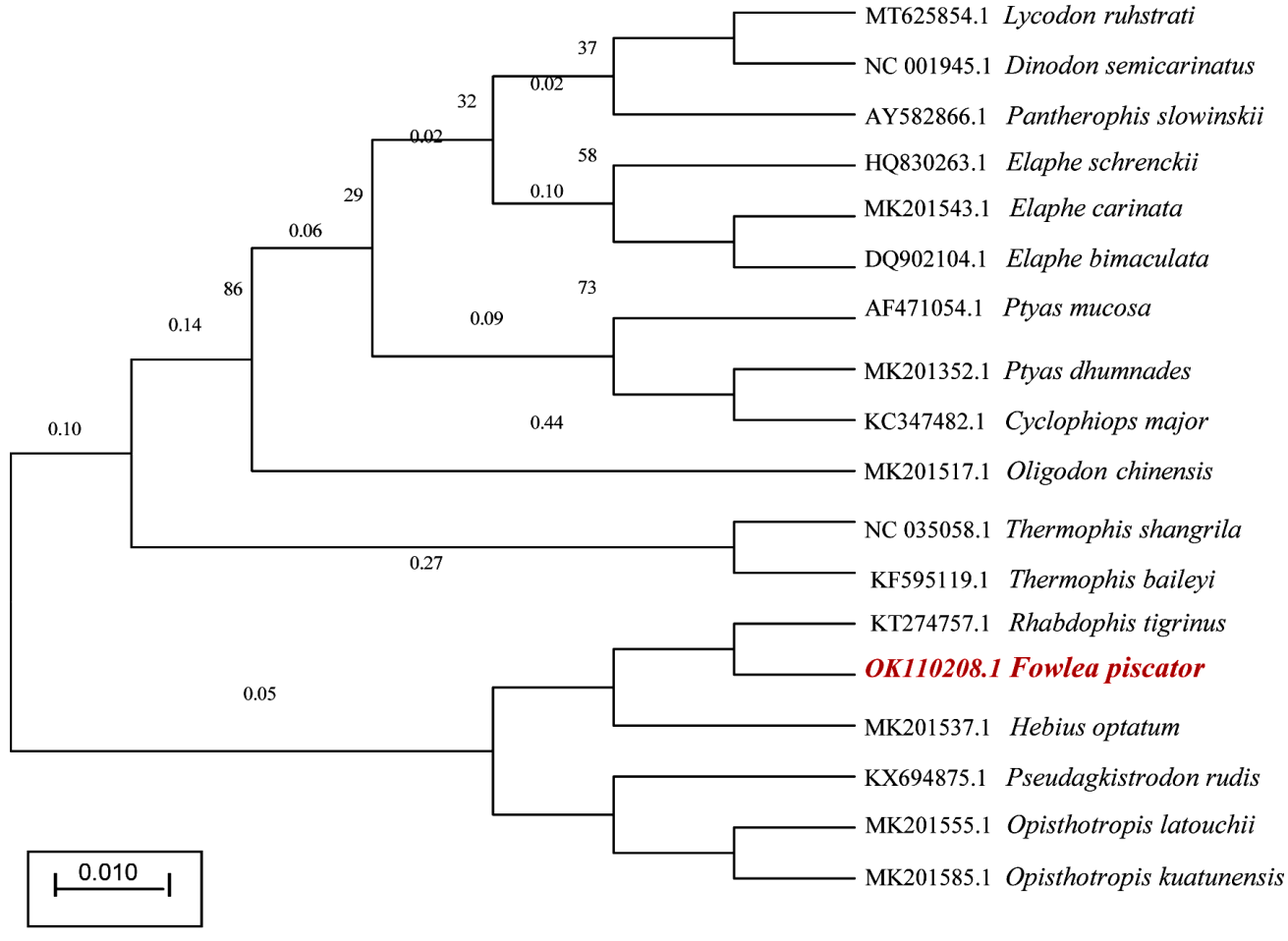


Figure 4.6: The Phylogenetic tree constructed using the Maximum Likelihood (ML) program based on the Tamura-Nei model analysis involving Cytochrome *b* gene sequences from 18 Colubridae snake species after eliminating all gaps and missing data in MEGA7. It consist the accession numbers of retrieved sequences. The bootstrap values are shown for each nodes based on 1000 replicates.

4.4 Conclusion

The application of mtDNA in Forensic wildlife cases, evolutionary genomics and hybridization of closely related species are well established. The complete mitochondrial genome sequences of a least concerned Asiatic Water Snakes, *Fowlea piscator*, whose previous phylogenetic position was doubtful has been represented in this study. The main quality of a mitochondrial DNA is that they can resist DNA degradation for a long period of time and from that minimum quantity of degraded samples the source species can be identified if the complete mitochondrial sequencing is available in GenBank data base. The sequences of Asiatic Water snake thus generated in this study can be BLAST searched for identification with unknown, extremely degraded samples where long and complete sequencing products can not be achieved and the other molecular markers are not effective. The mitogenome obtained here enriches the genomic resources available for further evolutionary studies, and can provide genomic resources for sub family Natricinae and family Cloubridae studies. The results were in complete agreement with the previously established snake phylogeny. It is expected that the data thus generated on protein coding genes, start/end codons and increased amount of mitochondrial sequence data generated in this study, is likely to be useful in various conservation genetics, population related and forensic identification studies of this species in future as most of the time samples are degraded or altered.

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CHAPTER V

DEVELOPMENT OF MICROSATELLITE MARKERS

"You can't cross the sea merely by standing and staring at the water."

~Rabindranath Tagore

Microsatellites are Short Tandem Repeat (STR) or Simple Sequence Tandem Repeats (SSTRs) of di-, tri- tetra- or pentanucleotides. Microsatellites are the most popular and versatile genetic marker with myriads of applications in population genetics, conservation biology, and evolutionary biology. They are useful markers at a wide range of scales of analysis. The major advantages of microsatellite markers are, they are locus-specific (in contrast to multi-locus markers such as minisatellites or RAPDs), Co-dominant (heterozygotes can be distinguished from homozygotes, in contrast to RAPDs and AFLPs which are "binary, 0/1"), PCR-based (means we need only tiny amounts of tissue; works on highly degraded or "ancient" DNA), highly polymorphic ("hypervariable") provides considerable pattern and useful at a range of scales from individual identification to fine-scale phylogenies. Until recently, they were the most important tool in mapping genomes, such as the widely utilized human genome mapping (Kocher *et al.,* 1998). They serve a role in biomedical diagnosis as markers for certain disease conditions. That is, certain microsatellite alleles are associated (through genetic linkage) with certain mutations in coding regions of the DNA that can cause a variety of medical disorders. They have also become the primary marker for DNA testing in forensics (court) contexts both for human and wildlife cases (Evett and Weir, 1998). The reason for this prevalence as a forensic marker is their high specificity. Match identities for microsatellite profiles can be very high. In a biological/evolutionary context they are useful as markers for parentage analysis (Chapman *et al.,* 1999). They can also be used to address questions concerning degree of relatedness of individuals or groups. For captive or endangered species, microsatellites can serve as tools to evaluate inbreeding levels (F_{IS}). From there we can move up to the genetic structure of subpopulations and populations using tools such as *F*-statistics and genetic distances (Knight *et al*., 1998). They can be used to assess demographic history (e.g., to look for evidence of population bottlenecks), to assess effective population size (N_e) and to assess the magnitude and directionality of gene flow between populations. Microsatellites provide data suitable for phylogeographic studies that seek to explain the concordant biogeographic and genetic histories of the floras and faunas of large-scale regions. They are also useful for fine-scale phylogenies up to the level of closely related species. An overview by Selkoe and Toonen (2006) provides a useful practical guide to the use of microsatellites as genetic markers. They are able to characterize allele through differences in the size of PCR products (Wright and Bentzen, 1995).

5.1 The Different Application of Microsatellite Markers

The different application of such microsatellite markers are described below;

5.1.1 Forensic Science and DNA Fingerprinting

Microsatellites are widely used for forensic identification, as Short Tandem Repeat (STR) loci are widely used for forensic identification and relatedness testing, and are predominant genetic markers in this area of application. In forensic identification cases, the goal is to typically link a suspect with biological samples found at the crime scene. PCR amplified microsatellites are very powerful markers because they are locus specific, co-dominant, occur in large number, hyper variable, allow the unambiguous identification of the alleles and data thus generated can be easily distributed and reproduce among different laboratories (Carlsson *et al.*, 1999). The co-dominant nature of the marker helps in the heterozygous allele identification and tagging (Norris *et al.*, 1999). Each microsatellite is usually flanked by unique sequences that can be used as primer binding sites for amplification using PCR technique (Smith *et al.*, 1998). This features made the use of microsatellites attractive for forensic applications.

5.1.2 Conservation Genetics

It can be used to detect sudden changes in population, effects of population fragmentation and interaction of different populations. Microsatellites are useful in the identification of new and incipient populations (Li, 2002, 2003). Low level of genetic variation mostly hampers the studies of endangered species. To assess the severity of restrictions in endangered populations, a highly polymorphic class of markers is needed. Microsatellites were found to be informative in several species which showed null variation at other genetic markers (Taylor *et al.*, 1994).

5.1.3 Population Genetic Studies

By looking at the variation of microsatellites in the population, inferences can be made about population structures and differences, genetic drift, genetic bottlenecks, and even the date of a common ancestor (Jarne and Lagoda, 1996; Fandon, 2004). Highly polymorphic microsatellite markers have great potential utility as genetic tags

for use in wildlife conservation. They are powerful DNA markers for quantifying genetic variations within and between populations of species (P M *et al.*, 2009).

5.1.4 Cross Species Amplification of Microsatellites

A major obstacle for microsatellite analysis was the need to develop PCR primers for each species. However, the fact that flanking sequences of some microsatellite loci are conserved among related taxa so that primers developed for one species can be used to amplify homologous loci in related species, it can significantly promote the widespread use of microsatellites (Schloterer *et al.*, 1991). Such an approach can circumvent extensive preliminary work necessary to develop PCR primers for individual loci that continues to stand in the way of quick and widespread application of single locus microsatellite markers, reducing the cost of developing similar markers in related species (P M, 2014).

5.1.5 Genetic Map Construction

The development of molecular marker and subsequent identification of many marker loci has caused new and improved interest in genetic mapping. Genetic map construction requires selection of the most appropriate mapping population(s), then calculating pair wise recombination frequencies using these population(s) which leads to the establishment of linkage groups, estimating map distances and determining map order. Since large mapping populations are often characterized by different marker systems, map construction has become computerized. Computer packages such as 'Linkage 1' (Suiter *et al.*, 1983), 'GMendel' (Echt *et al.*, 1992), 'Mapmaker' (Lander and Botstein, 1986; Lander *et al.*, 1987), 'MapManager' (Manly and Elliot, 1991), and 'JoinMap' (Stam, 1993) have been developed to aid in the analysis of genetic data for map construction. These programs use data obtained from segregating populations to estimate recombination frequencies that are then used to determine the linear arrangement of genetic markers by minimizing recombination events (Jack *et al.*, 1996).

Thus this chapter is based on isolation and characterisation of microsatellite markers from snake species to study their population structure for their conservation purposes as well as for forensic perspective.

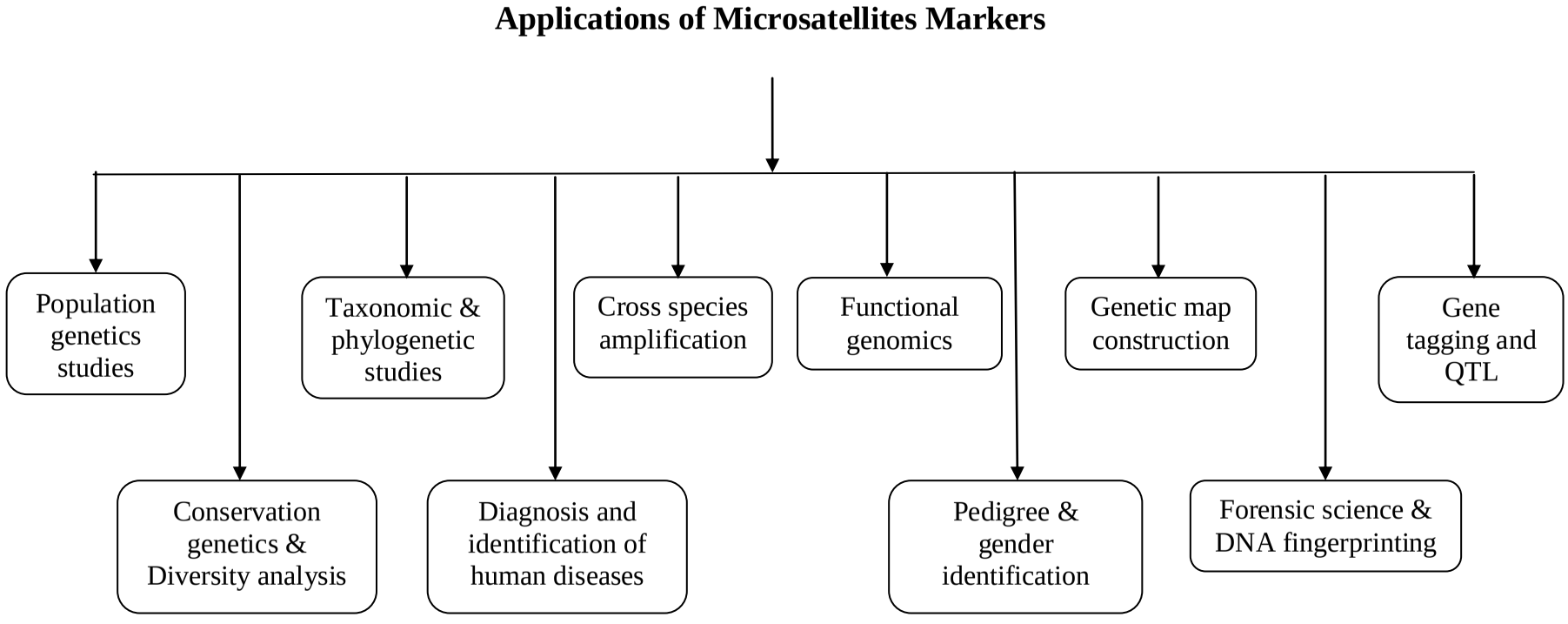

Figure 5.1: A schematic diagram showing different applications of microsatellites markers in different fields.

Isolation and Characterization of Novel Polymorphic Microsatellite DNA Markers for Indian Rat Snake (*Ptyas mucosa*)

5.2.1 Introduction

Ptyas mucosa, commonly known as the Indian Rat Snake, Oriental Rat Snake, or Dhaman, is a common species of Colubridae snake family and Colubrinae subfamily of snakes, found in parts of South and Southeast Asia. These are non-venomous and fast-moving large snakes, growing to 2 mt (6.6 ft) and occasionally even to 3 mt (9.8 ft). Their colour varies from pale browns to nearly black. Indian rat snakes are diurnal. They survive on variety of prey and are frequently found in urban areas where rodents thrive. They occupy farmland, forest floors, rice paddies and wetlands, where they prey upon small insects, reptiles, and mammals (Whitekar *et al.*, 2004). Adult members of this species emit a growling sound and inflate their necks when threatened. This adaptation may represent mimicry of the King Cobra or Indian Cobra and often mistaken for a venomous snake and killed (Das, 2000).

Figure 5.2: A Rat Snake (*Ptyas mucosa*) in the wild

DNA based genetic markers demonstrate high levels of intraspecific differences and therefore, are important tools for investigating a variety of questions at the population level. Microsatellite DNA markers seem to be ideal for these types of population studies because they represent loci that have co-dominant alleles, and the genotypic information is logistically easy to obtain compare to certain other DNA-based

markers (Li, 2002, 2003; Remya *et al.*, 2010). The Indian Rat snake (*Ptyas mucosa*) has been one of the important species in the class of Reptiles, listed in the Appendix II of CITES 2016 list, and according to 2016 IUCN Red list these species are at lower risk. The information gathered from the field of genetics/molecular biology could be of immense value for conservation efforts of this species. In this regard, there is always a need to build genomic data base for better understanding of evolutionary relationships (Boore JL, 1999), populations management and conservation status of a species.

In view of the above, it is considered that the conservation and rebuilding goals are urgently required. Here, we described isolation and characterization of 12 new microsatellite loci for Rat snake, which will enable investigation of the population genetic characteristics of this species throughout its distribution.

5.2.2 Materials and Methods

Biological samples like tissue, bone, blood and Saliva samples of *Ptyas mucosa* (N=20) were collected from Wildlife Rescue Centre and Transit Facility, Salt Lake, Kolkata, India; from Zoological Garden, Alipore, Kolkata, India and from the repository of Central Forensic Science Laboratory, Kolkata which were collected from Snake Park, Kolkata, India and Chennai Snake Park, Chennai, India. Whole Genomic DNA extraction from the samples were carried out by Qia tissue DNA extraction kit (Qiagen, Valencia, A) as per the manufacturer's procedure.

5.2.2.1 Microsatellites Isolation using Glen & Schable 2005 Protocol, Described Below

5.2.2.1.1 STEP-I Restriction Enzyme Digestion

Digest 1µg genomic DNA from two good quality isolated DNA with suites of restriction enzymes to get appropriate sized fragments (i.e., between ~400- ~1200bp).

Recipe

Rsa I or BstU I*

- 2.50µl NEB 10xLigase Buffer (note: heat briefly to 50°c to get all components in solution)
- 0.25µl 100x BSA
- 0.25 µl5M NaCl (50 mM final)
- 1.00µl Rsa I or BstU I
- 20.0 µl genomic DNA (100 ng/ µL).

❖ To make master mixes for Rsa I and BstU I, multiply the volume of each of the components in the preceding list by the number of DNA samples to be digested, plus half a sample to account for pipetting error and add to a 1.5-ml tube.

❖ Prepare the restriction enzyme digest for Rsa I and BstU I by adding 5 µl of master mix into a new tube (0.2 ml or 0.5 ml depending on thermal cycler available) and use a thermal cycler for all incubations. Add 20 µl of DNA to each tube. Pipette up and down to mix the solution.

❖ Incubate all samples (Rsa I and Bst UI) at 37°c for 30–60 min.

❖ While the restriction digest is incubating, pour a 1% agarose gel, including ethidium bromide (Sambrook and Russell 2001).

❖ Set aside a small aliquot (4 µl) of the digested DNA.

❖ Run on 1% agarose gel to check completeness of digestion; run undigested DNA as control.

❖ Pick enzyme combination that gives ~500bp fragments and digest ~10µg at 37c.

❖ Immediately proceed for ligation step.

5.2.2.1.2 STEP- II Ligating Linkers to DNA Fragments

Preparation of double-stranded (ds) SuperSNX linkers

Mix equal volumes of equal molar amounts of SuperSNX24 and SuperSNX24+4p primers (e.g., 100 µl of 10µM each). Add salt to a final concentration of 100 mM (i.e., 4 µl of 5M NaCl for 200 µl of primers). Heat this mixture to 95°c, and let it cool slowly to room temperature to form the ds SuperSNX linkers.

SuperSNX24 Forward: 5’GTTTAAGGCCTAGCTAGCAGAATC

SuperSNX24+4P Reverse: 5’pGATTCTGCTAGCTAGGCCTTAAACAAAA

Recipe for Linker-Ligation

- 7.0µl ds SuperSNX linkers (5µM each; see (Hamilton et al. 1999)
- 10.0µl digested and dephosphorylated DNA (from Step I above)
- 1.0µl 10x Ligase buffer
- 1.0µl T4 DNA ligase (400U/µl)
- 1.0µl Xmn I (20U)
- Incubate at room temperature for 2 hrs or ideally at 16°c overnight.

While the ligation is proceeding, run the small aliquots of restriction enzyme digested DNA (from Step I) on the 1% gel to ensure the DNA samples were successfully digested. A successful reaction should yield a smear of fragments at or below 1000bp. To ensure ligation was successful, perform a PCR on the linker ligation using the following recipe for a 25µl reaction.

Recipe for PCR

- 2.0µl ligation template.
- 2.5µl 10x PCR buffer (optimal buffer for Taq used below)
- 2.5µl BSA (250µg/ml)
- 2.0µl MgCl2 (25 mM final concentration)
- 1.5µl dNTPs (2.5mM each)
- 0.2µl Taq (5 units/µl)
- 1.3µl SuperSNX-24 (10µM final concentration)
- 13.0µl H2O

A. Control reaction contains linker and digested/dephosphorylated DNA.

B. PCR Cycling condition: 95°C for 2min; 20 cycles of 95°C for 20 sec, 60°C for 20 sec, 72° for 1.5 min. Hold at 15°C.

(Note: the same program as the enrichment recovery (step IV below) may be used, but it takes longer).

C. Run 4µl of PCR product on a 1.0% agarose gel to see if the linker-ligation was successful using a 100bp ladder as a size standard. A successful reaction should yield a smear of fragments centered at approximately 500bp. This PCR product can be used for enrichment if sufficient amounts of original linker-ligated DNA are available.

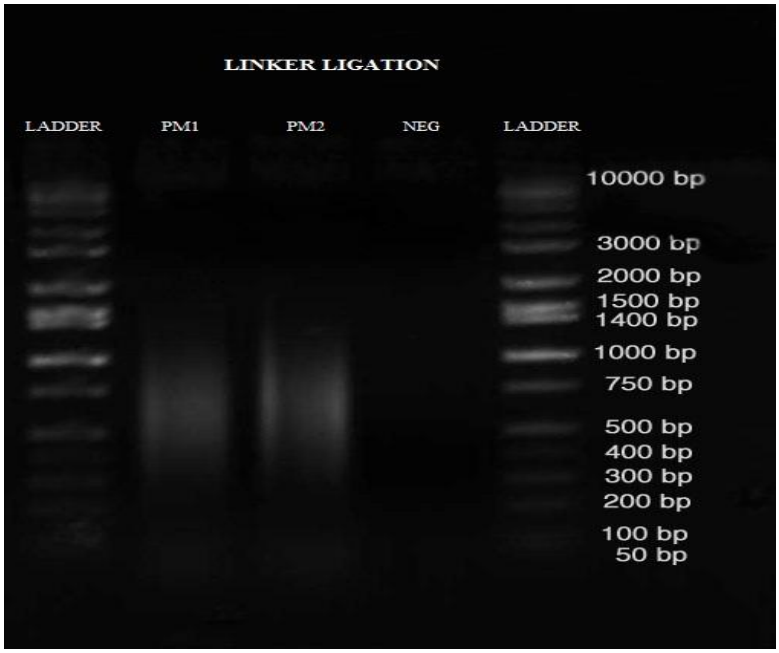

Figure 5.3: 1% Agarose gel electrophoresis run after successful Linker Ligation

5.2.2.1.3 STEP- III Dynabeads Enrichment for Microsatellite - Containing DNA Fragments

Materials and Solutions

I. **Washed Dynabeads:** Wash twice in TE [10 mM Tris pH 8.0, 2 mM EDTA] and twice in 1x Hyb solution. Note: each 50µl of Streptavidin M-280 Dynabeads (Invitrogen [formerly Dynal]; catalogue No. 112-05D) can capture 100 pmol of biotinylated oligo. It is critical to have an excess of bead capacity relative to the amount of biotin/oligo added. If beads from other manufacturers are used, the amount of beads should be adjusted to account for variation in biotin-binding capacity.

II. **2x Hyb Solution:** 12x SSC, 0.2% SDS (warmed; stock solution 20x SSC, 3.0 M NaCl, 0.3 M Sodium citrate, pH 7.0).

III. **1x Hyb Solution:** 6x SSC, 0.1% SDS (warmed to get everything into solution).

IV. **Washing Solutions:** I) 2x SSC, 0.1% SDS (warmed to get everything into solution). II) 1x SSC, 0.1% SDS (warmed to get everything into solution).

V. **Biotinylated oligos:** Mixtures of 3’ biotinylated oligos are used with this protocol. 3’ labeling is used because it has the highest efficiency of labeling (each oligo synthesis starts with a biotin). A large number of oligos may be used in a mix together when their lengths are varied to achieve similar melting

temperatures (Tms). We used oligos purified by standard desalting methods (i.e., no additional purification by high-performance liquid chromatography [HPLC], gels, etc.), because we ordered large numbers of oligos and the additional purification would be quite expensive. The critical factor to keep in mind when using biotinylated oligos purified by standard desalting methods is that the solution will contain many ‘‘free’’ biotins, so it is critical to ensure the amount of biotin (estimated from the oligo concentration) added is far less than the bead-binding capacity. Below are 3 generally used biotinylated oligo for enrichment.

a. $(AG)_{12,}$ $(TG)_{12,}$ $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12,}$ $(ATC)_8$

b. $(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACAG)_6$, $(ACCT)_6$, $(ACTC)_6$, $(ACTG)_6$

c. $(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(ACAT)_8$, $(AGAT)_8$

VI. **NaOAc EDTA Solution:** To a 50-ml conical flask, make 20 ml of 3 M NaOAc from the dry chemical stock. Do not adjust the pH. Add 20 ml of 500 mM EDTA, pH 8.0. This makes a solution that is 1.5 M NaOAc and 250 mM EDTA. Aliquot into 1.5-ml microcentrifuge tubes and/or 0.2-ml strip tubes and freeze.

Detailed Steps

5.2.2.1.3.1 Hybridizations (In a 0.2 ml PCR tube)

- 25µl 2x Hyb solution (warmed to get everything into solution)
- 10.0µl biotinylated microsatellite probe (mix of oligos at 1µM each)
- 10.0µl linker-ligated DNA (or PCR product if <2 g DNA initially used)
- 5.0µl dH2O

Use thermal cycler program **OLIGOHYB**. This program denatures the DNA+probe mixture at 95°C for 5 min. It then quickly ramps to 70°C and steps down 0.2°C every 5sec for 99 cycles (i.e., 70°C for 5sec, 69.8°C for 5sec, 69.6°C for 5sec, ...down to 50.2°C), and stays at 50°C for 10 min. It then ramps down 0.5°C every a5sec for 20 cycles (i.e., 50°C for 5sec, 49.5for 5sec, 49for 5sec, ... down to 40°C), and finally quickly ramps down to 15° C. The idea is to denature everything, quickly go to a temperature slightly above the annealing temperatures of the oligos in the mixes used, and then slowly decrease, allowing the oligos the opportunity to hybridize with DNA

fragments that they most closely match (i.e., hopefully, long perfect repeats) when the solution is at or near the oligo's Tm.

5.2.2.1.3.2 Preparation of M-280 Streptavidin-coated Dynabeads (Invitrogen, formerly Dynal. Inc, Oslo, Norway).

- ❖ While the DNA–probe mixture is in the thermal cycler, wash 50µl of Dynabeads (Invitrogen, formerly Dynal. Inc, Oslo, Norway). Resuspend the beads in their original tube, and transfer to a 1.5-ml tube. Add 250µl of TE and Shake.
- ❖ Place tubes in the Magnetic Particle Concentrator (MPC) (Dynal. Inc, Oslo, Norway). Repeat with TE, and twice with 1x Hyb solution. Resuspend the final beads in 150µ l of 1x Hyb solution.
- ❖ Add 300µl Binding &Wash (B &W) buffer [10mM Tris-Cl, 1mM EDTA, 1M NaCl]
- ❖ Pulse-spin your DNA+probe mix and add all of it to the 150µl of washed, resuspended Dynabeads (i.e., to the 1.5ml tube).
- ❖ Incubate on rotator or sideways in orbital shaker on slow speed at room temperature for 30 or more min.
- ❖ Capture beads using the MPC. Remove the supernatant by pipetting with a P200 pipetter (Optional: Save supernatant for troubleshooting purposes).

5.2.2.1.3.3 Washing

- ❖ Wash the Dynabeads two times with 400µl with washing solution I each time using the MPC to collect the beads and removing the supernatant by pipetting with a P200 pipetter (which can be saved for troubleshooting purposes). Resuspend beads well (i.e., flick or gently vortex) in next wash each time.
- ❖ Wash two additional times using 400µl washing solution II.
- ❖ Wash two final times using washing solution II and heating the solution to within 5–10°C of the Tm for the oligo mix used (usually 45°C or 50°C)
- ❖ Add 200µl TLE, vortex, and incubate at 95°C for 5 min. Label a new tube while incubating. Capture beads using the MPC. Quickly remove the supernatant by pipetting to the new tube. This supernatant contains the enriched fragments (i.e., ''the gold'').
- ❖ Add 22µl of NaOAc/EDTA solution (see ''Recipe'' above). Mix by pipetting up and down.

5.2.2.1.3.4 Ethanol Precipitation of Supernatants

- ❖ Add 444µl of 95% EtOH. Mix by inverting the tube and place on ice for 15 min or more (or store in the -20°C freezer for as long as desired).
- ❖ Centrifuge at full speed for 10 min, discard supernatant and add approximately 0.5 ml of 70% EtOH. Centrifuge for 1 min.
- ❖ Carefully pipette off all the supernatant and air-dry the sample. If there is any visible trace of EtOH, pulse-spin the tube and use a pipette to remove any residual EtOH. Dry until there is no trace (smell) of EtOH left.
- ❖ Resuspend the pellet in 25µl of TLE. This is the ‘‘pure gold.’’ Let the pellet hydrate while setting up PCRs (at least 20 min). It may be best to allow for overnight rehydration to be sure that the DNA is in solution. Inadequate rehydration is the most common reason for failure of the next step.

5.2.2.1.4 STEP- IV PCR Recovery of Microsatellite Enriched DNA

Perform PCR on supernatant to recover the enriched DNA fragments:

- 2.5µl 10x PCR buffer (optimal buffer for Taq used below)
- 2.5µl BSA (25µg/ml final)
- 1.5µl dNTPs (2.5 mM each)
- 1.3µl SuperSNX-24 (10µM)
- 2.0µl MgCl2 (25 mM)
- 13.0µl dH2O
- 0.2µl Taq DNA polymerase (5 units/l)
- 2.0µl eluted DNA fragments (‘‘pure gold’’)

A. PCR Cycling conditions: 95°C for 2 min; then 25 cycles of 95°C for 20 sec., 60°C for 20 sec., 72°C for 1.5 min; then 72°C for 30 min; then hold at 15

B. Run 4µl of PCR product on a 1.0% minigel next to a 100-bp ladder as a standard to verify whether DNA recovery was successful (The smear of fragments should be visible, centered at approximately 500 bp.) The rest of the PCR product can be used for cloning.

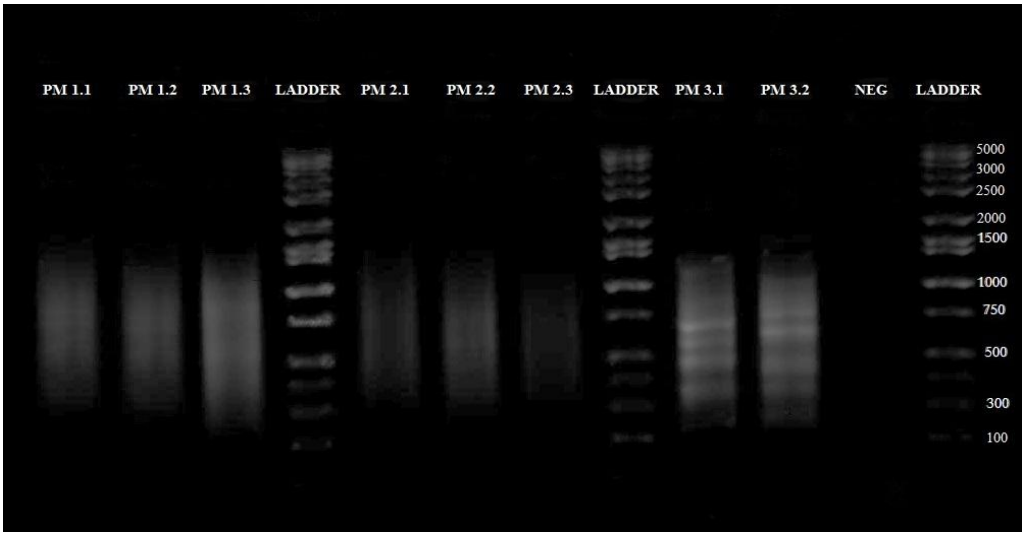

Figure 5.4: 1% Agarose gel electrophoresis results of successful Dynabeads enrichments

Three different experiments with three different DNA samples from same species i.e. PM or *Ptyas mcosa* are shown in Figure 5.3. The PM 1 and PM 2 enrichments are for standard enrichments using 3 different mixes of biotinylated oligos, The PM 3 enrichments are from a limited pool of starting DNA. Note the "bands" that appear in PM 3 are indicative of few repetitive elements dominating the enrichments.

5.2.2.1.5 STEP- V Ligating Enriched DNA into Plasmids and Transforming

TOPO cloning is a molecular technique in which DNA fragments are cloned into specific vectors without the requirement for DNA ligases. For TOPO TA cloning, the Taq polymerase has a non-template-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3'-end of the PCR products. This characteristic is exploited in TOPO-cloning. For Blunt End TOPO cloning, the recipient vector does not have overhangs and blunt-ended DNA fragments can be cloned.

The technique utilises the inherent biological activity of DNA topoisomerase I which helps in cleaving and rejoining supercoiled DNA ends to facilitate replication. Vaccina virus topoisomerase I specifically recognises DNA sequence 5´-(C/T) CCTT-3'. During replication, the enzyme digests DNA specifically at this sequence, unwinds the DNA and re-ligates it again at the 3' phosphate group of the thymidine base.

The idea is to place one fragment of the DNA into one vector and to do this for as many fragments as possible. Once ligated into the vector, the DNA is known as an insert. This protocol assumes that it is best to use the fastest and most reliable method

available for TOPO cloning. Both the TOPO TA Cloning® Kit containing pCR® 2.1-TOPO® with TOP 10 cells (catalog K4500-40) as well as the kit with TOP 10F cells (catalog K4550-40) have successfully been used with this protocol.

Follow the directions supplied with Invitrogen's TOPO-TA cloning kit exactly

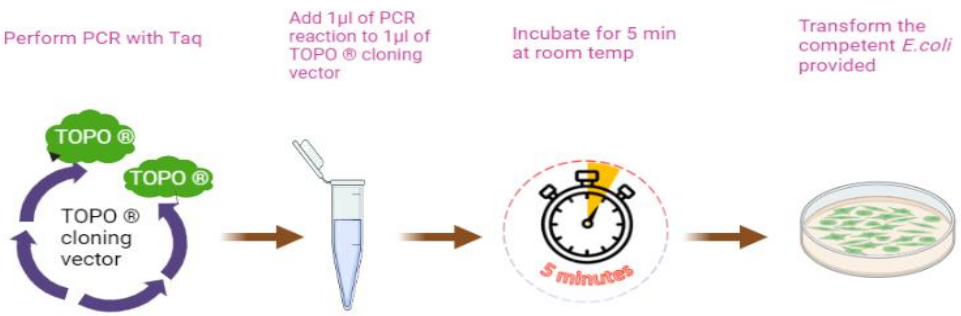

Figure 5.5: A graphical representation of directions for performing Invitrogen's TOPO-TA cloning kit.

5.2.2.1.6 STEP- VI PCR and Storing Positive Colonies

Day 1

1. Count the number of blue (or black, if S-Gal was used) colonies and the number of positive (white) colonies on each plate. If more than a few hundred are present, simply note that fact rather than trying to count each colony. The proportion of colonies with inserts (i.e., vector ligation efficiency) can be determined from the number of white colonies divided by the total number of colonies.
2. Prepare 50 ml of LB broth with ampicillin by adding 50µl of ampicillin (50 mg/ml stock) to a 50ml conical tube full of broth. Add 300µl of LB broth + ampicillin to each well of a sterilized 0.65 ml deep-well plate.
3. Lift isolated white colonies from the LB plate using the end of a sterile toothpick and transfer each colony to one well of the sterilized deep-well plate (spin the toothpick in your fingers while the end with the colony is immersed in the LB broth).
4. Cover the 96 deep-well plate loosely with Saran Wrap or a loose fitting 96-well mat. Incubate overnight at 37°C with semi vigorous shaking. It is often beneficial to incubate an additional 24 h (~40 h total) to achieve high-density cell growth.

Day 2

Pick white colonies and Set up the following 25µl PCR to check for inserts: For one 96-well tray, add the following to a clean V-bottom trough:

- 2.50µl BSA (250 g/ml)
- 2.50µl 10x PCR reaction buffer
- 1.00µl 10µM M13 forward primer
- 1.00µl 10µM M13 reverse primer
- 2.00µl 25 mM MgCl2
- 1.50µl 2.5mM dNTPs (2.5mM each)
- 12.80µl dH2O
- 0.20µl Taq DNA polymerase (2.5 Units/µl)
- 1.50µl DNA template (Bacteria grown up in LB broth)

Note: Store bacteria colonies in LB broth at 4°C until PCR product has been observed.

1. PCR cycling condition: 95°C for 3 min; then 35 cycles of 95°C for 20 sec.,50°C for 20 sec., 72°c for 1.5 min. Hold at 15°C.
2. After PCR the product will need to be examined for the presence of inserts in each plasmid.
3. Run 2µl of the M 13/bacterial PCR product on the agarose gel along with a 100bp ladder and 2µl of several different Lambda concentration standards (λ10ng/µl, λ25ng/µl, λ50ng/µl and λ100ng/µl). A 10µl multi-channel pipette may be used to save time. It loads every other lane, so it is important to keep notes on where each sample is located.
4. Run at 80 Volts for approximately 30-40 min.
5. Examine PCR results using a visual imaging system and save the results. Ensure that bands are clearly visible, but that they are not saturated/over-integrated (red). The desired insert range is from 300-1000bp.

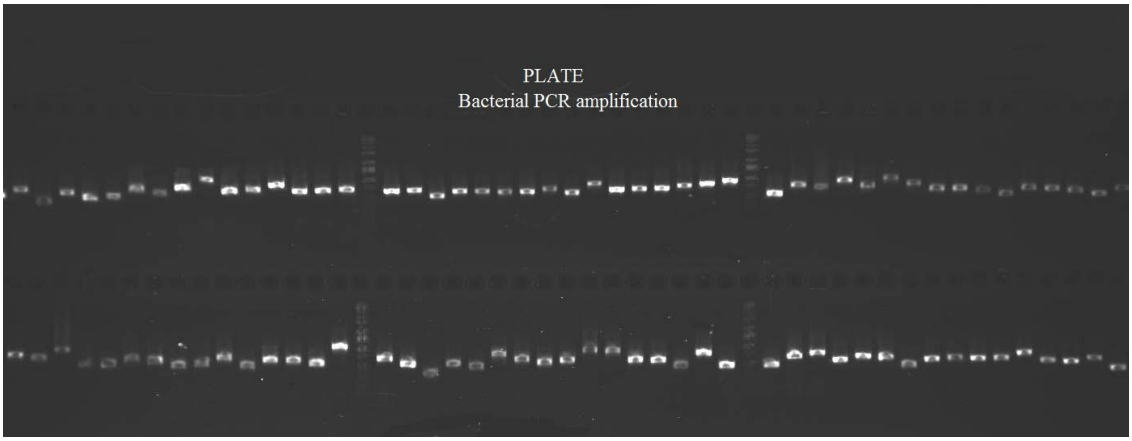

Figure 5.6: Typical PCR results for 96-well plates of clones. Each row has 48 PCR products and 2 size standards.

5.2.2.1.7 STEP- VII Prepare PCR samples for Sequencing using ExoSAP

To determine PCR product concentration and size and to purify the PCR product for subsequent sequencing. There are many ways to prepare PCR products for sequencing. If the PCR products are good and strong, dilution (i.e., using no more than 0.5 l of PCR product) is efficient and usually works well. However, cleaning the PCR using exonuclease I and SAP (or commercially available ExoSAP) is a preferred option, which improves consistency and quality of PCR products.

Recipe:
Add 1µl ExoSAP ready mixture to 6–10µl of PCR product.
Incubate the samples at 37°C for 15 min, 80°C for 15 min, and then hold at 15°C. The samples are now purified and ready for use as sequencing reaction template.

5.2.2.1.8 STEP- VIII DNA Cycle Sequencing Reactions

To determine the DNA sequence of the fragments that contain microsatellite repeats Cycle sequencing was performed.
Reaction Mix Preparation:

- 3.5µl 2.5x Sequencing buffer
- 0.5µl BigDye Terminator v 3.1 mix
- 1.0µl Primer (3.3µM)
- 2.0µl DNA template
- 3.0µl H2O (adjust volume to make 10.0µl)

Cycle sequencing conditions: ≥39 cycles at 96°C for 10sec, 50°C for 5sec, 60°C for 4 min. Hold at 15°C Note: No initial denaturation is necessary.

5.2.2.1.9 STEP- IX Precipitation of Sequencing Reactions

1. If evaporation has occurred in any of the tubes, add dH2O until it matches the others. Total volume should be about 10µl.
2. Add 1µl of 1.5M NaOAc and 250mM of EDTA (pH 8.0), using the 0.5–10.0µl multichannel pipetter and mix by pipetting up and down (i.e., sklooshing). (Note: 1.5M NaOAc pH should not be adjusted to pH 5.2).
3. Add 40µl of 95% ethanol using the 5–50µl multichannel pipetter (dripping down the sides of the tubes, tips do not need to be changed between samples).
4. Recap the tubes, invert several times, and incubate for 15 min at -20°C.
5. Centrifuge at 1,500xG for 45 min.
6. Remove caps, setting them aside on a clean Kim wipe.
7. Ensure the 96 deep-well block (note: Use the 96 deep-well blocks with square holes; these came from a Qiagen DNA prep kit) is dry by whipping out any liquids.
8. Carefully place a dry 96 deep-well block (note: Use the 96 deep-well blocks with square holes; these came from a Qiagen DNA prep kit) over the top of the tubes and flip (i.e., invert), leaving the tube holder in place over the tubes.
9. Centrifuge at 300xG for 1 min, balancing with an empty deep-well block and tube holder.
10. Carefully pull the tubes straight up, off the 96 deep-well block. If any of the tubes ‘‘stick,’’ put them back in to the holder in the correct orientation.
11. Recap the tubes and store them at 20 (non–frost-free freezer) until ready to sequence.

Concluding Remarks

1. At this point, the DNA can be sequenced on several commercially available DNA sequencers (e.g., ABI, Amersham Biosciences, or Spectrumedix).
2. Following DNA sequencing, vector and linker sequences should be removed.

5.2.2.2. PCR Amplification

Primers were designed to amplify 12 microsatellites loci for potential developmental genetic studies by using Primer 3 software and the Forward of each primer was 5’

fluorescently labeled with FAM. PCR was performed in a total volume of 10µl containing at least 50ng template DNA, 0.5µM of each primer, 1.0µl of dNTPs (2.5mM each) (MBI Fermentas, Glen Burnie, MD), 1.0µl of 10X buffer (containing 20mM Tris-HCl, pH 9.0, and 100mM KCl, 20mM MgCl2) (Genet Bio, Korea), and 0.5µl of Exprime *Taq*™ DNA polymerase (5U/µl) (Genet Bio, Korea). All PCR reactions began with a 94°C denaturation (5 min), followed by specific annealing temperature for 30 sec, extension at 72°C for 30 sec, and final extension at 72°C for 7 min. The number of cycles and the annealing temperature for each primer sets are given in Table 5.1.

5.2.2.3 Genotyping and Data Analysis

Genotyping was done on 3100 Genetic Analyzer (Applied Biosystems) using LIZ 500 size standard and results were analyzed using GeneMapper® 3.2 (Applied Biosystems). For further study of allelic diversity, data for these twelve microsatellite markers was tested in GeneMapper ® 3.2 (Applied Biosystems) software and observed (Hobs), expected (Hexp) heterozygosity and polymorphic information content (PIC) data analysis was calculated by using CERVUS version 3.0.7 (Kalinowski *et al.,* 2007).

5.2.3 Results and Discussions

Twelve loci showed polymorphism among the 20 individuals of Indian Rat snake, *Ptyas mucosa*. Of these 12 loci, six were di nucleotide repeats (R_1, R_5, R_6, R_7, R_11), five were tri nucleotide repeats (R_6, R_7, R_10, R_12), seven were tetra nucleotide repeats (R_2, R_4, R_5, R_7, R_8) and two were penta nucleotide repeats (R_3, R_9). Details about their repeat motifs, annealing temperature, allele range, cycle numbers and accession numbers are given in Table 5.1. The number of alleles per locus was ranged from 3 to 10. The observed heterozygosity (H_{Obs}) ranged from 0.789 to 1.000 whereas expected heterozygosity (H_{Exp}) ranged from 0.578 to 0.904. The average expected heterozygosity for all 12 microsatellite loci was observed to be 0.796 (Table 5.2) and average polymorphic information content (PIC) was 0.740. No deviations from the HWE were observed for the 12 loci in this study. Low to moderate levels of genetic variability within the population were observed. It may be due to their captive breeding or being small in sampling numbers, or it may be due to population decline. The 12 microsatellite loci's resolve and provide robust, high resolution tools for the efficient genetic evaluation of the studied species *Ptyas*

mucosa. They could be also helpful for capable genetic studies like linkage analysis, construction of molecular linkage maps and marker-assisted breeding, a valuable tool to assessing the population genetics of this species throughout its range. This is believed to be a significant first step to contribute to understand the wild population structure of the species and their conservation strategies. The data generated in this study were deposited in NCBI GenBank with accession numbers: KF723594-KF723600, KF723602-KF723606. These sequences could be retrieved as the reference sequence for the conservation studies of Rat snake or *Ptyas mucosa*. This is expected to be helpful for breeding programs on this species.

Table 5.1: Characteristics of 12 Microsatellite loci developed for *Ptyas mucosa* from India. Locus name, primers sequences, repeat motif and locus information.

Locus	Primer Sequence (5'-3')	Repeat Motifs	Ta°C	Allele Range (bp)	No.of cycles	Accession No.
R_1	F-TCG TGG TAG AGA CTT TGA CAG C R-ATT TGG GAG CAG ATG TTT ATG G	$(TG)_7 (GT)_8$	49	344-394	30	KF723594
R_2	F-CTT GGA AGC AAA TCT GAA ATC C R-GAA TGG GGT TTT AAT GGG AAG T	$(TATG)_6 (TATT)_{10} (CATT)_4$	52	321-370	30	KF723595
R_3	F- AGC ATA GGC ACC AAA GAC AAA T R-TTA ATC CGC AGG GAA GAT GTA T	$(ATTCT)_{18}$	47	313-354	30	KF723596
R_4	F-ACA ACC TAC ATG GTT TGG GAA C R-CAG AAT CAC CCT GAA ATA AGC C	$(AGAT)_{12}$	47	274-329	30	KF723597
R_5	F-TCT CCC TTG CTC GTT CTC TTT A R-GCC GTA TTT GAA GCT GGT AGA A	$(GAAA)_{15} (GA)_6$	52	281-313	32	KF723598
R_6	F-CCT GAG TCC TTC GGG AGT AGA R-CCA GCG CTA TGG AGA CAA ATA	$(AAC)_4 (TG)_4$	51	371-398	30	KF723599
R_7	F-ACA CTC TCT CCC ACA TGC TGA R-ACA TCA ACT GCA TCA ACC AAT C	$(TACA)_6 (CAT)_4 (TA)_4$	51	370-388	30	KF723600
R_8	F- TTA AAC CTG CAA CAC CAT CAT C R-TTG GAC CTC ACT TTG AAC AGA A	$(TTCC)_{12}$	48	362-390	30	KF723602
R_9	F-TGC AAG TGT CTC CTA CAA CAG A R-AGA TTT CTT CCC AAC CTC CTT T	$(GTTTT)_8$	51	330-345	30	KF723603
R_10	F- TGC AAG TGT CTC CTA CAA CAG A R- AGA TTT CTT CCC AAC CTC CTT T	$(ATG)_{13}$	48	367-389	32	KF723604
R_11	F- TCA TAG TGT CCT GGT TTG TAG CC R- CTT CTT GTC TGG GGT CAG TCT T	$(AC)_{10}$	48	346-363	30	KF723605
R_12	F- TAC TGG GGT GGA TCT TTT CTC T R- CAG GAT TTT AAC CAG GAG GAC TT	$(TAG)_5 (ATG)_5$	51	354-382	30	KF723606

Table 5.2: Microsatellite locus information including sample size (N), observed number of alleles (K), observed heterozygosity (Hobs), expected heterozygosity (Hexp), and polymorphic content (PIC) for *Ptyas mucosa*

Locus	N	K	H_{obs}	H_{exp}	PIC
R_1	20	9	0.950	0.867	0.826
R_2	20	8	0.900	0.859	0.817
R_3	20	10	0.950	0.904	0.869
R_4	20	8	1.000	0.894	0.857
R_5	20	8	0.950	0.872	0.832
R_6	20	3	1.000	0.578	0.469
R_7	20	10	0.789	0.845	0.802
R_8	20	6	1.000	0.729	0.665
R_9	20	6	0.850	0.826	0.777
R_10	20	10	1.000	0.859	0.817
R_11	17	8	0.941	0.809	0.755
R_12	16	3	1.000	0.627	0.530

5.2.3.1 A Comparative Study on Characteristics of Microsatellite Markers Developed from 3 Snake Species of Genus Ptyas

To understand the use and future forensic application of these newly developed microsatellite markers a comparative study was performed by collecting data from the available dataset of 3 different species from the genus Ptyas and they are *Ptyas mucosa* (data from this study), *Ptyas dhumandes* (Wang *et al.*, 2014) and *Ptyas korros* (Zheng *et al.* 2014). We tried to find out the similarities and differences among the microsatellite markers. The annealing temperature of only one primer from each species was nearly close to 50^{o}c. So those primers can be used to design multiplex marker for the identification of all the three Ptyas species with the target of 3 different microsatellite sequencing regions and corresponding identification of individual specimens. Besides we looked for similar microsatellite patterns and locus numbers whether they are same for all the species or not. The 'GT' and 'AC' microsatellite sequences were found to be common among all three Ptyas snake species with different repeat numbers. Table 5.3 to 5.5 describes the characteristics of all three Ptyas species whose datasets are available in public database NCBI (GenBank Accession no.s are given in the table) This small comparative study is expected to give a site for the strategy of development of common STR kit for identification of different snake species upto individual level but for achieving that goal further study with ample number of samples is required cause illegally traded samples are more often chemically treated and morphologically altered which eventually results into the isolation of degraded DNA from them.

Table 5.3: A comparative study on characteristics of Microsatellite markers developed from 3 snake species of Genus Ptyas

NCBI GenBank Accession No.			Primer Sequence (5'-3')		
PK	**PD**	**PM**	***Ptyas korros* (PK)**	***Ptyas dhumandes* (PD)**	***Ptyas mucosa* (PM)**
KF439726	KJ000644	KF723594	F: CACTACCAAGAATTCATAAGCC R: ATAAATCTGGCAAAGCTCTCTC	F: CACATGGCTTGAGATCCTGAG R: GGGACTCGTTTGACAACCTAC	F-TCGTGGTAGAGACTTTGACAGC R-ATTTGGGAGCAGATGTTTATGG
KF439727	KJ000645	KF723595	F: ACAATCGGTATTCTGCCAAGG R: GGAGCCCAAGAGACTTCAAC	F: TGGGTGTCACTTCCGAGAG R: TGTATGATTCAGCCTGAGGTTG	F-CTTGGAAGCAAATCTGAAATCC R-GAATGGGGTTTTAATGGGAAGT
KF439728	KJ000646	KF723596	F: GCAACATTTGTGTGAGGCTAC R: CTACCGTCCCTGTCCTACTG	F: GCAGGGAGGAGGAGGTTC R: AAGGCTGTCGTGTGAATGG	F- AGCATAGGCACCAAAGACAAAT R-TTAATCCGCAGGGAAGATGTAT
KF439729	KJ000647	KF723597	F: CGGGCGGATCACTAGGTC R: CGGTAGGTAGGGAAGGAAGG	F: GTGTTATTCGCCATCAAACCTC R: CTCTCTCCATCTCCATCCTCTC	F-ACAACCTACATGGTTTGGGAAC R-CAGAATCACCCTGAAATAAGCC
KF439730	KJ000648	KF723598	F: GGGAGGAGATGGGTAAGAGAG R: TTGGTAGGGAATGGATGGAAAC	F: AAGGTGAGATGGTTCTAAGTGC R: CCAGTCATTTAGAGGCTGTTTG	F-TCTCCCTTGCTCGTTCTCTTTA R-GCCGTATTTGAAGCTGGTAGAA
KF439731	KJ000649	KF723599	F: GGAAGAAGGTACTGGCACAAG R: CTGGTAGTGGAGAGAAGGAATG	F: CTTCAGGCTATGTCTTCTCCAG R: AGAAAATCTAGGGGTGTGTGTG	F-CCTGAGTCCTTCGGGAGTAGA R-CCAGCGCTATGGAGACAAATA
KF439732	KJ000650	KF723601	F: GAGTTCAAGTCATGCCTTAGTC R: CAACACATCTTCCTCCTCCAG	F: CAGGCAGTTCCAGCAGTG R: TCTCTCCATCTCCATCCTCTC	F-ACACTCTCTCCCACATGCTGA R-ACATCAACTGCATCAACCAATC
KF439733	KJ000651	KF723602	F: GCCCTTCAGCTATACACACAC R: GAAATGGGGTACAGCAGCAG	F: TTGTAATGGCTGCTGAAGGAG R: AGTGTGCGTATCCATATCTCTC	F- TTAAACCTGCAACACCATCATC R-TTGGACCTCACTTTGAACAGAA
KF439738	KJ000652	KF723603	F: TCTATGTGAGAGACAGTGTGTG R: CCTGACAGTGAGTAGTGAGTAG	F: GAACTTTACTGAGTGCTCTATC R: AAATCCCATTCGCCATCTTC	F-TGCAAGTGTCTCCTACAACAGA R-AGA TTTCTTCCCAACCTCCTTT
KF439739	KJ000653	KF723604	F: GTTAGCCCTGATAACACACAC R: GTTCTCTTTCTCCCCTTCCTC	F: GTAAACTCGGATCGTGCTTTTG R: GTCGTTCTCGCCCATTTCG	F- TGC AAGTGTCTCCTACAACAGA R- AGA TTTCTTCCCAACCTCCTTT
NA	NA	KF723605	NA	NA	F- TCA TAGTGTCCTGGTTTGTAGCC R- CTTCTTGTCTGGGGTCAGTCTT
NA	NA	KF723606	NA	NA	F- TACTGGGGTGGATCTTTTCTCT R- CAGGATTTTAACCAGGAGGACTT

Table 5.4: A comparative study on characteristics of Microsatellite markers developed from 3 snake species of Genus Ptyas

NCBI GenBank Accession No.			Repeat Motifs			Allele Range (bp)			Na		
PK	**PD**	**PM**	**PK**	**PD**	**PM**	**PK**	**PD**	**PM**	**PK**	**PD**	**PM**
KF439726	KJ000644	KF723594	$(AG)_{26}$	$(GA)_{29}$	$(TG)_7(GT)_8$	178–192	167–227	344-394	6	23	3
KF439727	KJ000645	KF723595	$(GT)_{16.5}$	$(CA)_{34}$	$(TATG)_6$ $(TATT)_{10}$ $(CATT)_4$	198–222	101–211	321-370	8	16	7
KF439728	KJ000646	KF723596	$(TATC)_{16.8}$	$(CA)_{13.5}$	$(ATTCT)_{18}$	188–246	155–201	313-354	10	15	4
KF439729	KJ000647	KF723597	$(AG)_{26.5}$	$(AGAT)_{16.8}$	$(AGAT)_{12}$	154–174	159–201	274-329	4	15	7
KF439730	KJ000648	KF723598	$(AGAT)_{17.3}$	$(GT)_{20.5}$	$(GAAA)_{15}$ $(GA)_6$	145–187	147–179	281-293	11	14	6
KF439731	KJ000649	KF723599	$(AC)_{23.5}$	$(AC)_{25}$	$(AAC)_4(TG)_4$	160–178	157–187	371-398	6	12	4
KF439732	KJ000650	KF723600	$(TC)_{41.5}$	$(ATAG)_{16.3}$	$(TACA)_6$ $(CAT)_4$ $(TA)_4$	130–180	179–247	370-388	14	18	4
KF439733	KJ000651	KF723602	$(AC)_{14}$	$(AG)_{32.5}$	$(TTCC)_{12}$	99–121	191–263	362-398	6	20	5
KF439738	KJ000652	KF723603	$(GT)_{29}$	$(GT)_{24}$	$(GTTTT)_8$	159–169	211–237	330-345	5	14	5
KF439739	KJ000653	KF723604	$(AC)_{19.5}$ $(AG)_{35.5}$	$(CA)_{16}$	$(ATG)_{13}$	115–123	215–233	377-389	6	6	4
NA	NA	KF723605	NA	NA	$(AC)_{10}$	NA	NA	346-356	NA	NA	4
NA	NA	KF723606	NA	NA	$(TAG)_5$ $(ATG)_5$	NA	NA	354-387	NA	NA	3

Table 5.5: A comparative study on characteristics of Microsatellite markers developed from 3 snake species of Genus Ptyas

NCBI GenBank Accession No.			Ta (^{o}C)			H_{obs}			H_{exp}			PIC		
PK	**PD**	**PM**	**PK**	**PD**	**PM**	**PK**	**PD**	**PM**	**PK**	**PD**	**PM**	**PK**	**PD**	**PM**
KF439726	KJ000644	KF723594	58	57.5	49	0.574	0.642	1.000	0.635	0.853	0.641	0.589	0.841	0.555
KF439727	KJ000645	KF723595	50	49.5	52	0.778	0.536	1.000	0.602	0.891	0.871	0.537	0.875	0.822
KF439728	KJ000646	KF723596	58	52.8	47	0.722	0.651	1.000	0.796	0.829	0.644	0.760	0.812	0.554
KF439729	KJ000647	KF723597	47	63.4	47	0.500	0.612	0.950	0.419	0.858	0.840	0.348	0.839	0.795
KF439730	KJ000648	KF723598	61	61	52	0.907	0.752	1.000	0.854	0.86	0.741	0.827	0.842	0.672
KF439731	KJ000649	KF723599	58	58	51	0.741	0.621	1.000	0.671	0.724	0.579	0.618	0.693	0.464
KF439732	KJ000650	KF723600	65	55.2	51	0.296	0.519	1.000	0.868	0.900	0.693	0.847	0.888	0.606
KF439733	KJ000651	KF723602	65	61	48	0.759	0.635	1.000	0.555	0.914	0.624	0.479	0.905	0.535
KF439738	KJ000652	KF723603	58	58	51	1.000	0.778	1.000	0.659	0.882	0.692	0.591	0.868	0.631
KF439739	KJ000653	KF723604	50	46.8	48	1.000	0.574	1.000	0.546	0.550	0.667	0.435	0.502	0.584
NA	NA	KF723605	NA	NA	48	NA	NA	1.000	NA	NA	0.613	NA	NA	0.515
NA	NA	KF723606	NA	NA	51	NA	NA	1.000	NA	NA	0.629	NA	NA	0.535

5.2.4 Conclusion

Microsatellites have proven to be an extremely valuable tool for conservation study in many organisms. Characterisation of microsatellite marker helps the conservation biologist in the study of animal family as well as their applications span over different areas ranging from ancient history studies, forensic DNA studies, genome mapping, population genetics and true hybrid identification. Due to this investigations using microsatellite markers have increased in recent years despite being severely limited by the high costs involved in the construction of genomic libraries. Our study is a significant first step that should help to establish conservation strategies and contribute to an understanding of the structure of wild, vestige populations for *Ptyas mucosa* snake species. In addition, the current study sequence data were deposited in NCBI GenBank under the Accession no. KF723594 to KF723600 and KF723602 to KF723604. These sequences could be retrieved as the reference sequence for the conservation studies of Rat Snake or *Ptyas mucosa.* It can be used to detect sudden changes in Ptyas population, their geographic distribution and rate of mutations. The effects of population fragmentation and interaction with other populations can also be detected through these microsatellite markers. Besides the polymorphic microsatellite markers have prospective utility as genetic tags for use in Ptyas conservation. They can be powerful DNA markers for quantifying genetic variations within and between populations of Ptyas species. This data along with the other technologies like captive breeding and sperm cryopreservation can be integrated into a package for conserving genetic diversity of them. This is expected that this data will help the conservation biologists for the study of *Ptyas mucosa's* ancestral history, migration history and geographical distribution range as well as in developing STR markers for their individual identification.

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CHAPTER VI

SUMMARY

&

SCOPE of FUTURE WORK

Ever since the evolution of life on earth, many species have become extinct and many are on the verge of extinction. The rate of extinction of wildlife has now become exponential mainly due to population explosion and rapid urbanization resulting in their natural habitat loss as well as due to illegal trafficking of wildlife parts and products resulting in their killing and poaching. Hence, to stop further extinction of such valuables from the nature, the Conservation Geneticists and the Wildlife Forensic Scientists together are trying to build the bridge by making strategies for their in-situ conservation, breeding etc. as well as curbing the illegal hunting and trading of wildlife by developing techniques for their identification from confiscated items followed by prosecution of criminals.

In this thesis through an extensive literature review the area of our research work on Indian snake families have been chosen as they are being illegally poached, trafficked and traded for their venom, skin and meat to different South Asian countries. They are listed in Schedule I and II of WLPA as well as in Appendix I, II and III of CITES. Here a keen and diligent attempt has been made for the conservation and forensic identification of some Indian snake species through the molecular genetics study. It explains about the different available forensically informative molecular markers and the need for their development at present day.

By the development of an economic, fast and robust multiplex PCR method identification of four (4) types of Indian snake species using multiplex PCR reaction followed by gel electrophoresis analysis has been performed. It will help in proper identification of the species without stepping through complex sequencing techniques and BLAST searching. It is believed that approaches for such techniques with other species should be made through research to get sequencing-free outcomes especially for those countries where the economy has taken a back seat.

Next, by performing sequencing using NGS followed by annotation, the complete mitogenome for the Asiatic Water snake, which is an important creature for balancing aquatic ecosystem of our environment a small contribution has been made towards building their genomic database as well as studying phylogenetic position. Although this species counted as least concern for conservation at present but due to their lucrative skin and nutritious meat their demand is increasing fast which in turn results as a new item into the illegal trafficking market. In this study we report for the first time the complete mitogenome of the species *Fowlea piscator* or previously known as *Xenochrophis piscator* through Next Generation Sequencing process. The result

shows a typical snake mitogenome structure of 17Kb consisting of 37 genes. It also supports the non-monophyletic evidence of the species. It is expected to be useful in increasing the knowledge of additional mitochondrial genes which can be used as potential markers in forensic analysis as well as enhancing the nucleotide database for similarity searches using the BLAST tool during the identification process leading to the punishment of the perpetrator.

Lastly, the process of isolation and characterization of novel polymorphic microsatellite markers from the Indian Rat snake or *Ptyas mucosa* has been elaborately described. The genetic differentiation has also been explained using these newly developed microsatellite markers. Low levels of genetic variability within the population were observed. It may be due to their captive breeding or being small in sampling numbers, or it may be due to population decline. By using these markers we can address issues like structure of population level, degree of discrimination between individuals, relationship among each other, etc. However, mutations in repeat units and flanking regions may result in data problems for population genetics studies. Hence, any absolute conclusion in this regard would require further analysis using extensive sampling of the species from across India and then from other continents where they are found.

Despite of facing many obstacles during work, especially availability of desired species, financial constraints, available expertise and limited time frame alongside working with those animals which are counted as threat to human life due to their self defensive nature, it is important to remain determined to get the work done. After overcoming those hurdles, the markers developed here through this study proved to be rapid and robust for snake species identification as well as evolutionary studies of Indian snake species. Therefore, the use of these newly developed markers is sincerely recommended. The author hopes these small but significant findings could be helpful for phylogeny and conservation studies of these antiquities and strongly wish that the contribution for the identification of some specific Indian snake species in the wild will be accepted by the court of law.

CHAPTER VII

LIST OF PUBLICATIONS

LIST OF ORIGINAL PUBLICATIONS

Mitra, I., Roy, S., Haque, I. Combating Illegal Wildlife Trading Through Wildlife Forensics: from Recognition to Current Laboratory Techniques. CBI Bulletein, Govt. of India.

Mitra, I., Roy, S., Haque, I. Application of molecular markers in wildlife DNA forensic investigations. J Forensic Sci Med 2018;4: 156-60. DOI: 10.4103/jfsm.jfsm_23_18.

Mitra, I., Roy, S., Haque, I. Asian Crocodile Poaching: A review of molecular techniques developed for forensic identification. J Forensic Sci Med (In press) DOI: 10.4103/jfsm.jfsm_49_21.

Mitra, I., Roy, S., Haque, I. Forensic identification of four Indian snake species using single Multiplex PCR. Journal of Applied Biology and Biotechnology. (In press) DOI: 10.4103/jfsm.jfsm_50_21.

Mitra, I., Roy, S., Haque, I. The complete mitochondrial genome study and phylogenetic analysis of Asiatic Water Snake (*Fowlea piscator*) using "Next-Generation Sequencing" technology". Mitochondrial DNA Part B (Under revision).

Roy, S., **Mitra, I**., Dinesh, D., Dried Umbilical Cord and Cord Blood Sample: Reliable Non Invasive Method of Sample Collection than Usual Invasive Method from Neonates for Forensic Identification. Int J Forens Sci 2022, 7(2): 000261. DOI: 10.23880/ijfsc-16000261.

ABSTRACTS/MANUSCRPTS SUBMITTED IN CONFERENCES

Mitra, I., Roy, S., Haque, I. Wildlife DNA Fingerprinting: An Initiative for Prosecution of Crocodile Trade Offenders. 23rd All India Forensic Science Conference, Bhopal. 2015. [Oral presentation].

Mitra, I., Roy, S., Haque, I. Forensic Identification of some Endangered Snake Species using Multiplex PCR. 24th All India Forensic Science Conference, Ahmadabad. 2018. [Oral presentation].

Roy, S., Mandi, A., **Mitra, I.,** Haque, I. Autosomal STR Typing of DNA extracted from Cigarette butt and its significance. 23rd All India Forensic Science Conference, Bhopal. 2015. [Oral presentation].

Roy, S., **Mitra, I.,** Haque, I. Consequence of Forensic DNA Fingerprinting in Criminal Proceedings. All India Police Science Conference, 2016.

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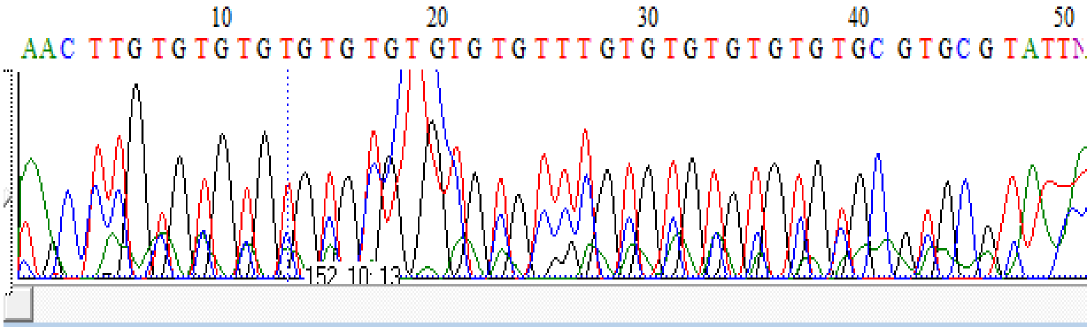

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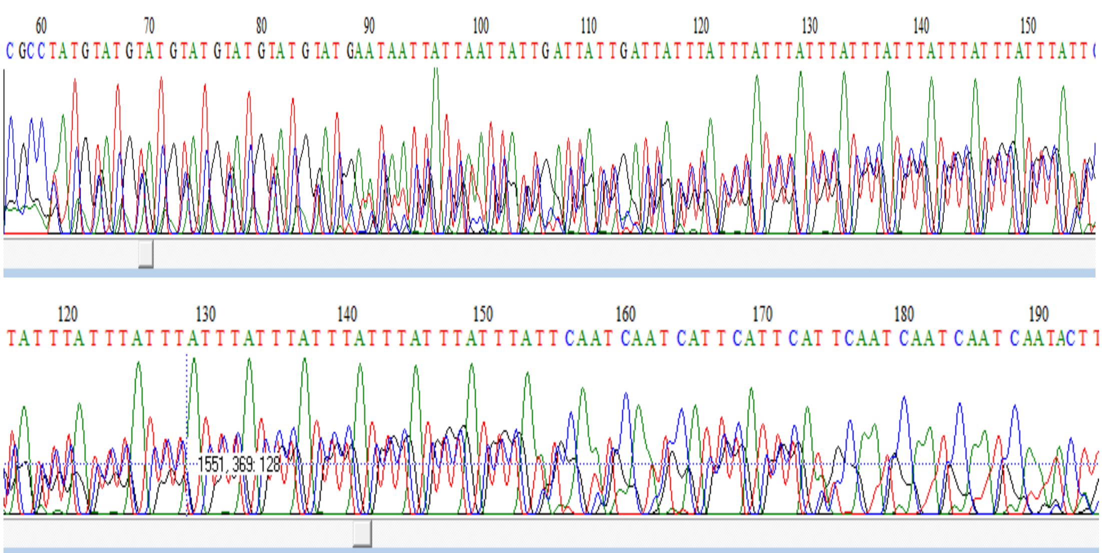

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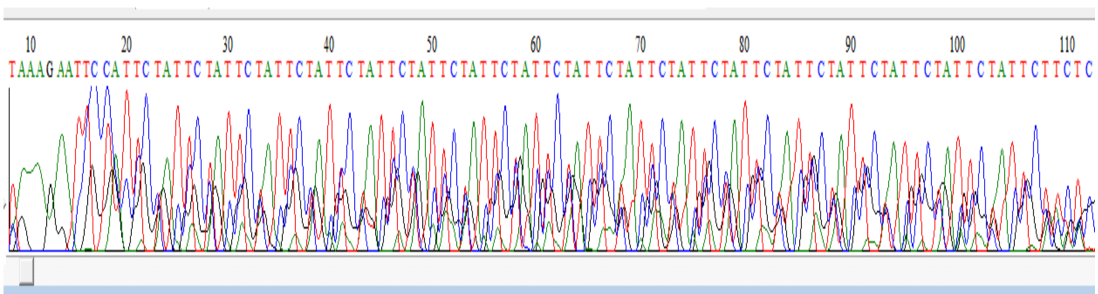

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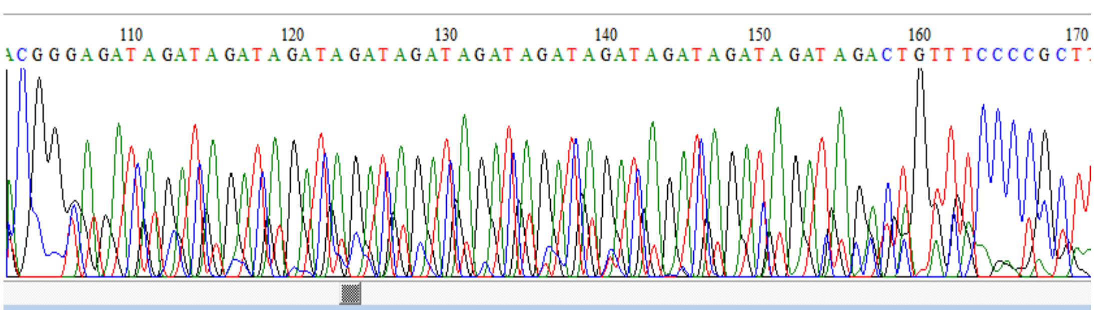

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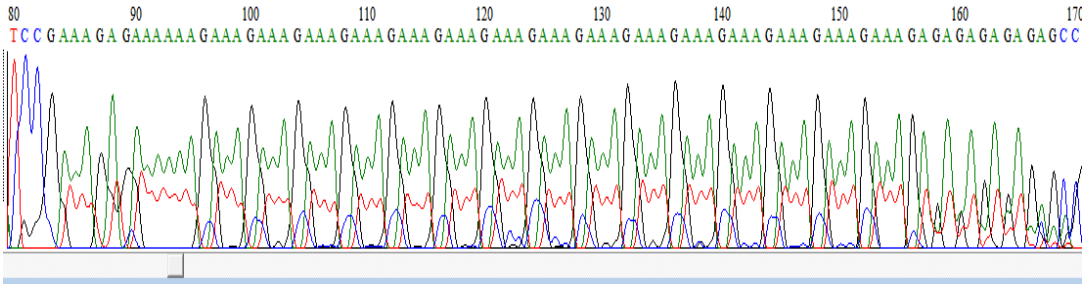

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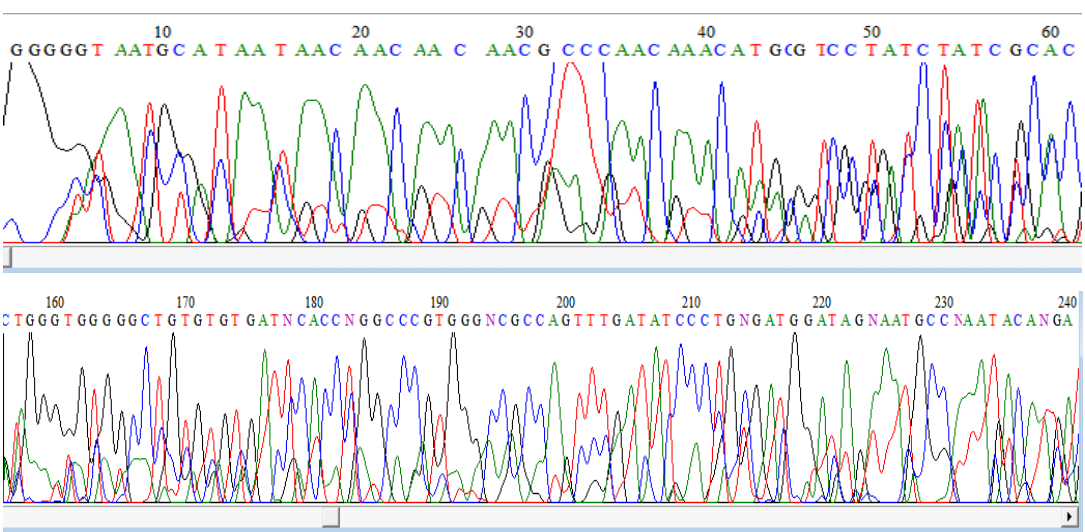

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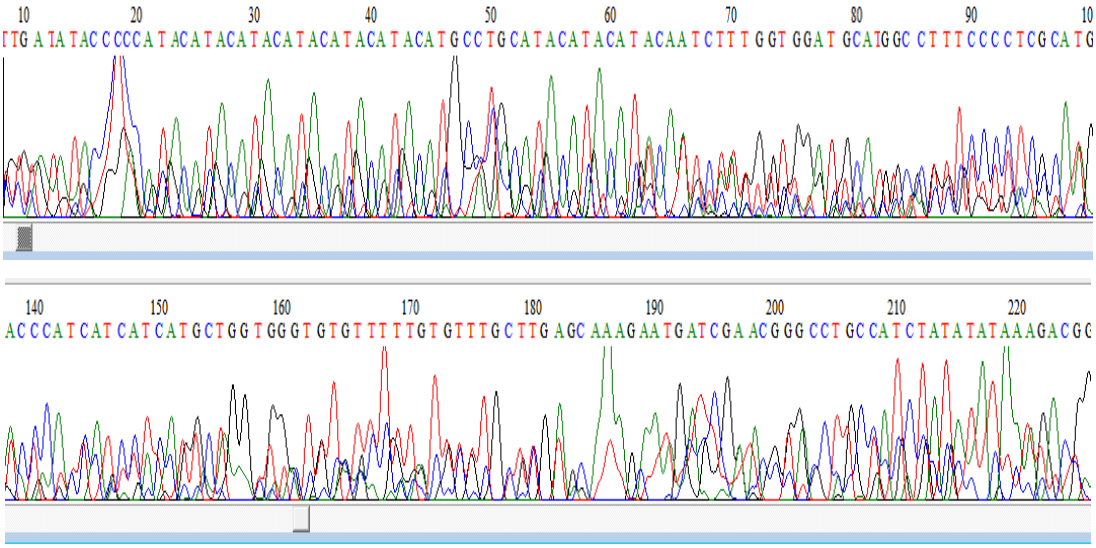

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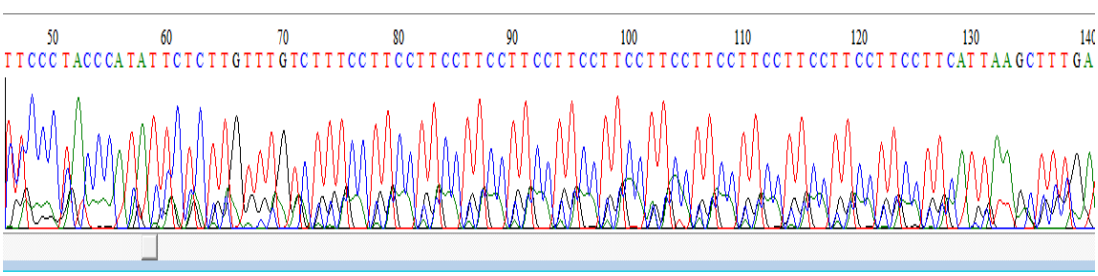

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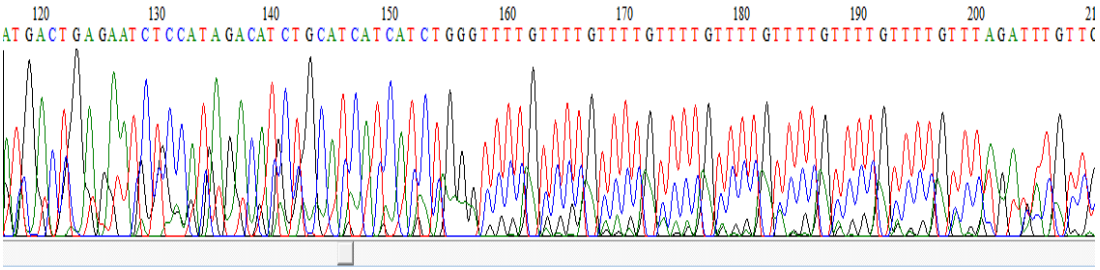

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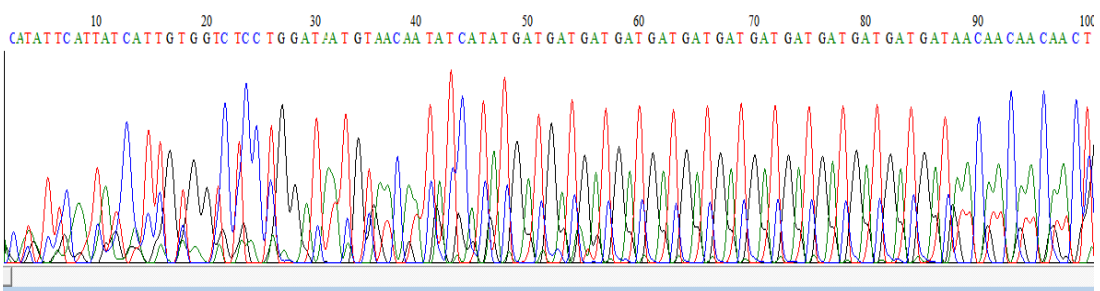

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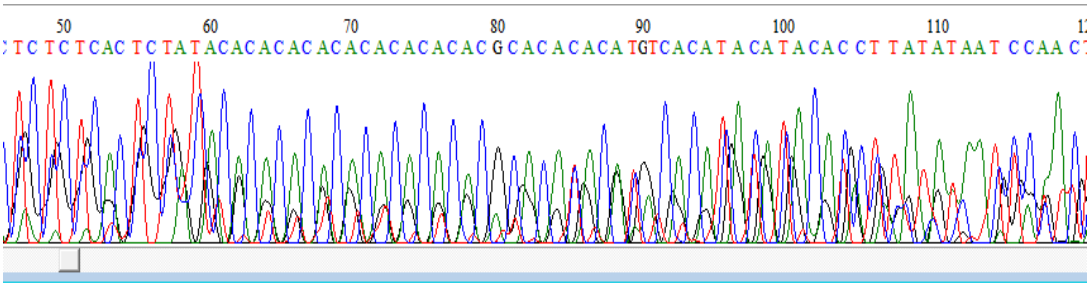

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