

**“IDENTIFICATION OF FORENSICALLY INFORMATIVE
MARKERS AND PHYLOGENY IN SOME THREATENED
SPECIES OF SNAKES AND LIZARDS IN INDIA”**

*Thesis submitted for the
DEGREE OF DOCTORATE OF PHILOSOPHY (SCIENCE)
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2025

CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled “**IDENTIFICATION OF FORENSICALLY INFORMATIVE MARKERS AND PHYLOGENY IN SOME THREATENED SPECIES OF SNAKES AND LIZARDS IN INDIA**” Submitted by Sri **D Dinesh** who got his name registered on **JULY 12th 2016**, with **Index number: 122/16/Life Sc. /25** for the award of Ph.D. (Science) degree of Jadavpur University, is absolutely based upon his own work under the supervision of **Dr. Soma Roy**, Assistant Director & Scientist ‘C’, Central Forensic Science Laboratory, Kolkata and 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 no 935(5)/WL/4R-6/2015 dated 21st February, 2017
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DEDICATED

TO MY

FATHER, MOTHER AND
BROTHER

D V PRASAD RAO

D K VASUNDARI

D V WARUN

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"Appreciation can make a day—even change a life. Your willingness to put it into words is all that is necessary." – **Margaret Cousins**

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ABSTRACT

Global trade in snakes and their products is extensive, involving millions of individuals and their products annually. Approximately 6.2 million live snakes scheduled under Convention on International Trade in Endangered Species (CITES) were traded between the years 1975 to 2018. During the same period, 34-million snake skin were traded. Despite the increase in captive breeding, the majority of the snakes that are traded are from the wild sourced accounting for 60% of the trade. These figures highlight the significant scale of snake and trade of its products emphasizing the importance of monitoring, regulating the conservation of snake population.

Apart from snakes, one other important reptile that is traded as pets and for its products are lizards. Skin of monitor lizards is the most traded for the fashion industry. Among the reptile species scheduled on the IUCN red list, 21% of those involved as vulnerable while 5% is deemed as a high-risk category.

A key basis for wildlife investigations is definitely advancements in forensic technology. Combating the illegal trade of snake and lizard byproducts, which compromises biodiversity and endangers species, molecular identification using DNA has been a vital weapon in hand. For processed or fragmented materials, traditional morphological identification is usually ineffective; thus, DNA analysis is the most precise technique for species identification. With their great sensitivity and specificity, DNA-based methods let one differentiate closely related species. DNA barcoding is one of the molecular techniques used in identification since it sequences homogeneous nucleotide areas and match them to databases. Next-generation sequencing (NGS) technologies have revolutionized molecular biology and made high-throughput sequencing, incredibly accurate and efficient feasible. NGS has become a valuable technique in wildlife forensics for species identification, especially for reptiles like snakes and lizards since it can precisely identify species from

biological samples such blood, scales and tissue. This is critical for both law enforcement and conservation activities.

This thesis explores the whole genome of *Chrysopelea ornata*, commonly known as the ornate flying snake, one of the rarest species in the snake family, renowned for its unique gliding ability between trees. It examines whole genome, microsatellites, proteins, and gene annotation, along with the sequencing of its first complete mitogenome and phylogenetic analysis. Furthermore, the study investigates forensically informative nucleotide sequences (FINS) of *Chrysopelea ornata* and *Varanus bengalensis*, essential for species identification. The Bengal monitor (*Varanus bengalensis*) is categorized under Near Threatened (NT) on the Red List of International Union for Conservation of Nature (IUCN) as of 2018, therefore underlining the possible risk of endangerment should ineffective conservation actions be taken. Declared in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), the species is under the greatest degree of protection since international trade is forbidden except in very rare cases. In India, it is safeguarded under Schedule I, Part II of the Wildlife Protection Act of 1972, ensuring stringent legal protection. It also explores the oral microbiota of the varanus lizards and the findings of harmful bacteria and fungi. The molecular genetic data presented in this study hold significant forensic value, aiding in the accurate identification of these species and contributing to conservation and wildlife protection efforts. Thesis has been divided into chapters explaining experiments.

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CHAPTER I

INTRODUCTION



"The wildlife and its habitat cannot speak, so we must and we will." – **Theodore Roosevelt**

1.1 Wildlife Protection & Sustainability

Wildlife is a vital component of the Earth's ecosystems. Animals, plants, and microorganisms contribute to ecosystem such as pollination, nutrient cycling, disease control, and climate regulation (Sodhi, N. S., 2010). Protecting wildlife helps to maintain these crucial ecological functions. Despite their importance, wildlife faces numerous intimidations, compelled by human actions. Environmental loss due to deforestation, urbanization is one of the most significant challenges.(Laurance et al., 2014). Illegal wildlife trade is another major concern. Poaching of ivory, horns, and exotic pets remains to drive species towards extinction.(Challender & MacMillan, 2014). To address these threats, several conservation strategies have been implemented globally. Establishing areas that are protected such as wildlife reserves and national parks has proven effective in being safe habitats for endangered species (Watson et al., 2014). Positive results have also come from community-based conservation projects involving nearby people in wildlife protection. By providing economic incentives for conservation, therefore lowering poaching, and encouraging sustainable tourism, programs like Namibia's community conservancies empower local populations.(Naidoo et al., 2016). The Society for Conservation Biology's Asia Section pointed up major obstacles to the preservation of Asian biodiversity. They underlined that the area is going through a dynamic but challenging phase with fast urban growth and fast economic development across many countries—especially because of major infrastructure investments in India and China. Two main issues they underlined are managing the sale of threatened plant and animal species and using conservation biology to improve knowledge of zoonotic disease transmission, which is intimately related with the wildlife trade.(McNEELY et al., 2009). Local extinctions of traded species and notable population decreases have resulted from the exploitation of animals to satisfy this demand. According to a recent meta-analysis, traded species saw a 62% average decline in abundance in trade-active regions.(Morton et al., 2021). Comprising civil society and governmental agencies committed to preserving environment and advancing ecological development, the International Union for Conservation of Nature (IUCN) is a worldwide membership organization. Their works center on evaluating the state of nature, its resources, and the required actions to preserve

them. First created in 1964, the IUCN Red List of Threatened Species is well known for assessing the global species conservation situation. It is the most all-encompassing worldwide inventory of species' states of conservation. Though it is believed that around 6 million species remain undiscovered, the IUCN Red List (2021) notes that over 1.3 million species—including microbes, plants, and animals have been reported.

1.1.1 Wildlife protection and sustainability of India

India is one of the most biodiverse countries, with a rich variety of flora and fauna. It is home to almost 8 percent of the world's recorded species, including mammals, birds, reptiles, amphibians and insects. It has got the diverse ecosystems from Himalayas in the north up to tropical rain forests in the south. The country's unique geography and climatic conditions have led to a diverse range of habitats and an astonishing diversity of wildlife species. India accounts for 45,500 plant species and 91,000 animal species (MOEFCC (2021), n.d.) including some of the most prominent species such as Asiatic lion (*Panthera leo persica*), Indian elephant (*Elephas maximus indicus*), Indian rhinoceros (*Rhinoceros unicornis*) and Bengal tiger (*Panthera tigris tigris*). Indian peafowl (*Pavo cristatus*), Great Indian bustard (*Ardeotis nigriceps*) and Sarus crane (*Grus antigone*) Naga king cobra (*Ophiophagus hannah*), Gharial (*Gavialis gangeticus*) and India star tortoise (*Geochelone elegans*). Purple frog (*Nasikabatrachus sahyadrensis*), Indian bullfrog (*Hoplobatrachus tigerinus*). India has several government-run conservation programmes to protect its rich biodiversity. India has over 100 national parks and 550 wildlife sanctuaries, with popular reserves like Jim Corbett National Park and Kaziranga National Park. (WII (2022), n.d.) and Project initiatives -- like Project Tiger (1973) which has played an important role in protecting tiger populations via reserve areas. Project Elephant (1992) is for protection of elephants and their habitats. By implementing the Crocodile Conservation Project (1975), crocodile populations have rebounded. You, along with the Wildlife Protection Act (1972) which serves as a legislative mechanism for the conservation of species and their habitats. The act forbids trade in more than 1,800 species of wild animals, plants, and their derivatives. India has also been a CITES (Convention on International Trade in Endangered Species of Fauna and Flora) member since 1976, an international agreement to ensure that international trade in wild animals and plants does not threaten their survival. The role of

trade in species extinction is still largely undocumented (Jenkins et al., 2014). However, some species are particularly vulnerable to overharvest, especially those that have long lifespans, long generation times, and low reproductive rates, including all tortoises and most large lizards ((Reznick et al., 2002). India, a signatory to CITES, ranks among the top 20 countries for wildlife trafficking and among the top 10 for wildlife trafficking by air. India is one of the major source, transit and destination countries for trafficked wildlife and wildlife products. The two major channels of international wildlife trafficking to and from India are through the country's long international border along the Northeast, or via its airports, with the airports at Chennai and Mumbai emerging as the epicentres of this illegal trade (Anusha, n.d.)

1.1.2 Conservation through Genomics

Genetic analysis for the identification of non-human evidence developed shortly after the initial application of DNA fingerprinting in human forensic investigations.(Gill et al., 1985). The discovery that the same technique could be used for familial identification in birds (Burke & Bruford, 1987)quickly prompted the adoption of these methods to confirm captive breeding claims in instances where there was suspicion of wild bird theft (Fleming et al., 2011). Principal challenges in wildlife conservation encompass the identification of populations and conservation units, estimation of population size and connectivity, detection of hybridization, assessment of population persistence and adaptability to environmental changes, and comprehension of the factors affecting this potential. Genetic data are essential in resolving these difficulties and formulating efficient management strategies. The progress in genomics has transformed population genetics, rendering high-throughput sequencing attainable for the examination of many organisms, including rare and challenging species in natural populations.(Luikart et al., 2018; Rajora, 2019; Supple & Shapiro, 2018). Genomic approaches are essential tools for gaining fundamental insights into wildlife biology, including population dynamics, and for informing conservation and management efforts for wildlife populations and their habitats. (Hohenlohe et al., 2021) Concurrently, the potential of genomics approaches brings novel concerns for researchers managing and deriving meaning from these voluminous genomic datasets. Conventional conservation genetics approaches used in wildlife have included allozyme analysis,

microsatellite genotyping, and mitochondrial DNA sequencing to generate valuable insights into natural populations (Allendorf, 2017).

1.1.3 Forensics in wildlife conservation

Wildlife forensics is a scientific discipline that aids law enforcement by identifying wildlife samples collected during criminal investigations or connecting wildlife crimes to those responsible.(Huffman et al., 2011). The primary goal of any criminal investigation, regardless of the reason, is to identify a suspect and establish a connection between the suspect and the illegal activity being investigated. While identifying the species or individual of wildlife can help confirm if a crime has occurred and is useful in cases where a suspect claims that seized goods are of legal origin, it doesn't always establish a direct link to the suspect. Although strict laws and penalties are in place, perpetrators are frequently acquitted due to insufficient evidence from accusers regarding species identification. Therefore, accurate animal identification, whether for scheduled species or not, is crucial in legal proceedings.(Gupta, 2012; Verma & Goswami, 2014; Verma & Singh, 2003a). Sales of wildlife products derived from hunted animals range in processed forms including chopped meat, tanned skins, bones, horns, and ivory goods(K et al., 2017). Often the lack of clear physical traits renders identification dubious. Therefore, conservation policies depend on a fast and accurate species-level identification system to follow the source animal of evidence seized from poachers.(Kumar et al., 2019).

1.1.4 Molecular Forensic identification

Forensic DNA techniques serve as a valuable tool for law enforcement in combating wildlife crime. Over the past several decades, these techniques have advanced significantly, making criminal investigations more efficient, cost-effective, and widely accessible.(Butler, J.M., 2005). Conservation genetics often uses information from mitochondrial DNA (mtDNA) sequences. The mitochondrial genome is a circular fragment of DNA that usually has 36 or 37 genes. This includes 2 genes for rRNAs, 22 for tRNAs, and 12 or 13 that make parts of proteins found in the inner mitochondrial membrane. Also, a part of the DNA that doesn't code for proteins, called the control region (CR), is very

important for the replication and transcription of mitochondrial DNA (mtDNA). The exons in the mtDNA circle are closely packed together with no gaps. Unlike nuclear DNA, mtDNA does not have histones, has fewer ways to heal itself, and changes (mutations) at a rate 5 to 10 times faster than nuclear DNA. mtDNA changes quicker than nuclear DNA, but its rate of change is different in various areas. This makes mtDNA useful for studying family tree connections. Some common primers have been created to help copy different parts of mitochondrial DNA in various species. In addition, because most cells have many copies of mtDNA, even small amounts of damaged tissue can provide good DNA patterns. mtDNA is important for conservation genetics because it helps with understanding population structures, sorting out species, finding hybrid species, and tracking illegal hunting of rare animals. Choosing the right computational tools is crucial for accurately studying evolutionary relationships using mitochondrial markers (Khan et al., 2008). First PCR-based markers extensively used for molecular fingerprinting were microsatellites; sometimes-referred to as simple sequence repeats (SSRs). These are small DNA segments in which a straightforward sequence motif usually 1–6 base pairs long is repeated up to about 100 times. Highly polymorphic, numerous, and very equally spaced throughout eukaryotic genomes, microsatellite markers Microsatellites, as co-dominant markers, let heterozygotes differ from homozygotes by varying the number of tandem repetitions, therefore producing varied sequence lengths. Their simple amplification by PCR, co-dominant inheritance, and high allelic diversity across loci help to explain their great utility. Still, most species lack known microsatellite loci because of the labor-intensive and complex character of microsatellite analysis. Although cross-species amplification is occasionally possible among closely related species, substantial mutation rates in the flanking regions make creating conserved primers for worldwide use difficult. Consequently, anytime a species is first investigated, new species-specific microsatellite markers have to be isolated a procedure that is expensive and time-consuming.(Arif et al., 2011a)

1.1.5 Forensically Informative Nucleotide Sequencing (FINS)

Forensically Informative Nucleotide Sequencing (FINS) is a molecular technique used in wildlife forensics to identify species by analyzing nucleotide sequences of specific genetic markers. This method relies on sequencing regions of DNA, such as mitochondrial or nuclear genes, and comparing them with reference databases to determine species identity. Due to its high accuracy and reliability, FINS is particularly useful in cases involving illegal wildlife trade, poaching, and species misidentification. Unlike traditional morphological identification, which may be inconclusive for processed animal products like meat, skin, or bones, FINS can provide definitive species identification even from degraded or minute DNA samples. The application of FINS in conservation and law enforcement has significantly improved the ability to trace the origin of wildlife products and strengthen legal cases against offenders. By utilizing bioinformatics tools and sequence alignment techniques, forensic experts can establish genetic differences between closely related species, ensuring precise identification. However, the effectiveness of FINS depends on the availability of comprehensive genetic reference databases, making it essential to continuously expand species-specific DNA sequences. As a powerful tool in wildlife forensics, FINS plays a crucial role in protecting endangered species and enforcing conservation laws by providing irrefutable genetic evidence in legal proceedings.

1.1.6 Microsatellites

Genomes contain abundant repeating sequences known as simple tandem repeats, comprising several contiguous copies of a certain pattern. (Richard et al., 2008a). This assessment is best conducted using polymorphic and informative markers. Microsatellites, also known as simple sequence repeats (SSRs), are short tandem repeat motifs (1–6 bases) present in both coding and non-coding regions of the genome, characterized by significant length polymorphism. Microsatellite markers are now a useful tool for studying germplasm and evolution, genetic linkage mapping, genetic diversity detection, and genetic structure analysis because of their many benefits. However, because enriched microsatellite libraries had to be created, conventional techniques for isolating and producing microsatellite

primers were expensive and time-consuming.(Zane et al., 2002). In recent years, next-generation sequencing (NGS) has revolutionized the study of microsatellite markers by offering high-throughput data generation at exceptional speed. NGS has been widely utilized in genomics-based approaches to efficiently and cost-effectively identify new microsatellite marker sequences in both animals and plants.(Capobianchi et al., 2013; Castoe, Poole, et al., 2012; Cheng et al., 2007; Miller et al., 2007; Triwitayakorn et al., 2011). Genome survey sequencing (GSS) on the NGS platform has demonstrated significant utility in identifying microsatellite markers across entire genomes, especially in non-model species. Several species have been studied for microsatellite marker development using GSS.(Adelyna et al., 2016; X. Lu et al., 2017; Zhou et al., 2013; Ziya Motalebipour et al., 2016)

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CHAPTER II

REVIEW OF LITERATURE

2.1 Introduction

Snakes, diverse groups of legless (Coates & Ruta, 2000) reptiles found within the suborder Serpentes, are essential for maintaining ecological balance around the world. With around 3,000 species (Vidal et al., 2006), snakes are commonly found in various habitats like forests, grasslands, deserts and wetlands around the world, except Antarctica. Their presence in the wild represents not only biodiversity, but environmental health. Snakes act as both predators and prey, helping to control the population of pests while providing a high-quality food source for many birds, mammals and other predators. It is often misconception and fear are the two basic element about snakes, these makes a human-snake conflict a broader human and snakes issue and in the same time an important issue about illegal trade and smuggling. The use of forensic science to identify and monitor smuggled snakes has become a critical tool in combatting wildlife crime, protecting biodiversity, and enforcing conservation laws. Although snakes are a poorly researched group since nothing is known about their population, distribution, or threats.

In their natural habitats, snakes play important ecological roles which are often overlooked. In their role as apex predators or mesopredators, snakes are essential for regulating the cycles of rodents, insects and other small animals that can cause significant agricultural loss or spread disease. In farmland, snakes like the rat snake play a critical role in controlling the population of rodents, which in turn reduces the need for chemical pest control agents. This organic pest-management practice helps the farmers and protects soil and biodiversity. On the left hand, snakes may also be eaten by some animals, like hawks and eagles, mongooses and even other snakes (like the king cobra, which eats other snakes). This feedback loop highlights their role in food webs, aiding environmental balance by controlling the populations of their different trophic levels.

Snakes play a critical role in medical research and biotech. Their venom, evolved to subdue prey and deter predators, consists of complex proteins and enzymes that have been researched for a variety of therapeutic applications. Drugs such as captopril, an ACE inhibitor used to treat hypertension, and anticoagulants to prevent thrombosis have been developed from compounds found in snake venom. Research on snake venom is

advancing the development of potential treatments for diseases like cancer, pain relief and neurological diseases. Additionally, snake venom is necessary as an intermediate product for the production of antivenoms that are important drugs for the patients bitten by a snake, especially in the remote and underdeveloped regions of the world, where snakebites are a significant public health issue.

Although they play vital ecological and medicinal roles, snakes are under threat from habitat destruction, climate change and illegal wildlife trafficking. Over the past few decades the trade of snakes (legal and illegal) has increased due to demand for exotic pets, traditional medicine, and luxury items such as snake skin products (Van Cao *et al.*, 2014). For instance, the ball python (*Python regius*), reticulated python (*Malayopython reticulatus*), and boa constrictor (*Boa constrictor*) are often sought in the pet trade, while species like the king cobra (*Ophiophagus hannah*) and green tree python (*Morelia viridis*) are prized for their incessant rarity and beauty. Additionally, snake skins are used to produce high-end fashion items like bags, shoes, and belts, particularly for species like the Burmese python (*Python bivittatus*).

Snake trafficking and smuggling are extremely concerning as it depletes these snake populations in the wild and disrupts local ecosystems. Many snakes are forcibly removed from their natural habitats using inhumane methods, leading to high rates of mortality during transit due to stress, dehydration, and poor husbandry. Smuggled snakes are often hidden among legitimate goods or even hidden inside luggage, making detection methods by police very difficult. According to recent reports by organisations like TRAFFIC (the wildlife trade monitoring network) and CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora), the smuggling of snakes has become a lucrative business, with thousands of specimens trafficked every year. One reticulated python skin can fetch hundreds of dollars on the underground market, while some rare live specimens can sell for up to thousands of dollars, depending on their rarity and demand.

The smuggling data paint a grim picture for the global snake trade. In Southeast Asia, particularly in Indonesia, Malaysia and Thailand, snakes are often poached and illegally trafficked to meet global demand. In West Africa, the ball python trade, particularly from

countries including Ghana and Togo, has been singled out for unsustainable practices, with thousands of snakes exported annually under questionable legal regimes. Even in India, despite the establishment of the Wildlife Protection Act, the illegal sale of cobra venom and live cobras for religious practices and traditional medicine has wiped out several species. The United States and Europe, meanwhile, are key destinations for imports of exotic snakes, fueled in part by the pet trade and the high-end fashion industry. Smugglers often exploit legal loopholes or use false documentation to launder illegally obtained snakes into legitimate markets.

Now as snake trafficking becomes more sophisticated, modern forensic techniques have already been brought in to help tackle the illegal trade. Identity and prosecution of wildlife crimes involving snakes depend heavily upon wildlife forensics, a specialised, recently developed arm of forensic science. Techniques such as DNA barcoding, genetic fingerprinting, and molecular phylogenetics are used, for example, to identify snake species, determine their geographical origin, and trace links between confiscated specimens and poaching hotspots. Genetic data are crucial for distinguishing captive-bred from wild-caught specimens, as traffickers often falsely claim that snakes are captive bred to avoid legal restrictions.

DNA barcoding is the analysis of a specific region of an organism's DNA, usually the cytochrome c oxidase I (COI) gene (*Xia et al., 2012*), to identify the species. This approach is particularly useful in cases where traditional physical identification is difficult, such as when analyzing food products made from snakes or fragmented specimens. For example, forensic scientists can use DNA barcoding to determine whether a seized piece of skin comes from a protected species, (like the Burmese python) or an unprotected species, helping to enforce the measures under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). In addition to identifying species, population genetics can determine where smuggled snakes originated, revealing information on poaching hotspots and trafficking routes. It is critical evidence for law enforcement agencies to intercept their activities and disrupt trafficking networks.

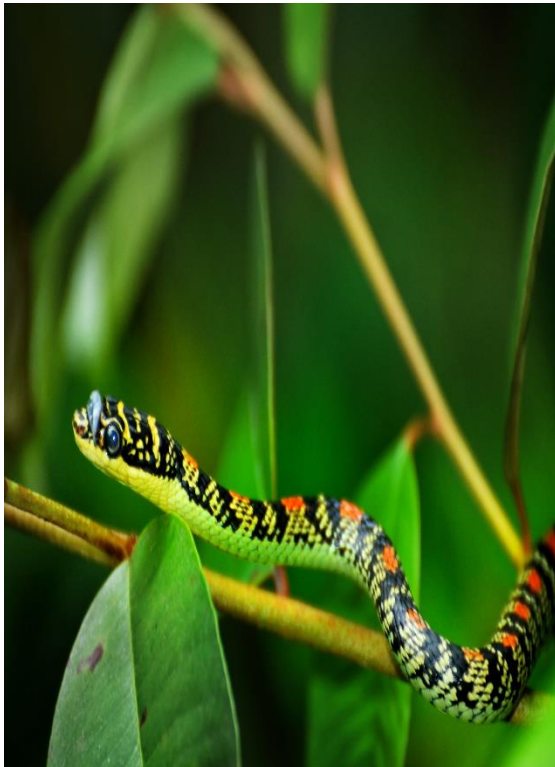
Forensic science also plays a role in oversight of legitimate trade to ensure that conservation regulations are followed. Many countries require permits and documentation for the legal trade of snakes, particularly species listed under CITES. To corroborate claims made by traders and breeders, forensic audits of trade records are performed in combination with genetic testing. In cases of discrepancies, forensic evidence may be used in a court to convict traffickers and impose penalties, thus acting as a deterrent to future crimes.

Forensic data not only helps law enforcement, but it also assists in conservation efforts by providing information about the population dynamics and genetic diversity of snake species. Understanding the genetic make-up of populations will help conservationists devise strategies to protect vital habitats, manage breeding programmes and mitigate the threat of illegal trade. Recognizing genetically unique populations of a species emphasizes the need for localized conservation strategies and genetic monitoring assesses long-term survival prospects of populations affected by poaching and habitat degradation.

2.2 Snake and lizards species chosen for this study

Chrysopelea ornata

Chrysopelea ornata is a rear-fanged (opisthoglyphous), mildly venomous colubrid snake found in South and Southeast Asia. And also being rightly called as golden tree snake, ornate flying snake, golden flying snake, this species is distributed in India (North Bengal), Bangladesh, Sri Lanka, Nepal, Myanmar, Thailand, western Malaysia, Laos, Cambodia, Vietnam, China (Hong Kong, Hainan and Yunnan) and introduced to Singapore. *Chrysopelea ornata* occurs in India from the Western Ghats through the Dangs, and Katernia Ghat in Uttar Pradesh, North Bihar, northern West Bengal and eastwards to Arunachal Pradesh. It is also found in the forests of Andaman islands. The snake is usually green with black cross-hatching and yellow or golden highlights. Though slender, its body is sturdier than other tree snakes. It has a flattened head, slender neck, a blunt snout and large eyes with round pupils.



Domain:	Eukaryota
Kingdom:	Animalia
Phylum:	Chordata
Class:	Reptilia
Order:	Squamata
Suborder:	Serpentes
Family:	Colubridae
Subfamily:	Ahaetuliinae
Genus:	Chrysopelea
Species:	C. ornata

Figure 2.3 *Chrysopelea ornata*

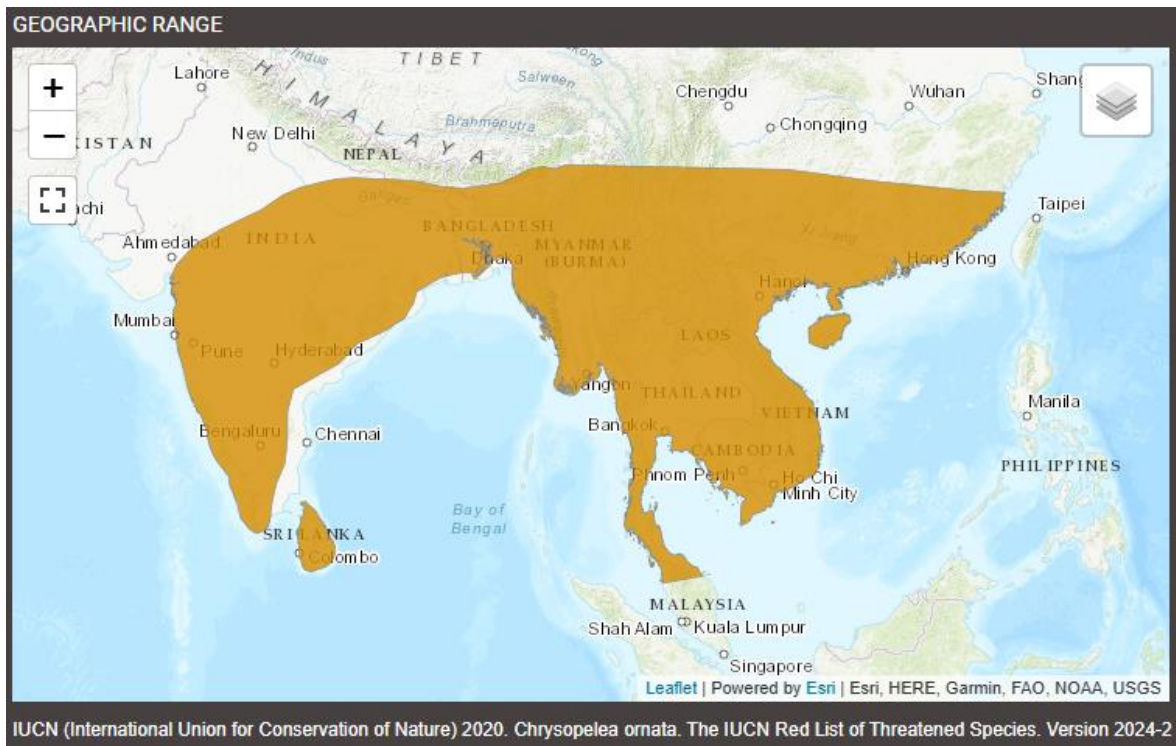


Figure 2.4 Geographical distribution of *chrysopelea ornata*

2.5 Bengal Monitor or *Varanus bengalensis*

The Bengal monitor (*Varanus bengalensis*), also known as the Indian monitor, is a monitor lizard species found extensively across the Indian subcontinent, as well as regions of Southeast Asia and West Asia. The Bengal monitor can grow up to 175 cm (69 in) in total length, with a snout-to-vent length (SVL) of 75 cm (30 in) and a tail measuring 100 cm (39 in). Usually larger than females, men can weigh up to 7.2 kg (16 lb). Heavier people can weigh this as well. The Bengal monitor is listed on Schedule I of the Wild Life (Protection) Act, 1972 and on Appendix I of CITES. Bengal Monitor Lizard *Varanus bengalensis* has most recently been assessed for *The IUCN Red List of Threatened Species* in 2018. *Varanus bengalensis* is listed as Near Threatened under criteria A2d. While all monitor lizards are now placed in a clade called the Toxicofera which are known to possess venom glands, there are no reports of the effects of venom in Bengal monitors other than a

very controversial case report of fatal renal failure as a result of envenomation from this species



Domain:	Eukaryota
Kingdom:	Animalia
Phylum:	Chordata
Class:	Reptilia
Order:	Squamata
Family:	Varanidae
Genus:	<i>Varanus</i>
Subgenus:	<i>Empagusia</i>
Species:	<i>V. bengalensis</i>

Figure no 2.6 Bengal Monitor (Dhruv Jain

https://upload.wikimedia.org/wikipedia/commons/7/72/Bengal_Monitor_Lizard.JPG)

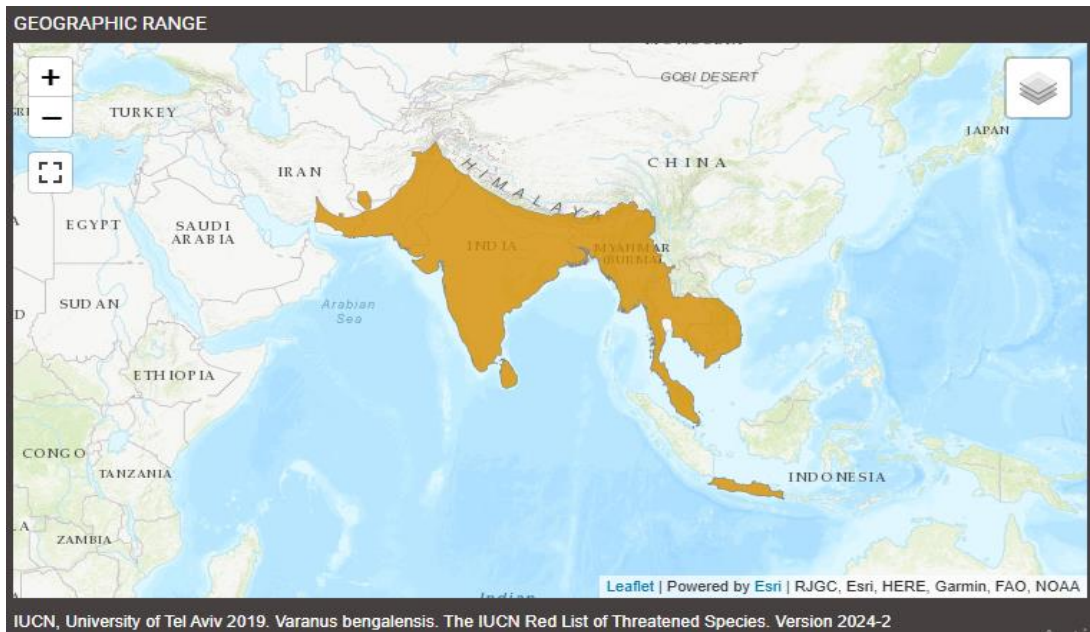


Figure no 2.7 Geographical distribution of *Varanus bengalensis*

2.8 Yellow monitor or *Varanus flavescens*

The yellow monitor (*Varanus flavescens*) or golden monitor is a monitor lizard native to South Asia. The yellow monitor is a medium-sized species, with total length, including the tail, ranging from 45 to 95 cm (18 to 37 in) and weighing up to 1.45 kg (3.2 lb). It presents slightly compressed subcorneal teeth. The length of the snout is slightly shorter than that from the front edge of the eye to the front edge of the ear, and the profile of the snout is somewhat convex. The canthus rostralis demarcation is well-defined. The yellow monitor inhabits the flood plains of the Indus, Ganges and Brahmaputra rivers in India, Pakistan, Nepal and Bangladesh. It lives in wet areas, on the edges of forests and near human settlements and agricultural land. It is a poor tree climber because of its short hindu toes. Yellow Monitor *Varanus flavescens* has most recently been assessed for *The IUCN Red List of Threatened Species* in 2019. *Varanus flavescens* is listed as Endangered under criteria A2cd.

Figure 2.9 Geographical distribution of *Varanus flavescens*

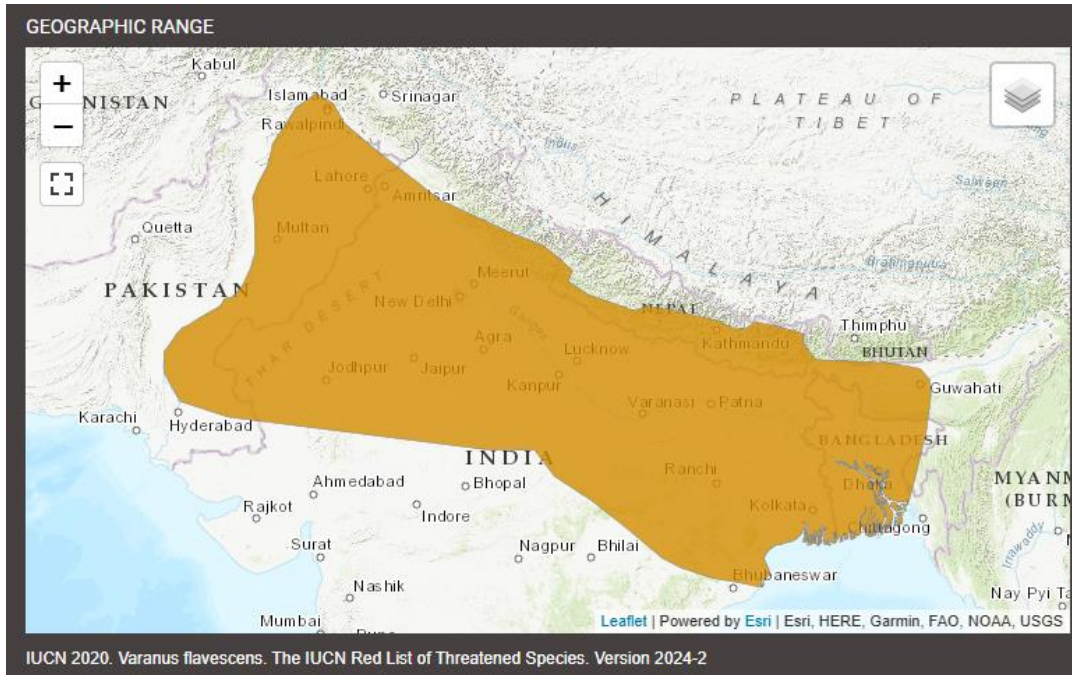


Figure 2.10 Yellow Monitor (Mohammad Tarik Hasan <https://www.inaturalist.org/photos/277760032>)

Domain:	Eukaryota
Kingdom:	Animalia
Phylum:	Chordata
Class:	Reptilia
Order:	Squamata
Family:	Varanidae
Genus:	<i>Varanus</i>
Subgenus:	<i>Empagusia</i>
Species:	<i>V. flavescens</i>

2.11 Review of literature

Forensic identification of the crime exhibits regarding snakes and lizards, usually include two types of analysis:

- i) Identification by forensically informative mitochondrial markers, and
- (ii) Development and characterization of microsatellites.

This section represents a brief overview of the studies on snakes that utilized mitochondrial and nuclear markers for species identification, especially in the legal assessment of snake species identity.

2.12 Identification using Forensically Informative Markers

Molecular genetics in forensic science is becoming more common in the identification and conservation of wildlife species including snakes and lizards. In this study, we highlight the importance of Forensically Informative Nucleotide Sequencing (FINS) and microsatellite markers in species identification, population genetics, and the enforcement of wildlife protection laws. FINS essentially differentiates species based on certain regions of the mitochondrial DNA (mtDNA). Mitochondrial genes, which are inherited through mothers and therefore evolve somewhat faster than nuclear genes, are optimal for species-level identification. Commonly amplified mtDNA regions in reptiles are cytochrome b (cyt b) (Weisburg et al., 1991) and 12S rRNA and 16S rRNA genes (Jarman et al., 2004). For instance, regarding Indian monitor lizards (*Varanus* species), the fragments cyt b, 12S rRNA and 16S rRNA genes were used to correctly identify four species from skin samples, in the first confirmed application of FINS for species authentication in illegal trade. Short Tandem Repeats (STRs), also known as microsatellite markers, are polymorphic regions of the genome comprising of repeating sequences of 2-6 base pairs of DNA. Such markers are useful for individual identification, population genetics and parentage analysis. For example, tetranucleotide microsatellite markers were developed for the Texas horned lizard (*Phrynosoma cornutum*), and non-invasive DNA sampling methods such as cloacal swabs

were employed to evaluate within-population genetic diversity. Overall, microsatellite markers have significantly aided in the investigation of population dynamics and phylogeography in snakes, as well as forensic work to trace illegal poaching and trade. Microsatellite profiles allow authorities to detect the geographic origin of confiscated specimens and the potential actors (i.e. individual animals) involved in wildlife crimes. The combination of FINS and microsatellite markers has one of the greatest implications for reptile forensics in legal scenarios, where it provides solid lines of evidence for the identification of species and individual organisms in forensic contexts. The genetic diversity observed among reptile species makes the development of species-specific primers for polymerase chain reaction (PCR) amplification complicated, while mutations in the primer-binding region can impede accurate species identification, especially in the absence of reference genetic databases for many reptile species. There is potential to adopt whole-genome approaches utilizing next-generation sequencing technologies, offering much broader genetic information for forensic purposes. Overall, forensically informative nucleotide sequences and microsatellite markers have realised a revolution in wildlife forensics, as applied to snakes and lizards, by enabling the population-sequenced individuals to which DNA remains of seizures or carcasses relate. Molecular tools ensure accuracy and reproducibility in species identification and individual distinction, acting as an effective component in addressing wildlife crime and promoting biodiversity conservation. Researchers have made incredible discoveries in areas such as whole genome sequencing (WGS), microsatellites and oral microbiota studies, greatly increasing our knowledge of genetics, evolution and health. Whole genome sequencing is a methodology capable of decoding the complete DNA sequences within an organism's genome, and thus allowing for an extensive data compilation that includes genetic variations, gene functions, and evolutionary connections. So in the field of wildlife conservation it serves to illuminate genetic diversity and population structures, particularly of endangered species, such as snakes and lizards, and wide genome sequencing (WGS) in forensic science is crucial to identify species, follow genetic lineages and monitor changes associated with diseases or traits. Microsatellites are highly polymorphic short tandem repeat (STRs) of DNA, and forensic uses of these microsatellites help to generate genetic fingerprints, as well as population studies and parentage research. Because their characters fluctuate, this

also makes them key in human genetics in paternity tests, and for the study of inherited disorders. The oral flora such as bacteria, fungus, viruses, protozoa are critical for oral and systemic health. Whereas dysbiosis or imbalances can lead to greater oral infections, such as caries and periodontitis, and increase susceptibility to diseases, such as cardiovascular disease and diabetes, healthy oral microbiome commensals hold pathogenic overgrowth in check, promote digestion, and regulate the immune system. Next-generation methods, such as whole genome sequencing (WGS) and microsatellite analysis, in this context, have contributed significant information regarding the dynamics and diversity of oral microbiota as they allow for product visualisation and functional gene identification based upon read sequencing, whereas microsatellites help assess the transmission and evolution of microbial strains within populations. These tools have wide applications in medical diagnostics, forensic science, and conservation biology, and together they help increase understanding of genetic and bacterial diversity.

Table 2.13 Published snake genome sequencing projects.

Common name	Scientific Name	Family	Notes
Boa constrictor	Boa constrictor	Boidae	GenB: PRJNA210004
Burmese python	Python bivittatus	Pythonidae	GenB: AEQU00000000
Garter snake	Thamnophis elegans	Colubridae	GenB: PRJNA210004
Garter snake	Thamnophis sirtalis	Colubridae	GenB: LFLD00000000
Corn snake	Pantherophis guttatus	Colubridae	GenB: JTLQ01000000

King cobra	<i>Ophiophagus hannah</i>	Elapidae	GenB: AZIM000000000
European adder	<i>Vipera berus berus</i>	Viperidae	Baylor College of Medicine, Human Genome Sequencing Center; GenB: JTGP000000000
Timber rattlesnake	<i>Crotalus horridus</i>	Viperidae	GenB: LVCR000000000.1
Speckled rattlesnake	<i>Crotalus mitchellii pyrrhus</i>	Viperidae	GenB: JPMF010000000
Pygmy rattlesnake ;	<i>Sistrurus miliarius</i>	Viperidae	GenB: PRJNA210004

(W. Liu et al., 2019) The complete genome sequence of the king cobra (*Ophiophagus hannah*) reports the genome-wide and coding region SSRs analysis and allows cross-species identification of microsatellite markers to further support the Chinese cobra microsatellite. A total of 411,290 SSRs were identified with a frequency of 38.03% of tetranucleotide repeats, followed by dinucleotides (23.03%), pentanucleotides (13.07%), mononucleotides (11.78%), trinucleotides (11.49%) and hexanucleotides (2.6%) in the *O. hannah* genome. 4,344 SSRs were found in (CDSs), among which tetranucleotides were top (52.79%).

(Castoe, Streicher, et al., 2012) Thousands of microsatellite loci from the venomous coralsnake (*Micrurus fulvius*), variability of select loci across populations and related species have been achieved. Next-generation DNA sequencing approaches are increasingly being used to identify microsatellite loci in non-model organisms in population genetics research. The overview emphasized that only a very limited number of studies evaluate the practical transfer from the development of biomarkers to their application among

various species and populations. Here we used 454 shotgun sequencing to evaluate the feasibility of developing microsatellite markers in coral snakes. From a single individual, a genomic shotgun library was produced, which provided ≈ 7.74 Mb in 26,831 reads that were employed to identify potentially amplifiable microsatellite loci (PALs). To verify these markers, we sampled 76 individuals across the geographic range of the species complex and tested whether the newly identified PALs were both amplifiable and polymorphic. Of the loci tested, approximately half were found to be easily amplifiable in all the individuals tested; 80% of the loci examined for variation were found to be polymorphic. This illustrates their usefulness as informative markers for studies in population genetics.

Identification of thousands of copperhead snake (*Agkistrodon contortrix*) microsatellite loci from modest amounts of 454 shotgun genome sequence was achieved by (Castoe et al., 2010). Randomly sampled a total of 26.8 Mbp (128,773 reads) of the copperhead genome (approximately 2% of the total genome of the species) using the 454 Genome Sequencer FLX next-generation sequencing platform. A total of 14,612 microsatellite loci were identified, and microsatellite loci were detected in 11.3% of the reads obtained. Out of these loci, 4,564 contained suitable flanking sequences for the design of PCR primers. This random sequencing approach was an effective and inexpensive means of identifying thousands of useful microsatellite loci from a species with no prior study.

(Rodionov et al., 2021) The study, Reliance on Multiple Microsatellite Analysis and Genome-Wide SNP Genotyping to Address Wildlife Crime: The Case of Poaching of a Caucasian tur (*Capra caucasica*) in Russian Mountain National Park, investigates the use of these techniques to solve wildlife-related crime, specifically the poaching of Caucasian tur (wild goat endemic to the Caucasian mountain range of Russia) in recent years. For the study, the goal was to identify biological evidence obtained from the crime scene and a suspect's car, and to see if the carcass was a wild Caucasian tur or a domestic goat, as the suspect claimed. Genetic forensic analysis consisted of microsatellite analysis and SNP-based genome-wide genotyping. For the genetic comparison between the carcass remains at the crime scene and the samples taken from the offender's car, fourteen microsatellite loci suggested by the International Society of Animal Genetics (ISAG) to develop a genetic

match, were used, while differences between domestic goats and the wild Caucasian tur were determined by Illumina Goat SNP50 BeadChip with over 53,000 SNPs. Microsatellite analysis conclusively supported that all this evidence belonged to a single animal, and SNP genotyping, corroborated by Principal Component Analysis (PCA) and admixture analysis, confirmed that the poached animal was indeed a Mid-Caucasian tur, thus palliating claim of the aligned to suspect. In their study, we demonstrated that a combination of microsatellite markers and genome-wide SNP genotyping in wildlife forensic applications can be a powerful tool to confirm poaching and assign the ecotype of the animal as well, encompassing both genetic and ecological genetics capability. This is the first study demonstrating use of DNA chips in wildlife forensic investigation of ungulates in Russia, establishing a standard of molecular genetic techniques for wildlife criminalistics, and emphasizing the need of harmonization of protocols for investigation and the establishment of genetic databases to improve the efficiency of wildlife law enforcement.

(Rajpoot et al., 2017) has identified forensically informative nucleotide sequence in some of the *varanus* species of lizards

(Jose et al., 2024) has amplified partial fragment of cytochrome b of Bengal monitor. In order to identify the genitals that are being smuggled.

(Hyde et al., 2016) oral microbiota of komodo dragons have isolated and examined along with skin microbes with comparison to stool microbiota to analyse the habitat.

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CHAPTER III

Integrative Whole Genome Analysis for Microsatellite Mapping and Protein Annotation in *Chrysopelea ornata*

3.1 Introduction

Wildlife forms an important part of the Earth's biodiversity, playing a crucial role in the preservation of ecological balance. The rich diversity of species, from tiny microbes to massive mammals, contributes to ecological health. However, human activity, habitat loss, illegal wildlife trade and climate change are major threats to wildlife. It is important to understand the importance of wildlife and its balance and conservation along with a degree in wildlife. Additionally, modern molecular techniques such as DNA analysis and Next-Generation Sequencing (NGS) have revolutionized the study of wildlife, allowing for species identification, population genetics, and conservation efforts. Wildlife refers to all living organisms that are non-domesticated in nature including mammals, birds, reptiles, insects and marine species. These are interacting within ecosystems maintain population control and nutrient cycling and stability. The loss of one species could reverberate through the entire ecosystem. This may lead to the overpopulation of prey species, thereby disturbing the ecological balance. The wildlife trade pertains to the commercial exchange of wild animals and their derivatives. This encompasses the commerce of exotic pets, animal derivatives (including ivory, rhinoceros horns, and pangolin scales), and live animals for consumption or traditional medicinal purposes. A considerable segment of wildlife trading is illicit and exacerbates biodiversity loss, despite some being legal and regulated. The illegal wildlife trade ranks among the most profitable black-market enterprises, frequently operated by organized crime syndicates. It results in significant repercussions, including species extinction from overexploitation. Wildlife balance denotes the equilibrium sustained in ecosystems by the interactions among various species and their environment. Anthropogenic disturbances, including deforestation, pollution, and hunting, undermine this equilibrium. Conservation initiatives seek to restore and sustain ecological balance through protected regions such as national parks and sanctuaries, habitat restoration endeavors like reforestation, anti-poaching strategies include legislation and ranger patrols, and captive breeding programs to rehabilitate endangered species. Governments and international organizations have implemented numerous laws and agreements to save animals. Examples of wildlife protection legislation include the Convention on International Trade in Endangered Species (CITES), which regulates the

international trade of endangered species; the US Endangered Species Act (ESA), which protects species at risk of extinction; India's Wild Life (Protection) Act (1972); and the European Union Wildlife Trade Regulations. Despite these regulations, challenges to enforcement remain due to corruption, a lack of resources and high market demand for wildlife products. Better enforcement, higher punishments for wildlife crime, and better cooperation internationally is crucial for effective conservation. Molecular approaches have transformed the study and conservation of wildlife. Traditional identification methods based on morphology are limited and problematic, especially in cases of incomplete remains or studied organic products such as processed animal products. This DNA-based methodology for molecular identification provide accurate identification of the species. DNA barcoding, which uses a short genetic fragment of a well-defined region of the genome, has been instrumental in detecting illegal wildlife products in trade, revealing vague or false labeling of products where species identification can make or break a product, and in species discrimination among closely related taxa.

Environmental DNA (eDNA) analysis identifies genetic material released by creatures into their surroundings, facilitating the monitoring of aquatic species without direct capture, the identification of invasive species, and the evaluation of biodiversity in a region without causing harm to organisms. DNA analysis has greatly enhanced wildlife conservation initiatives by offering insights into species genetics, population dynamics, and evolutionary lineage. It aids in comprehending genetic variety for the administration of breeding programs, forensic applications to identify and punish poachers, hybridization studies to regulate interbreeding, and species recovery initiatives to inform conservation strategies. Next-Generation Sequencing (NGS) has transformed animal genomics by facilitating swift and economical sequencing of whole genomes. Next Generation Sequencing (NGS) offers extensive understanding of animal genetics, adaptability, and evolutionary mechanisms. It is used in the field of wildlife disease surveillance to study pathogens that affect wildlife populations and track zoonotic diseases, help identify species and study evolution to resolve taxonomic uncertainties, do conservation genomics to identify genetic markers associated with benefitting from adaptations and resiliency, and monitor illegal wildlife trade through DNA metabarcoding. The forests and jungles are very crucial for wildlife

conservation which is efforts to protect endangered species of animals, plants and their habitat. However, issues like habitat loss, illegal wildlife trade and climate change continue to compromise the effectiveness of conservation efforts. Strengthening legislation regarding wildlife and enhancing enforcement must be coupled with new molecular techniques. These molecular identification and DNA-based applications provide powerful tools for species identification and population and forensic surveillance. The revolution of Next-Generation Sequencing markedly improved research capacities allowing to obtain deep information about genetics and evolution of the wildlife. Such approaches combining conservation and advanced molecular techniques have the potential to enhance biodiversity protection and mitigate threats to the wildlife. What is needed is a collective global effort from governments and scientists, conservationists and the public, to ensure a sustainable future for wildlife. Rising to the challenge of education, awareness and strengthened law enforcement will help preserve the precarious balance of our ecosystems for generations to come.

The Complete Genome Sequence of *Chrysopelea ornata*, Ornate Flying Snake

3.1.1 Introduction

Chrysopelea ornata the ornate flying snake is a beautiful and unusual species, which occurs in Southeast Asia, for its extraordinary gliding ability. It has a unique look, being a long, thin snake with a pattern of black and yellow scales. One of its most remarkable characteristics is its ability to glide from tree to tree, traveling distances of up to 100 meters. This special ability is enabled by its physical adaptations, which include an elongated ribcage and a flattened belly. By spreading out and flattening its body, the snake is able to increase its surface area, thus allowing the reptile to remain airborne longer. And when in flight, it moves up and down in an undulating motion, enabling lift and some stability. Unfortunately, this interesting species is often a target of illegal hunting and smuggling. An interesting species of living thing that always attracts the scientific community's attention is a unique flying carcass called the ornate flying snake (*Chrysopelea ornata*), which is native to Southeast Asia, thanks to its gliding ability and evolutionary adaptations. These include evolutionary adaptations both morphologically (e.g. limb reduction) and physiologically that maximize the propulsive surface a snake has available during gliding, classifying *C. ornata* as one of the few gliding snakes in the world³⁹²²³⁰. Only through recent advances in genomics have researchers been able to explore this species' genetic blueprint, culminating in the sequencing and analysis of its entire genome. Here we discuss how the whole genome of the flying tree snake, *Chrysopelea ornata*, provides great information about its evolutionary history, biological adaptations, ecological relations, and genetic basis of aerial locomotion. Whole-genome sequencing (WGS) delivers exhaustive information of the entire genome of an organism, allowing efficient identification of key genomic entities leading to the majority of biological functionalities of any living organism. In the case of *C. ornata*, genome deconvolution sheds light on the molecular underpinnings of unique life history traits, such as PSD, scale traits, metabolic traits, and ecological fitness traits. By comparing the genomes of *C. ornata* with those of other, closely-related snakes, scientists can isolate the genetic changes that have shaped the species during its long evolutionary history. Genes

in *C. ornata*, which are revealed through the genome, will be used to find microsatellites and proteins for identification.

The investigation of *C. ornata*'s genome has important implications for understanding the genetics of reptiles more broadly. Moreover, within snakes, their extreme physiological diversity has resulted in the ability to adapt and evolve (arborally, terrestrially, or aquatically) their body forms to specialize in a variety of ecosystems. One other notable feature of the *C. ornata* genome is its significance in understanding ecological adaptation and evolutionary theory. Exploring environmental interactions and life strategies, such as twohundred genotypes, in a horse species adapted for arboreal habitat. In recent years, with the rapid development of NGS technologies, whole-genome sequencing of *Chrysopelea ornata* has also become more efficient and economical. These genomic data can be analyzed with bioinformatics tools to assemble, annotate, and interpret functional elements in the genome, and, together with high-throughput sequencing, generate large-scale genomic data. Using this genomic data, the authors can then compare it to known snake genomes for the analysis of evolutionary trends, genetic divergences, and dictraits. Not to mention, the *C. ornata* genome represents not just a new player in reptilian genetics, but more data in multiple fields including evolutionary biology, biomechanics, and conservation science. Combining genomic information with ecological and behavioral studies would provide a holistic view of how this species adapted to its environment over time. The sequence data for *C. ornata* may also inspire technologies in the fields of biomimicry and robotics based on principles of gliding locomotion.

3.2 Materials and Methodology

The following materials and methodologies have been applied in this study

3.2.1 Sample collections

Biological sample of *Chrysopelea ornata* was collected from a road-killed specimen.

3.2.2 Isolation, qualitative and quantitative analysis of whole genome

DNA extraction was executed using the Qiagen DNaseasy genomic extraction kit (Qiagen Germany) as per manufacturer's protocol. A paired-end sequencing library was constructed using the Illumina TruSeq kit following the manufacturer's instructions. The DNA was fragmented, and adapters were ligated to the fragments, which were then amplified to generate sufficient material for sequencing. (Dinesh et al., 2023) The library was sequenced on an Illumina HiSeq platform in paired-end, 2×150 bp format, providing high-quality short reads. The resulting FASTQ files underwent trimming to remove adapter and primer sequences, along with low-quality regions, using Cutadapt version 2. This step ensured that only high-quality reads were used for the assembly. The trimmed sequences were then assembled into larger contigs using MaSuRCA (Maryland Super Read Cabog Assembler), a hybrid assembler designed for de novo genome assembly. MaSuRCA combines short and long-read data to improve assembly continuity, especially in repetitive regions. The final output was a high-quality genome assembly, which could be further analyzed and annotated for downstream research purposes.

3.2.3 Base Trimming

According to the quality report from FASTQC, the low quality bases from the reads were removed. Average Q30 (Phred value) score was used as a cutoff to remove low quality bases. In addition, if there any specific bias observed in base composition, those bases were also trimmed. The low quality bases were removed using custom-made Perl script.

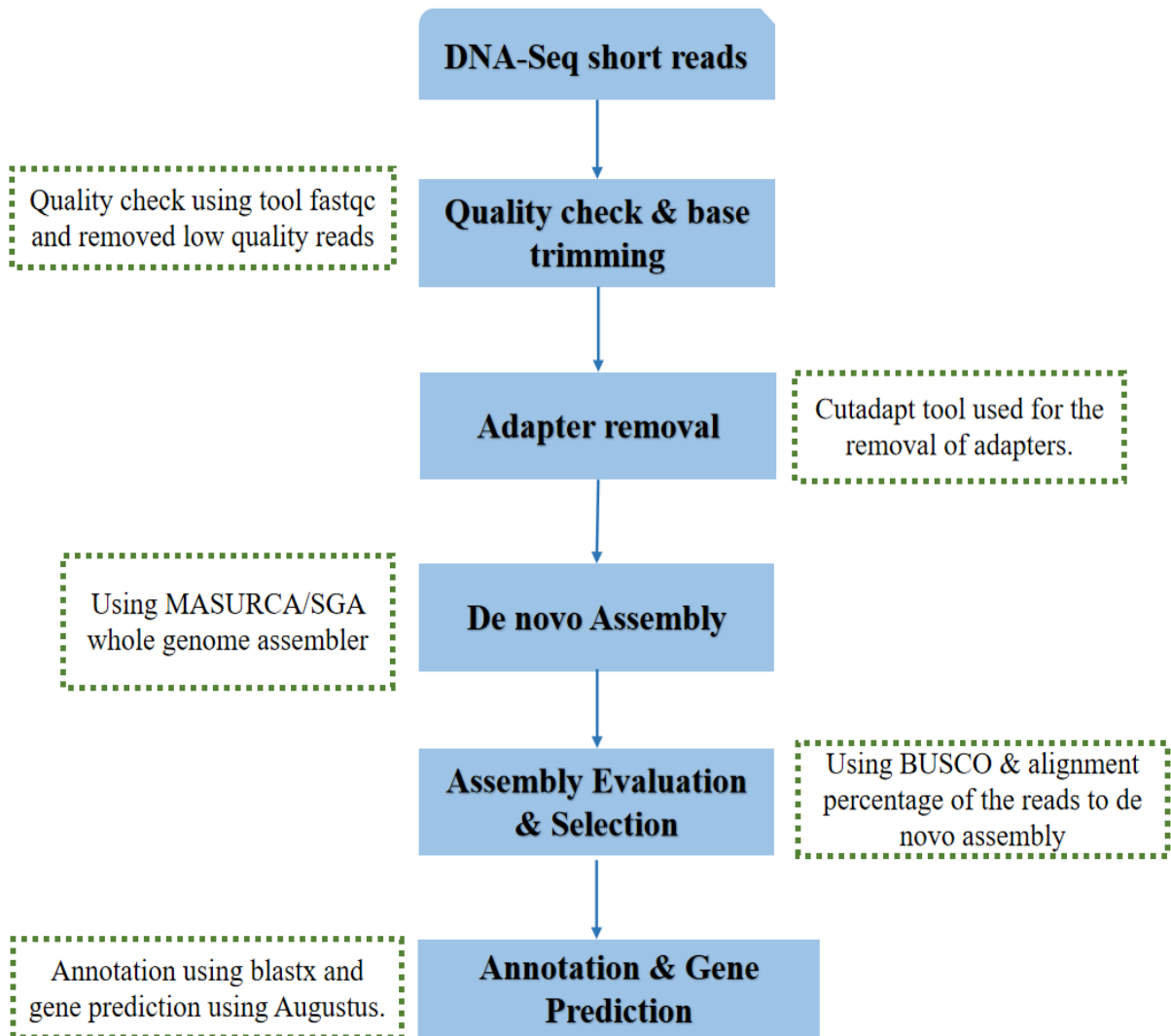


Figure 3.3 De novo WGS analysis pipeline

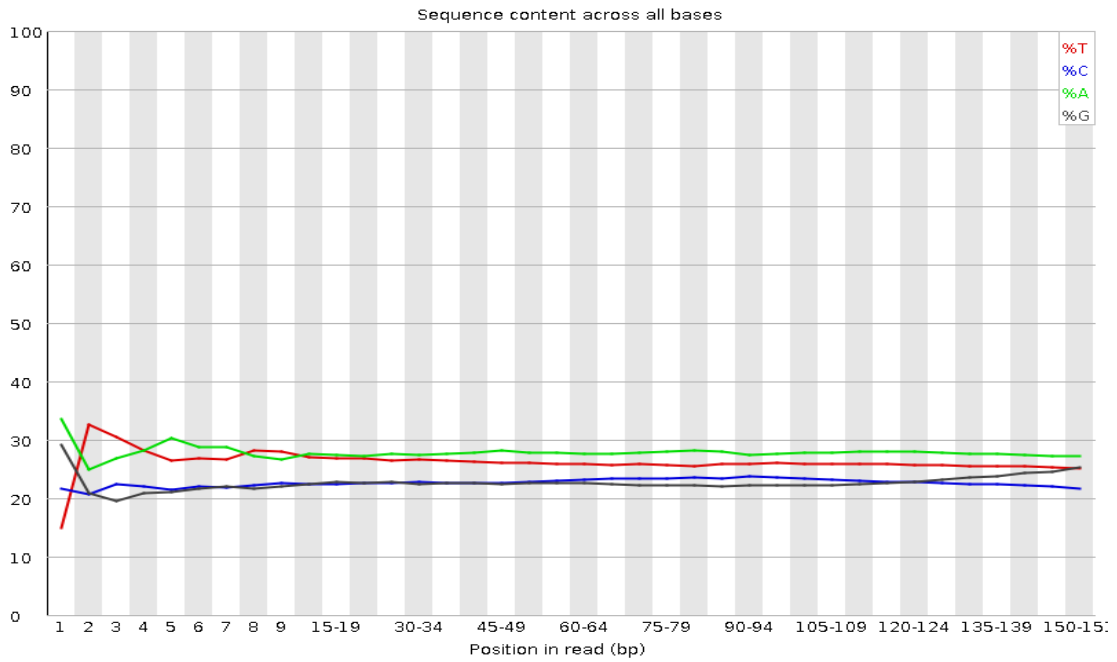


Figure 3.4 Average base composition of R1 reads of Sample

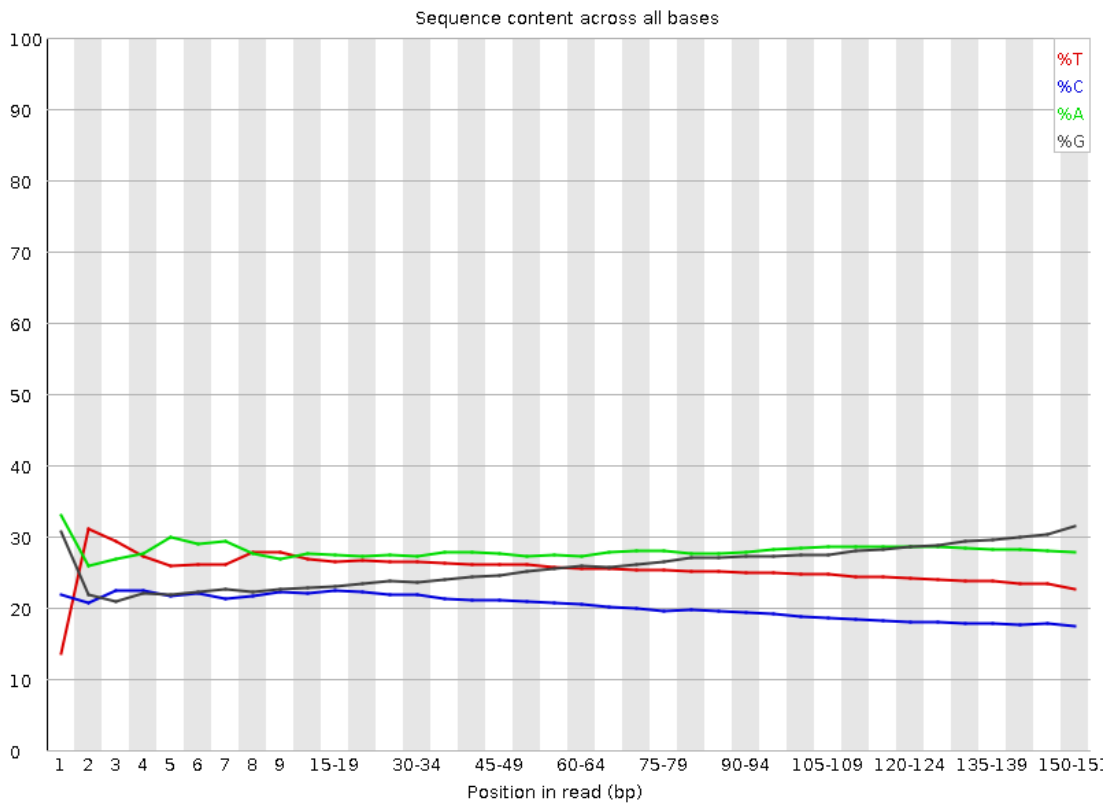


Figure 3.5 Average base composition of R2 reads of Sample

3.2.4 Adapter Removal

Another recurring problem in Illumina sequencing is adapter contamination in reads, where adapters can be present partially or completely within the read. We used the tool Cutadapt (version 2) (Martin, 2011) to remove adapter sequences from the based trimmed reads. After adapter trimming, we used a perl script to select the pre-processed reads having read length ≥ 30 bases, as the reads less than 30 bases does not make any influence in alignment process.

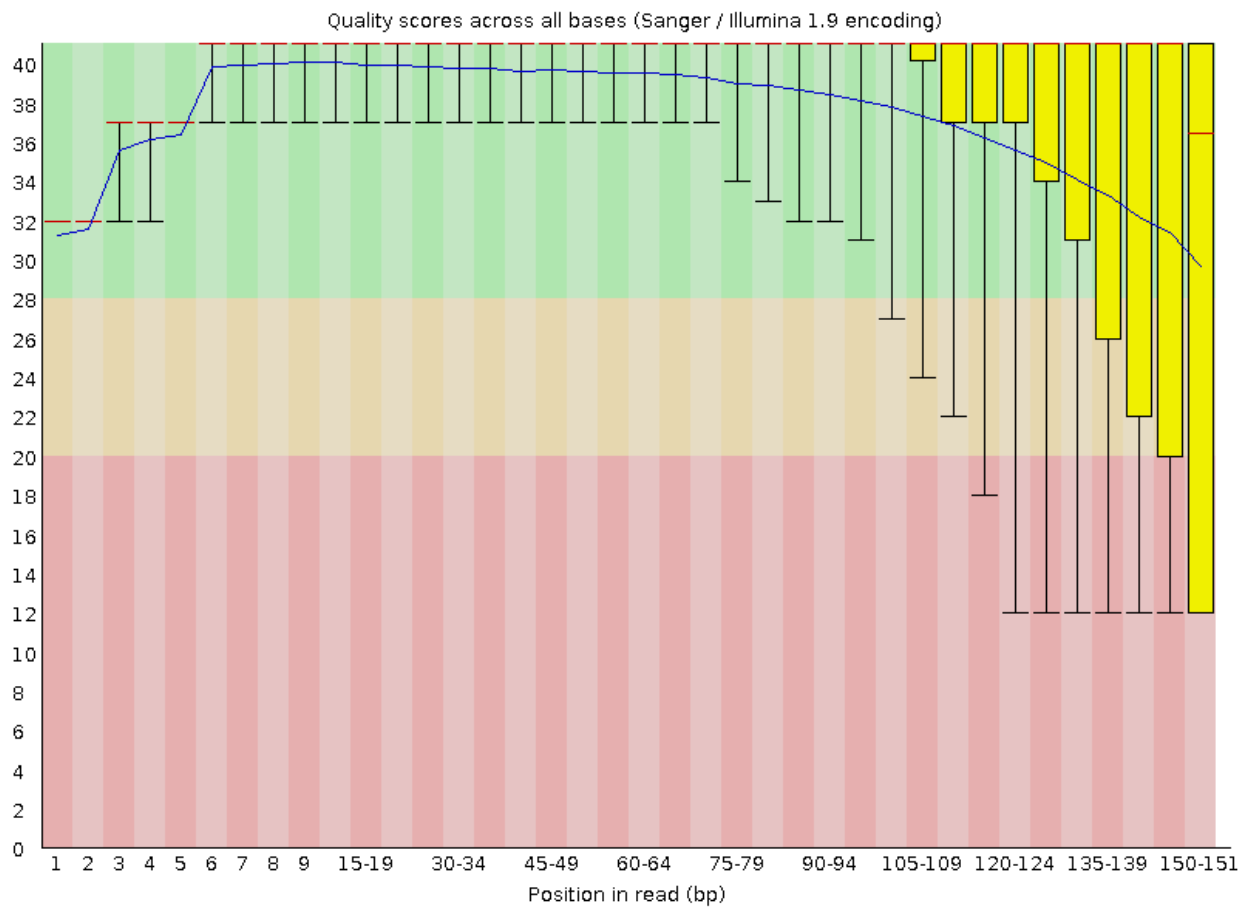


Figure 3.6 Average base quality of R1 reads of Sample

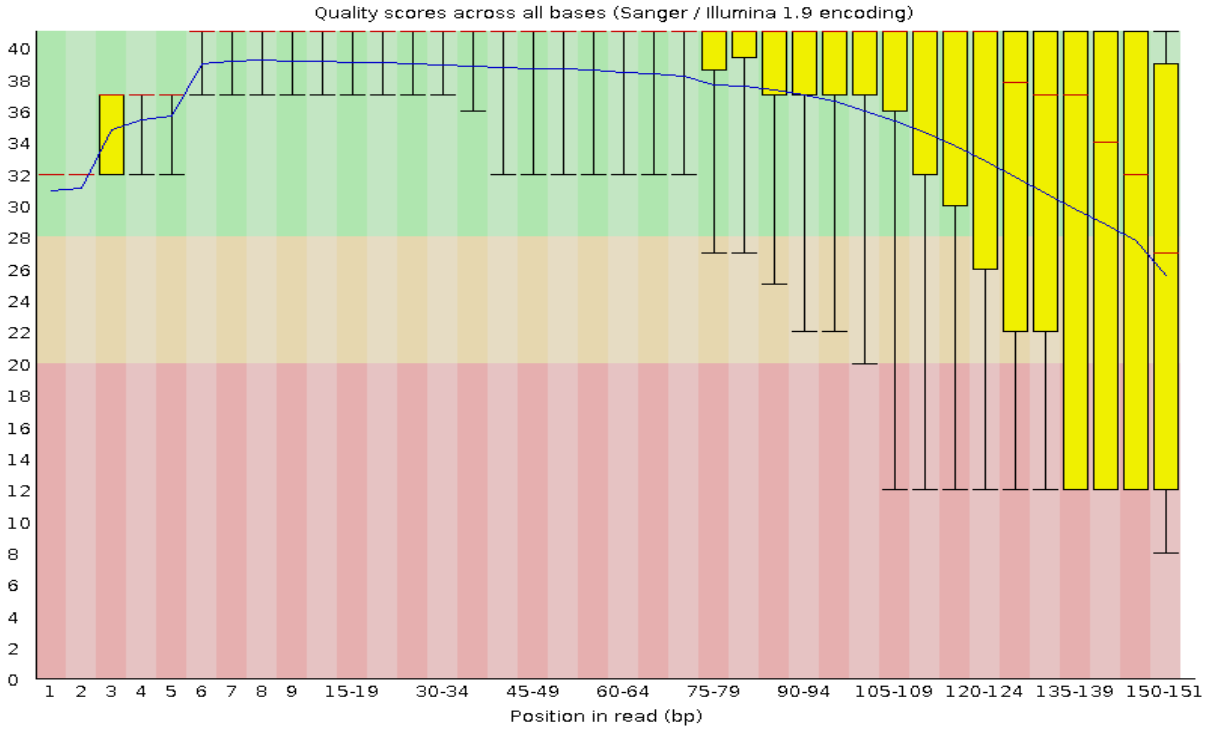


Figure 3.7 Average base quality of R2 reads of Sample

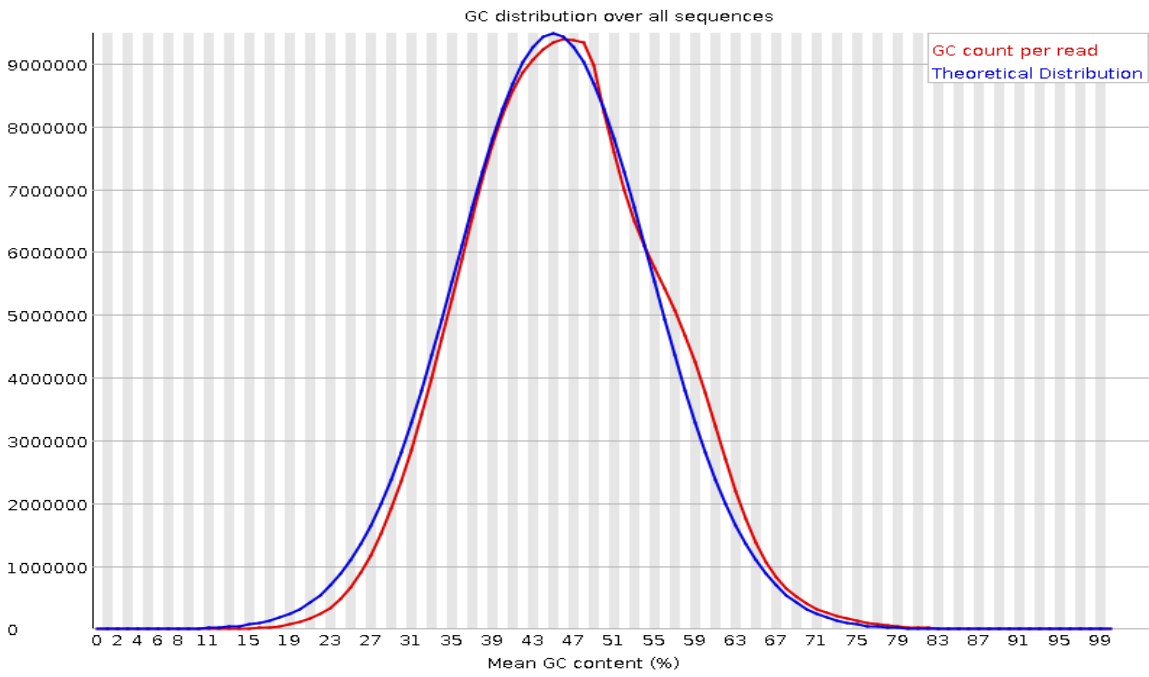


Figure 3.8 GC distribution of R1 reads of Sample

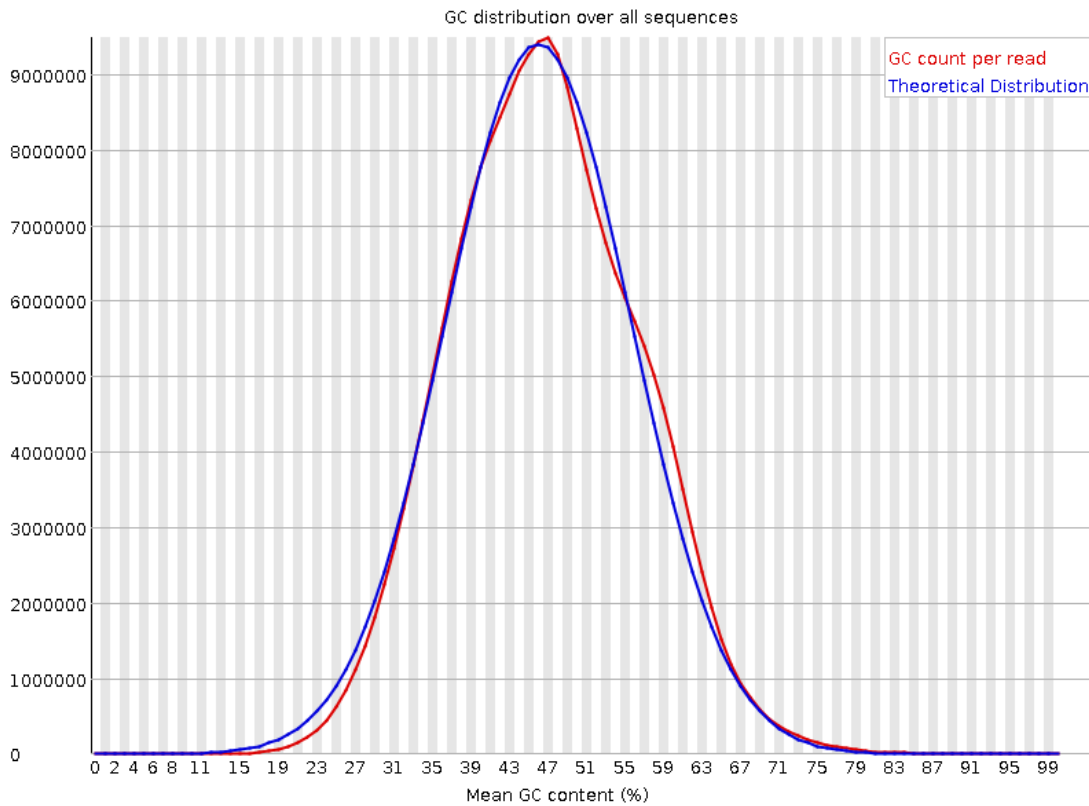


Figure 3.9 GC distribution of R2 reads of Sample

3.2.5 Alignment Evaluation & Selection

The evaluation of the level of genome completeness of the generated genome assemblies were done using BUSCO (Simão et al., 2015). In addition, the alignment percentage of the pre-processed reads to the generated de novo assemblies were done using bowtie2 (Langmead et al., 2009). The selection of the final de novo assembly is based on the bowtie mapping percentage and BUSCO results.

3.2.6 De novo Assembly

The pre-processed reads were assembled using de novo assemblers MaSuRCA (Maryland Super Read Cabog Assembler) (Zimin et al., 2013) using three K-Mer values (K=21,

K=35 and K=43), and SGA (String Graph Assembler) (Simpson & Durbin, 2012). using K-Mer values (K=21, K=35 and K=43). The MaSuRCA assembler combines the benefits of deBruijn graph and Overlap-Layout-Consensus assembly approaches. In addition, made a denovo assembly using the assembler SGA (String Graph Assembler), which is a de novo genome assembler based on the concept of string graphs. We also tried another assemblers Velvet, AByss and SOAPdenovo; which were failed because of insufficient memory. The de novo assembly statistics of MaSuRCA and SGA is given in table 3.1

Table no 3.10 De novo assembly statistics

De novo Assembly statistics				
Assembler	MaSuRCA			SGA
	KMER=43	KMER=35	KMER=21	
Total number of bases	1,358,199,940	1,332,632,993	982,755,146	1,083,357,561
Total sequences	1,136,049	2,966,658	2,781,466	1,671,136
Average	1,196	449	353	648
Largest contig	252,688	45,984	24,953	121,363
N50	1571,n=253816	809,n=453836	500,n=575022	800, n = 412344
N60	1282,n=349684	620,n=641958	398,n=795491	663, n = 561176
N70	1032,n=467745	445,n=894936	307,n=1076529	542, n = 741788
N80	801,n = 616817	282,n=1270155	224,n=1450631	433, n = 965234
N90	568,n = 816907	161,n=1904428	153,n=1981953	332,n=1250567
N100	128,n=1136049	49,n = 2966658	44,n = 2781466	200,n=1671136
N_count	1,012,155	248,672	231,964	2,357
Gaps	44,257	19,042	19,909	92

3.2.7 Data summary

Table 3.11 The fastq quality information

	Raw read information
Total Reads :	457426560
Total Paired Reads :	228,713,280
Raw Read Length :	151 x 2
Total Data :	69.07 (Gb)
Average GC% :	45.50%

3.2.8 Assembly Evaluation

We considered the MaSuRCA assembly with KMER=43 and SGA for assembly evaluation. The pre-processed reads were aligned to the selected assemblies using Bowtie2 and found **83.42%** alignment percentage with MaSuRCA assembly and **72.30%** alignment percentage with SGA assembly. The assembly evaluation using BUSCO (database: vertebrata_odb10) is currently going on. Once we obtain the BUSCO results, we can finalize our assembly.

3.2.9 Results and Data Availability

The genome sequence of *Chrysopelea ornata* is available on NCBI:

Genome:JAHWGE000000000

(<https://www.ncbi.nlm.nih.gov/nucleotide/JAHWGE000000000>)

BioProject: PRJNA742155 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA742155>)

BioSample:SAMN19929648

(<https://www.ncbi.nlm.nih.gov/biosample/SAMN19929648>).

Identification of Microsatellite Markers and Protein annotation across the Genome of ornate flying snake (*Chrysopelea ornata*)

3.12 Introduction

The global commerce of wildlife is a highly profitable sector valued at billions of dollars and serves as a significant contributor to the threat of species extinction.(Scheffers et al., 2019)The rampant illegal hunting of wildlife for the purpose of the pet trade is widespread and recognized as a major cause of the reduction in biodiversity. The global commerce in live reptiles as exotic pets is undergoing tremendous expansion; however, the impact on wild populations is still not well comprehended due to difficulties in monitoring. Although illegal poaching is causing a growing number of species to become extinct, there is a shortage of thorough research that measure the effect of capturing wild animals for the pet trade on their populations(*Scheffers et al., 2019; Tingley et al., 2017*) (*Bush et al., 2014; Harris et al., 2017*). *Chrysopelea ornata*, commonly known as the Golden tree snake, is a remarkable species of snake found in Southeast Asia. It is notable for its visually appealing features and exceptional ability to glide through the air.(Socha, 2002) Genetic markers that exhibit a high degree of variation and have the ability to accurately differentiate between individuals are commonly employed in forensic testing as a standard practice.(Butler, 2006; Jobling & Gill, 2004) Microsatellites, also known as short tandem repeats (STRs) or simple sequence repeats (SSRs) are repetitive DNA sequences consisting of tiny motifs (1-6 base pairs) that are repeated consecutively.(*Powell et al., 1995; Tautz, 1989*). Microsatellites typically consist of one or more distinct DNA patterns that range in size from one to six base pairs. These patterns vary in the frequency of repeated sequences among various species, subspecies, groups, and individuals. The approach described in this study was well suited for the development of polymorphic molecular markers used in genetic research (*Richard et al., 2008b*). The variations for repeats of these motifs, which have been associated with various alleles of the genes, result from errors that occur during DNA replication.(Burke & Bruford, 1987) The availability and examination of almost complete genome sequences from many organisms has produced useful knowledge regarding the distribution, evolution, and putative functions of microsatellites(Li et al., 2002). Animal forensic genetics is essential for revealing and presenting evidence in

criminal cases involving both farmed animals and wildlife species (Linacre, 2021). The application of microsatellites for DNA typing is the most effective and reliable technique for linking individual specimens to evidence. (Ogden et al., 2009a) Next-generation sequencing (NGS) recently represented a revolutionary technique; therefore, NGS has shared a novel perspective for the study of microsatellite markers. Its ability to rapidly generate high-throughput data also has a lot of positive aspects in promoting the development of genomics-based methods to discover new microsatellite markers in animal and plant. Time- and cost-effective as this has proven, however, this strategy only works if the initial phases are considered, which are unique to each company and thus must be developed iteratively and limited at each step. (*Capobianchi et al., 2013; Castoe et al., 2012; Cheng et al., 2007; Miller et al., 2007; Triwitayakorn et al., 2011*). This work aimed to investigate the entire *Chrysopa ornata* genome and find the microsatellites it contains.

The rapid advancement of next-generation sequencing technologies has provided exceptional opportunities for SSR marker development and genome-wide microsatellite analysis using the genome sequencing based approaches. With this methodology, the time spent in screening was greatly reduced, and the efficiency of the selection process improved. (Bergey et al., 2013). Forensic proteomics is a novel approach to forensic science, which applies proteomic technologies for forensic analysis and involves protein identification and story generation for potential usage in criminal findings. Unlike traditional forensic techniques, which often depend on DNA analysis, forensic proteomics explores the protein composition found in biological samples — hair, skin, blood or bones — to provide vital data about an individual's identity, age, state of health or cause of death. This means that proteins are more resistant to extreme environmental conditions than DNA, fulfilling a useful application in situations where DNA might be damaged. Utilizing techniques such as mass spectrometry and bioinformatics, proteomics allows for the direct analysis of the protein content from any biological sample, thus holding promise for solving cold cases, identifying remains, and understanding post-mortem modifications in detail. Forensic proteomics in its current form demonstrates the potential to supplement traditional forensic methods with the power of proteomics, and to provide greater specificity and sensitivity in the context of forensic science with potential repercussions.

3.12.1 MISA preparation:

Microsatellite identification across the whole genome was performed using the microsatellite identification tool MISA (Beier et al., 2017). In order to run microsatellite analysis with the command-line version of MISA, two input files need to be prepared: (i) a configuration file called 'MISA.Resolution': (i) a configuration file specifying the three parameters—SSR search parameters (SSR Search Params), compound SSR search parameter (Compound SSR search Params), output file type parameter (Output File type Param); and (ii) a multi-FASTA file covering a nucleotide series (i.e., sequence of DNA bases in a nucleotide) to be processed for microsatellite detection analysis. A set of criteria was used to discover optimal microsatellites. Minimum repeat counts were set as follows: for mononucleotide SSRs ≥ 12 ; dinucleotide SSRs ≥ 7 ; trinucleotide SSRs ≥ 5 ; for tetra-, penta-, and hexa nucleotide SSRs ≥ 4 . (Xu Y, 2016).

3.12.2 Results

Microsatellite distribution: 338,108 perfect microsatellites were identified in the *Chrysopelea ornata* genome assembly using MISA to scan the genome sequence. The distribution of six SSR categories in the *Chrysopelea ornata* genome as shown in Figure 1. Mononucleotide were most abundantly category accounting for 33% of total perfect SSR's followed by Tetranucleotide 24% , Dinucleotide 18% , Trinucleotide 15% , Pentanucleotide 9% and Hexanucleotide 1%.

3.12.3 Mononucleotide:

The most abundantly found repeats are A-39% , T-31%, C-15% and G-14%

3.12.4 Dinucleotides:

The most abundantly found repeats are AC, TG, GT, TC.

3.12.5. Trinucleotides:

The most abundantly found repeats are AAC, AAT, TTA, GAG, TCC.

3.12.6 Tetranucleotides:

The most abundantly found repeats are AAAT, TTTA, ATTT, AATA, AAGG, AGGA.

3.12.7 Pentanucleotides:

The most abundantly found repeats are ATTCT, AGAAT, TAGAA, GAATA, ATAGA, TATTC.

3.12.8 Hexanucleotides:

The most abundantly found repeats are CTTCTC, AGAAGG, AGGAGA, GAAGGA, GGAGAA, TCCTTC.

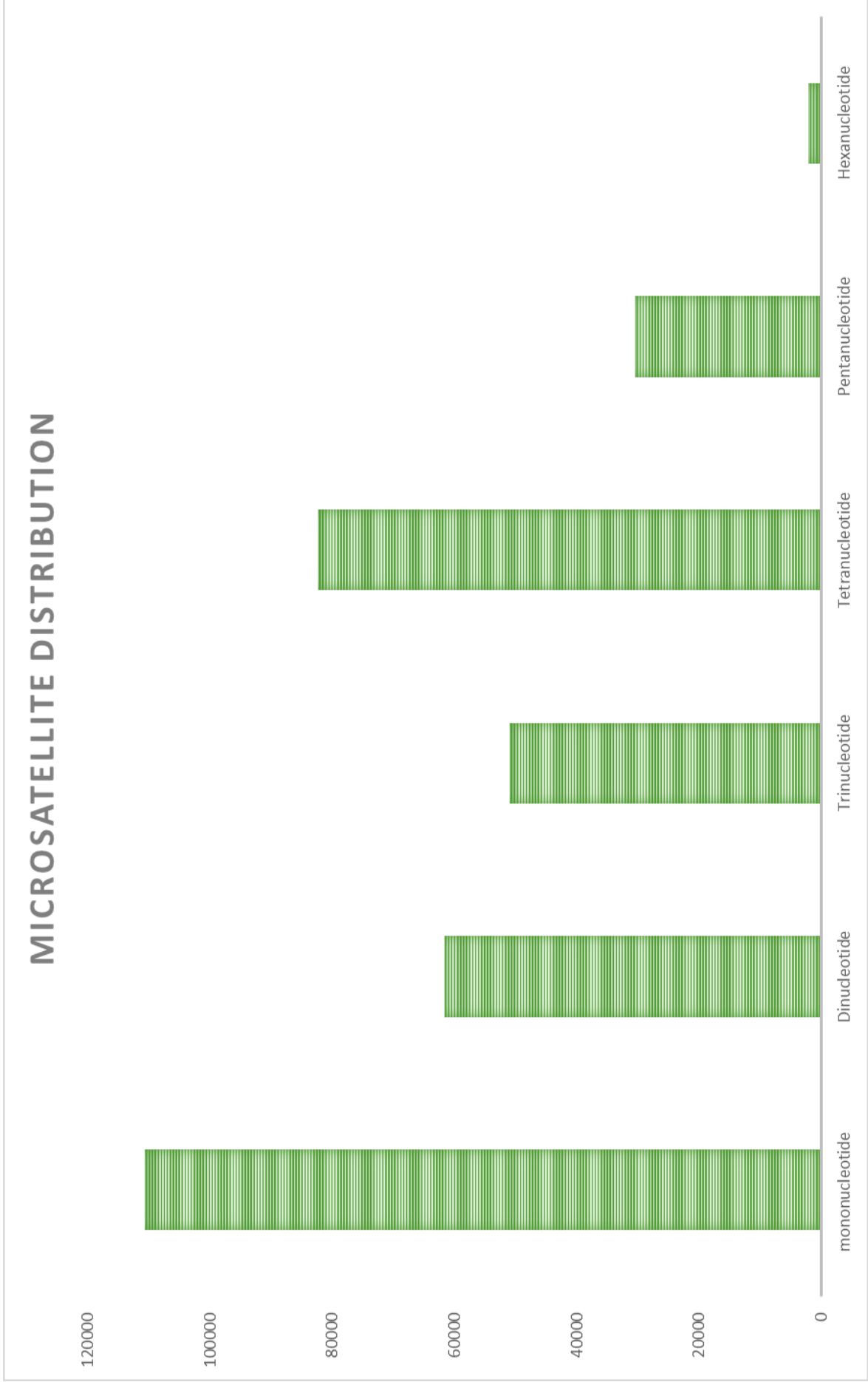


Figure 3.13 Distribution of SSR motifs in *chrysopelea ornata*

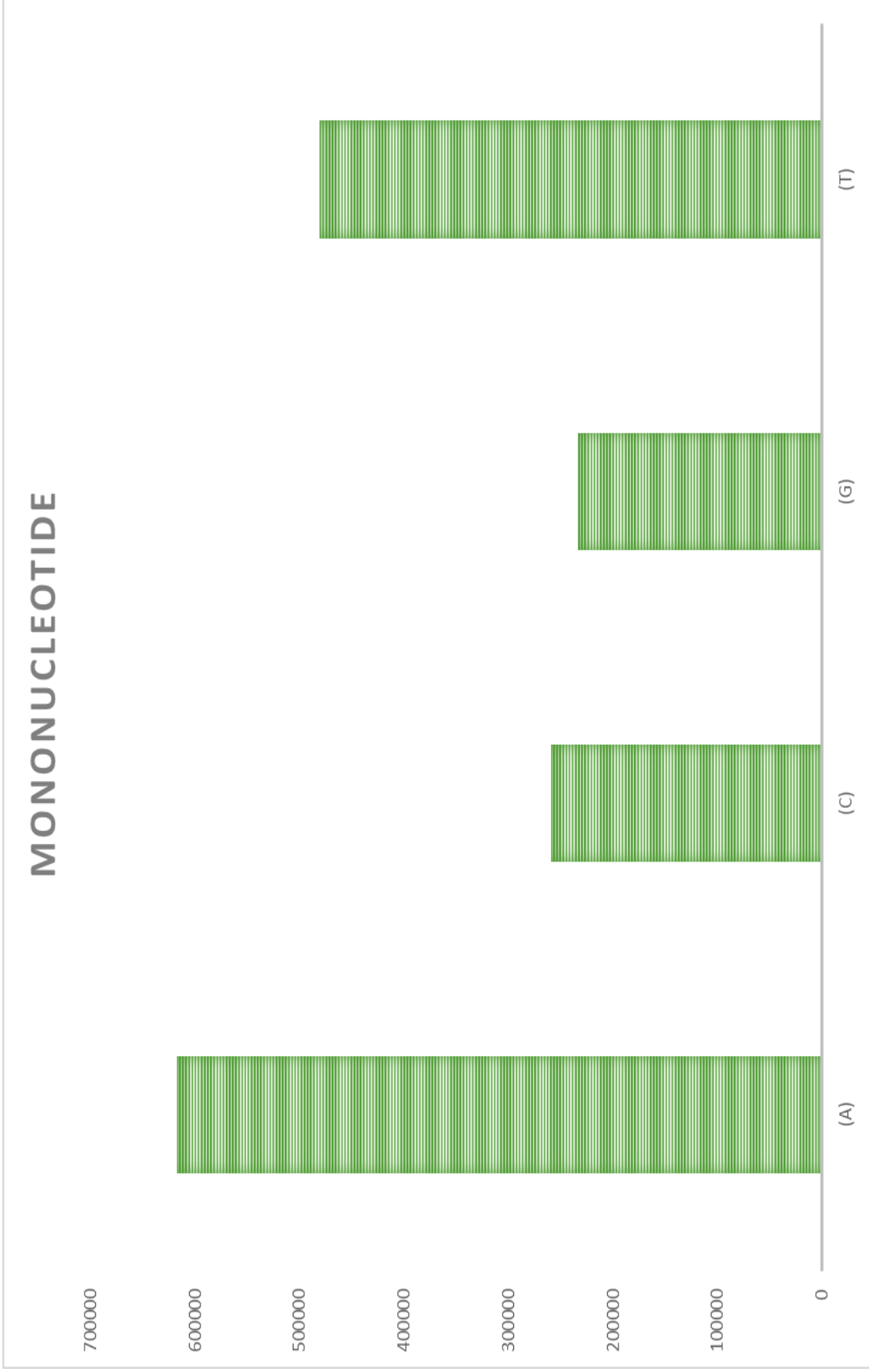


Figure 3.14 SSR distribution of Mononucleotides

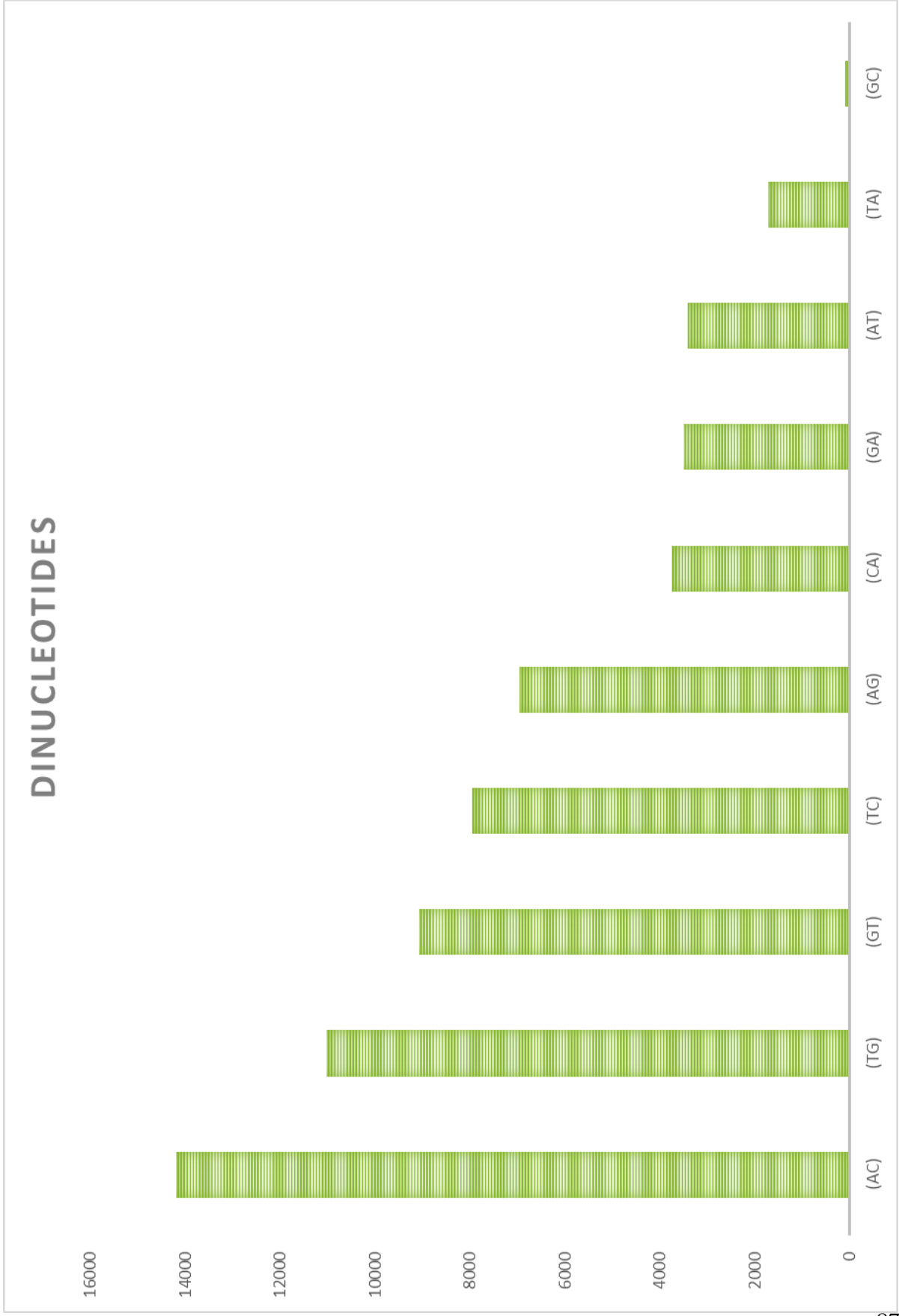


Figure 3.15 SSR distribution of dinucleotides

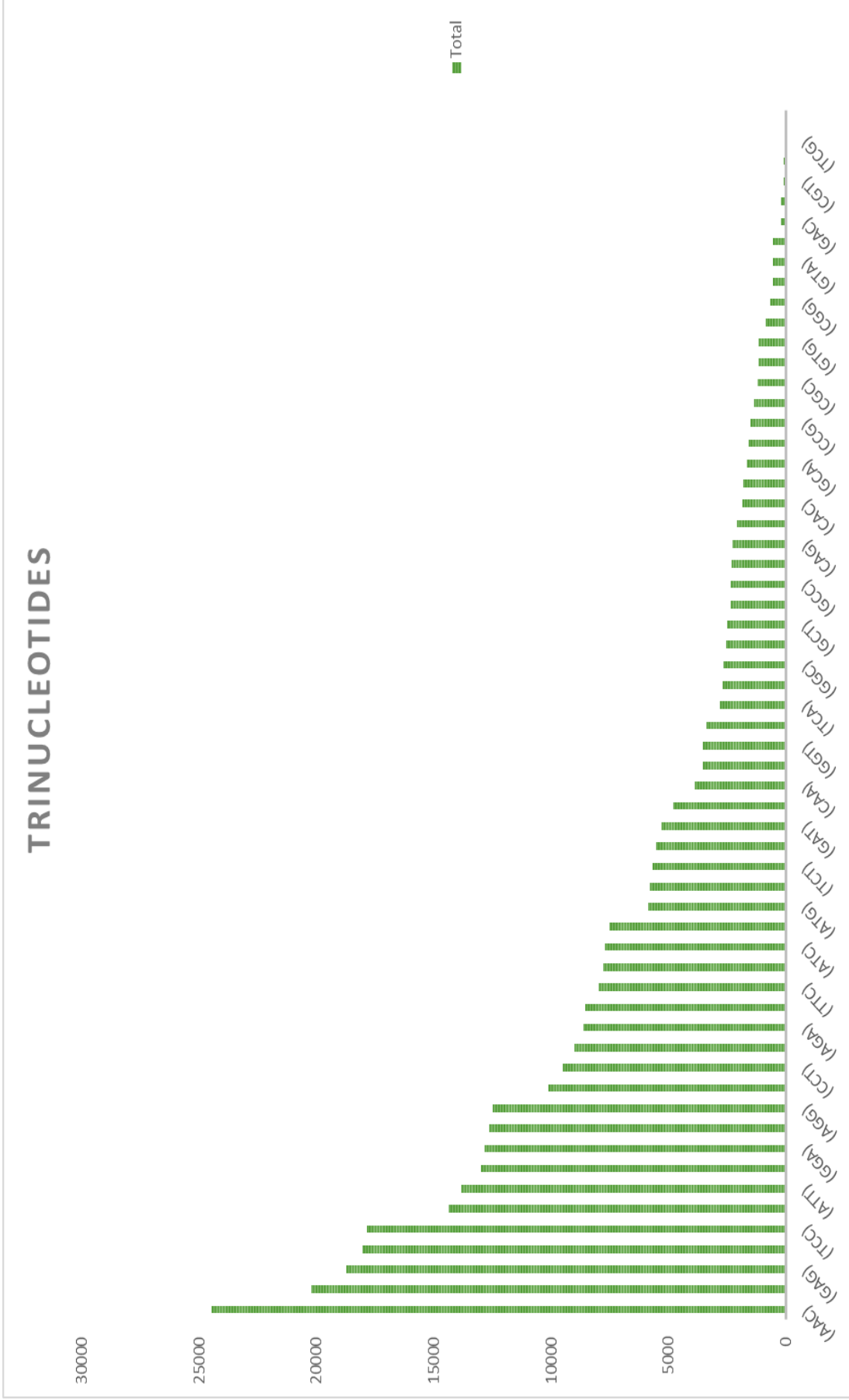


Figure 3.16 SSR distribution of trinucleotides

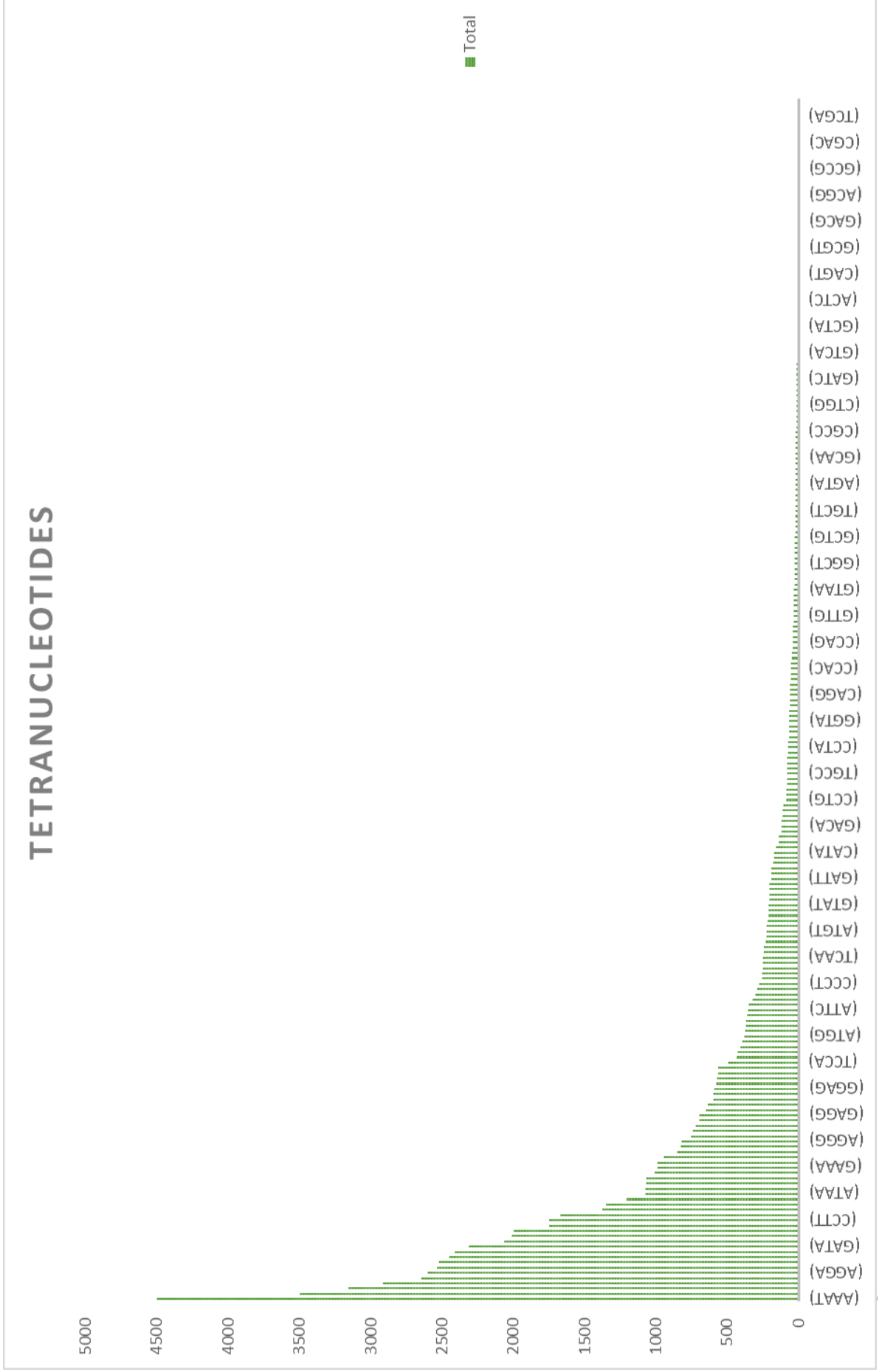


Figure 3.17 SSR distribution of tetranucleotides

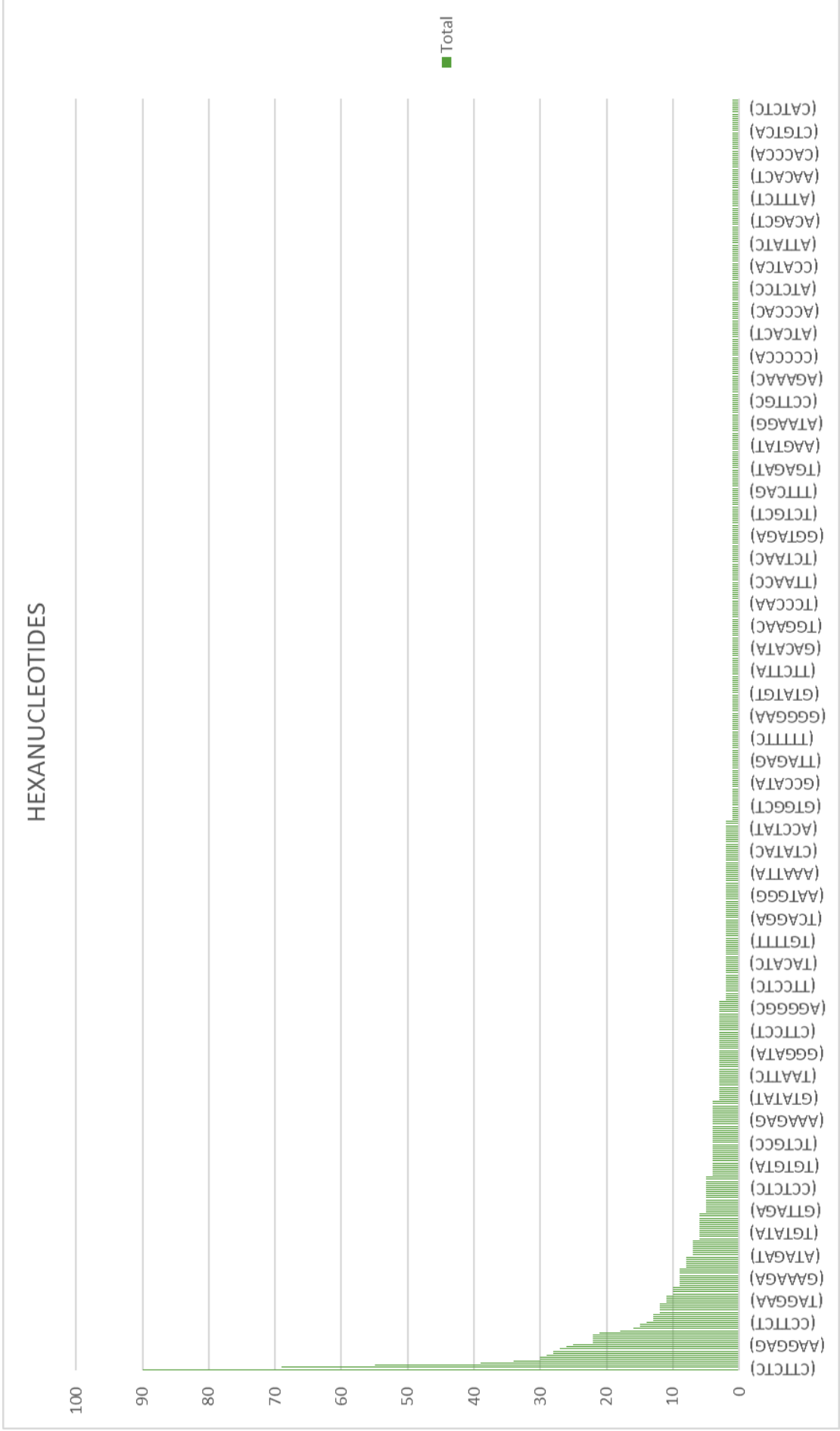


Figure 3.19 SSR distribution of Hexanucleotides

3.20 Uniprot Annotation

Uniprot (The UniProt Consortium, 2019) is an annotated protein sequence database maintained by the European Bioinformatics Institute (EBI). The database consists of protein sequence entries; each entry contains information on protein sequences, literature references, taxonomy, and annotation.

3.20.1 Results

8,510 Uniprot annotations are found.

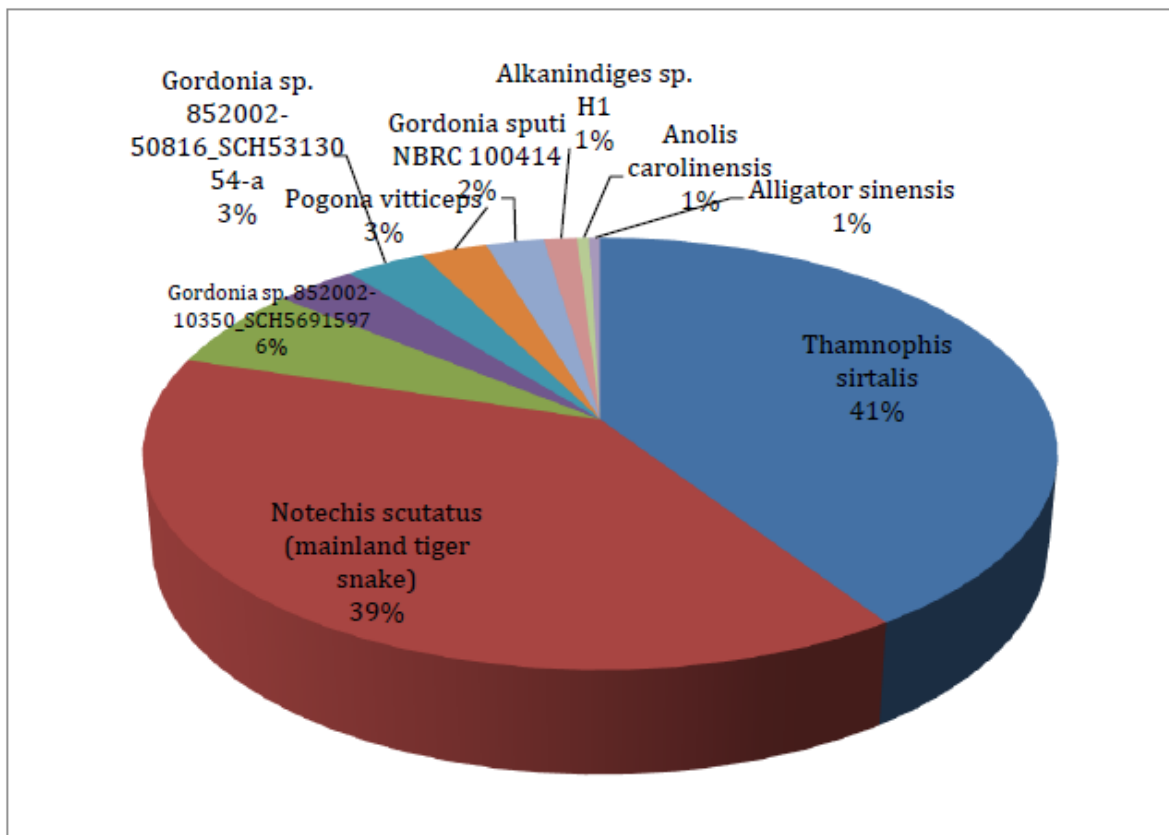


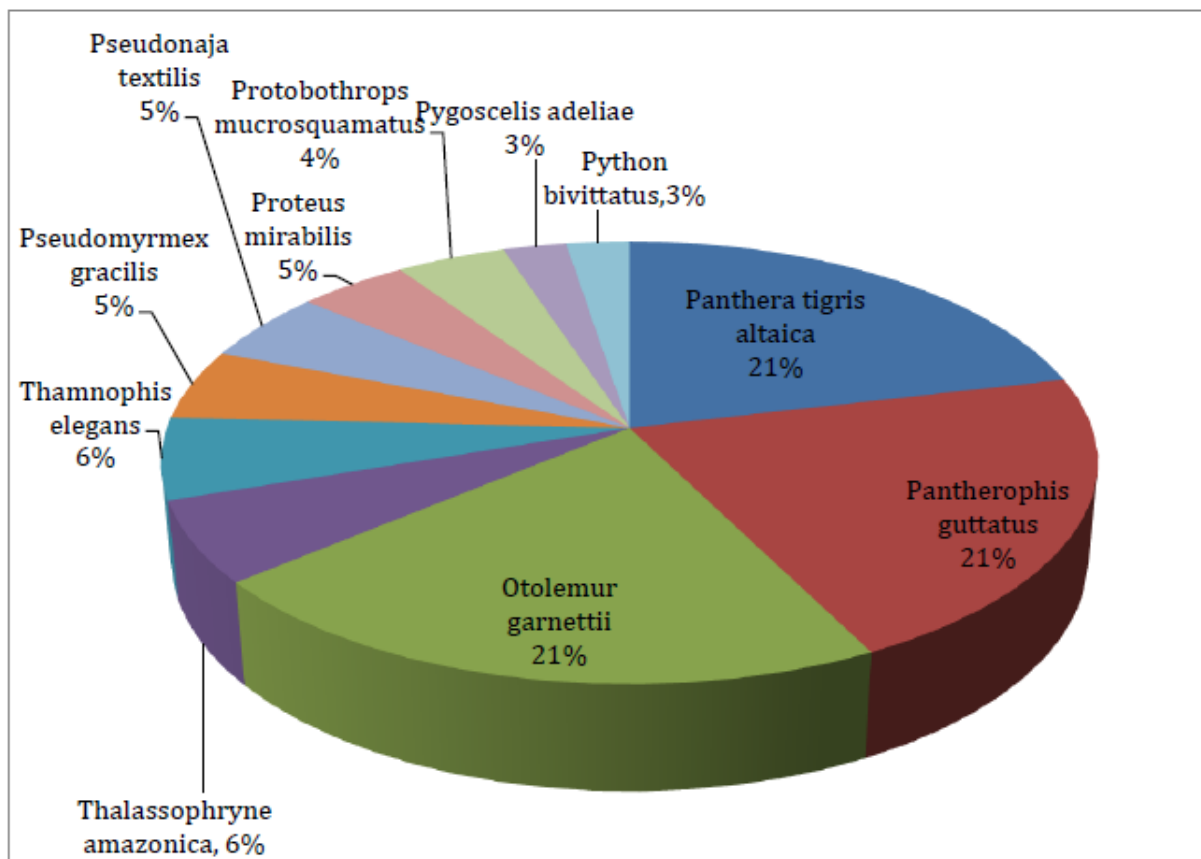
Figure 3.21 Composition of organisms from Uniprot

3.22 UniParc Annotation

UniParc (UniProt Archive) is a comprehensive and non-redundant database that contains most of the publicly available protein sequences in the world. It avoids redundancy by storing each unique sequence only once and giving it a stable and unique identifier (UPI) making it possible to identify the same protein from different source databases.

3.22.1 Results

A total 26195 Uniparc annotations are found.



3.23 Composition of organisms from Uniparc

3.24 Conclusion

We identified 338,108 perfect microsatellites (SSRs) in the *Chrysopelea ornata* genome, facilitating the understanding of the genetic structure of the gliding snake species. Mononucleotide repeats are the most prevalent types of SSRs and which represented 33%

of total SSRs is an indicator of its impact on genome structure and stability. Such high abundance could be associated with the process of genome evolution, such as replication slippage and mutational dynamics, and may account for the adaptability and ecological success of this species. The diversity of repeats was also up to 24 % for tetranucleotide repeats and 18 % for dinucleotide repeats, which could provide effective markers for advancing population genetics studies, phylogenetic analyses and conservation activities.

SSR type distribution (including mononucleotide-hexanucleotide) can provide valuable resources for further studies of genetic diversity in and between populations of *Chrysopelea ornata*. Wildlife researcher can utilize these microsatellites for gene flow, population structure, and evolutionary historical studies that are essential for species management and conservation. The overall low content of hexanucleotide repeats (1%) is consistent with patterns in other vertebrates, in which longer repeat motifs are generally under-represented.

From a genomic perspective, more than 8,510 Uniprot and 26,195 Uniparc annotations are identified for *Chrysopelea ornata*. These annotations can support the study of functional genomics, identifying correlations between individual genes and proteins and physiological traits, behaviors and their environmental adaptations. These mechanisms allow the species to glide differently than other animals, leading to biomechanical, evolutionary innovations and gathering more understanding of these differences could develop interest in evolutionary biology.

Such genomic resources can play a critical role in monitoring the genetic health of endangered species, identifying inbreeding, and informing conservation planning in the context of wildlife conservation. By identifying genetic markers connected to traits that promote disease resistance, adaptation to changes in the microenvironment, or reproductive success increases, the approach may influence targeted conservation management, especially where habitat fragmentation and climate change impacts occur. In summary, a comprehensive microsatellite analysis which establishes the basis for future studies to maintain the genetic diversity and ecological resilience of *Chrysopelea ornata*.

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Chapter IV

COMPLETE MITOCHONDRIAL GENOME: THE TOOL FOR PHYLOGENY AND NUCLEOTIDE SEQUENCE STUDY

The mitochondrial genome: a tiny part of eukaryotic cells that is critical for energy generation and metabolism. The mitochondrial genome in animals is central to enabling critical physiological processes necessary for survival and adaptation. Unlike the nuclear genome, inherited from both progenitors, mitochondrial DNA is inherited only among females, providing a unique tool for tracing ancestry and understanding evolutionary relationships between animal species. The distinct mismatch repair mechanism, the higher mutation rate, and ubiquitous presence of the mitochondrial genome in all cells make mtDNA invaluable across various scientific fields, including forensic science and phylogenetic studies. Animal mitochondrial genome consists of a circular double-stranded DNA molecule about 15,000-17,000 base pairs in length and encodes 37 genes related to mitochondrial function.

This set includes 13 oxidative phosphorylation related protein-coding genes, 22 tRNA genes, and 2 rRNA genes. The non-coding regulatory region, termed the D-loop, is the most variable part of the mitochondrial genome and often consists of the subject of genetic studies because of its high mutation rate. The chance of recovering mtDNA (from degraded or minor biological substances) is significantly raised, which in turn, makes this trait particularly advantageous in the forensic division of animal parts.

In forensic science, when nuclear DNA is insufficient or degraded, the mitochondrial genome serves as a powerful tool in identifying animals. It has applications in multiple forensic contexts, such as the identification of poached wildlife and victims of illegal animal trafficking, and the solving of historical and archaeological mysteries involving animal remains.

Mitochondrial DNA analysis is particularly useful for identifying remains that are old, decayed or otherwise unsuitable for nuclear DNA examination. Because mtDNA is maternally inherited it can be analysed between maternal relatives to enable identification; this is especially helpful in wildlife forensics to monitor poaching and illegal trade. In poaching as someone had only limited access to the lab now has only been the primary tool there to find the remains of animal where laboratory techniques couldn't sustain there Due to the high copy number of mtDNA, it is more likely that a usable sample can be derived

from the shafts of hair, bones and teeth. The mitochondrial genome is useful in various applications other than forensic biology, phylogenetic studies that contribute to understanding the evolutionary relationships of the animal kingdom. And its unique features make it such a great marker to study evolutionary history, species divergence and population genetics. Due to the relatively high mutation rate of the animal mitochondrial genome, a relatively close pair can still show some genetic differences. Researchers were able to create phylogenetic trees showing evolutionary relationships and divergence dates by comparing mtDNA sequences. This strategy has been key for resolving the evolutionary history of taxa including primates and reptiles. The idea of a molecular clock, which estimates when evolutionary events took place by measuring how many genetic changes had accrued based on the rate of changes, is largely based on mitochondrial DNA.

With fossil records and known dates of evolutionary events, scientists can use molecular clocks to accurately determine when animal species and populations diverged from each other. This strategy has yielded vital data about the timings of major evolutionary events, like when mammals diversified and the evolutionary lineage of birds. Mitochondrial genes are leading the way in terms of both exploration of genetic diversity, population structure and gene flow in animals, pioneering a new discipline within population genetics. By examining mtDNA variation both within and between groups, researchers can infer migrations, population bottlenecks and expansions. Genomic data of this kind is essential to aid in evolutionary history and genomic integrity elucidation in animal species under conservation and biodiversity considerations.

Using a relatively short and standardised segment of mtDNA for species identification the mitochondrial genome has been widely used for species identification and DNA barcoding of animals. In animals, the cytochrome c oxidase minigene (COI) has emerged as the gold standard for the barcode region. The advent of DNA barcoding transformed the field of taxonomy, allowing for the quick and reliable identification of both cryptic and morphologically similar taxa. This technique can be applied in biodiversity monitoring, environmental assessments and combatting wildlife poaching. Phylogeographic studies are commonly reliant on the use of mitochondrial genome data, a collection of additional material investigating the spatial distribution of genetic lineages. Studying mtDNA

differences in different geographical regions, scientists speculate the patterns of movement, population growth and the impact of environmental changes on distribution of species. This raises our appreciation for how historical contexts, including past glaciations or habitat fragmentation, can affect conservation of the animals we currently live with, and why.

Despite the usefulness of the mitochondrial genome in forensic science and phylogenetic reconstruction of animals, it has some drawbacks. This means mtDNA cannot distinguish even closely related individuals descended through maternal lineage. Heteroplasmy (the coexistence of multiple mtDNA variants in a single individual) further complicates interpretation. Ultimately, mtDNA analysis can still be a powerful tool in forensic and evolution studies, especially when combined with other genetic markers. The animal mitochondrial genome will open new opportunities for application in forensic and phylogenetic contexts in line with advancements in sequencing and analytical technologies for mitochondrial DNA.

The complete mitochondrial genome and phylogenetic analysis of the ornate flying snake *Chrysopelea ornata* (Shaw 1802) Colubridae

4.1 Introduction

Chrysopelea ornata (shaw1802) typically has green hues with accents of gold or yellow and black crosshatching. The body, albeit slim, is significantly larger compared to other tree snakes. The creature possesses a head that is flattened, accompanied by a neck that is restricted. Its nose is blunt, and its eyes are huge, featuring circular pupils (Daniel, 2002). Vertebrates capable of flying and gliding have developed wings that are bilaterally symmetrical, originating from their limbs, ribs, and feet. However, the snake body structure poses specific difficulties for efficient aerial movement. In order to function as a glider, a cylindrical snake without limbs must generate forces that push it into the air, sustain stability and forward speed during flight, and land safely without any harm. The *Chrysopelea* flying snakes, a tiny genus of colubrid snakes, have developed the capacity to glide with control in a forested habitat. Despite being recognized for more than a century (Daly, 1899) . In India, this species is listed on Schedule IV of the Indian Wildlife (Protection) Act, 1972. The size and trends of the *chrysopelea ornata* population are currently unknown due to a lack of sufficient data, but as of 2021, the species was listed as Least Concern on the IUCN Red List of Threatened Species.(IUCN, 2010)

4.2 Materials and Methodology

This study utilized the following materials and methodologies.

4.2.1 Sample Collection

Biological sample of *Chrysopelea ornata* was collected from a road-killed specimen.

4.2.2 Isolation and Analysis of Mitochondrial DNA

The genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Following the assessment of DNA concentration, the library was introduced to the Illumina platform for cluster creation and paired-end sequencing, facilitating the sequencing of template fragments from both termini.

The library molecules were affixed to corresponding adapter oligonucleotides on the paired-end flow cell. These adapters were explicitly engineered to enable the selective cleavage of both forward and reverse strands during sequencing.

The reverse strand used for sequencing the fragment from the other end. Determining the DNA concentration, the library was mixed into the flow cell of the Illumina platform where cluster creation (parallel amplification of the array of single molecule templates) was performed followed by paired end sequencing of the template fragments bidirectionally from both ends.

Library molecules were ligated to their associated adapter oligonucleotides on a paired-end flow cell. These adapters were custom designed for selective cleavage of forward and reverse strands during sequencing. The other reverse strand was then used for sequencing from the end of the DNA fragment. NGS was performed as followed: library preparation started with 100 ng of there isolated gDNA, which was enzymatically fragmented into smaller pieces. The fragmented DNA subsequently underwent an end-repair procedure to facilitate adapter ligation. Adapters were affixed to both terminal of the DNA fragments, incorporating sequences critical for attachment to the flow cell during sequencing. These adapters facilitated the binding of the fragments to regular Illumina sequencing primers for subsequent PCR amplification. A high-fidelity PCR amplification was conducted utilising HiFi PCR Master Mix (Takara Bio, Japan) to guarantee elevated yields. The resultant libraries had an average fragment size of 324 bp and were sequenced using the Illumina platform (Illumina, San Diego, USA) utilising 2x150 bp paired-end chemistry, generating roughly 1.5 to 2 GB of sequencing data. The raw data was processed using Trimmomatic v0.38 to remove adapter sequences and low-quality reads. (Bolger et al., 2014) The sequence was assembled into contigs using MetaSPAdes software (Nurk et al., 2017). Subsequently, gene annotation was performed using MitoZ v3.6 (Meng et al., 2019).

4.2.3 Phylogenetic analysis

The image represents a phylogenetic tree constructed from mitochondrial genome sequences of various snake species, likely using a maximum likelihood or neighbor-joining method to depict evolutionary relationships. Branch lengths reflect genetic distances, with shorter branches indicating closer relationships, while bootstrap values at each node provide statistical support for these relationships. High bootstrap values, such as 100, indicate strong confidence in specific evolutionary groupings, while lower values, like 42, suggest weaker support. Notably, *Ptyas dhumnades* (NC_028049.1 and KR812112.1) cluster together with 100% bootstrap support, highlighting their close genetic relationship or potential representation of the same species from different samples. *Chrysopelea ornata* (CMD60730.1) is shown as closely related to *Ptyas dhumnades*, suggesting a shared evolutionary lineage. Similarly, *Dinodon semicarinatus* (NC_001945.1) and *Boiga kraepelini* (NC_070009.1) form a well-supported cluster with a 98% bootstrap value, while *Elaphe davidi* (NC_025643.1) and *Elaphe quatuorlineata* (MK334307.1) group together with strong support (100%), indicating their close genetic similarity. A moderately supported cluster (96%) is observed between *Gonyosoma frenatum* (NC_057467.1) and *Pituophis catenifer sayi* (KU833245.2), suggesting some evolutionary relatedness. However, the branch grouping *Boiga kraepelini* and *Gonyosoma frenatum* shows a lower bootstrap value of 42, reflecting less confidence in this specific relationship. Overall, the tree illustrates how mitochondrial genome data can reveal evolutionary patterns among snake species, with high bootstrap values confirming robust relationships and weaker values indicating areas requiring further investigation. The scale bar (0.020) represents genetic distances, aiding in the interpretation of divergence levels between species.

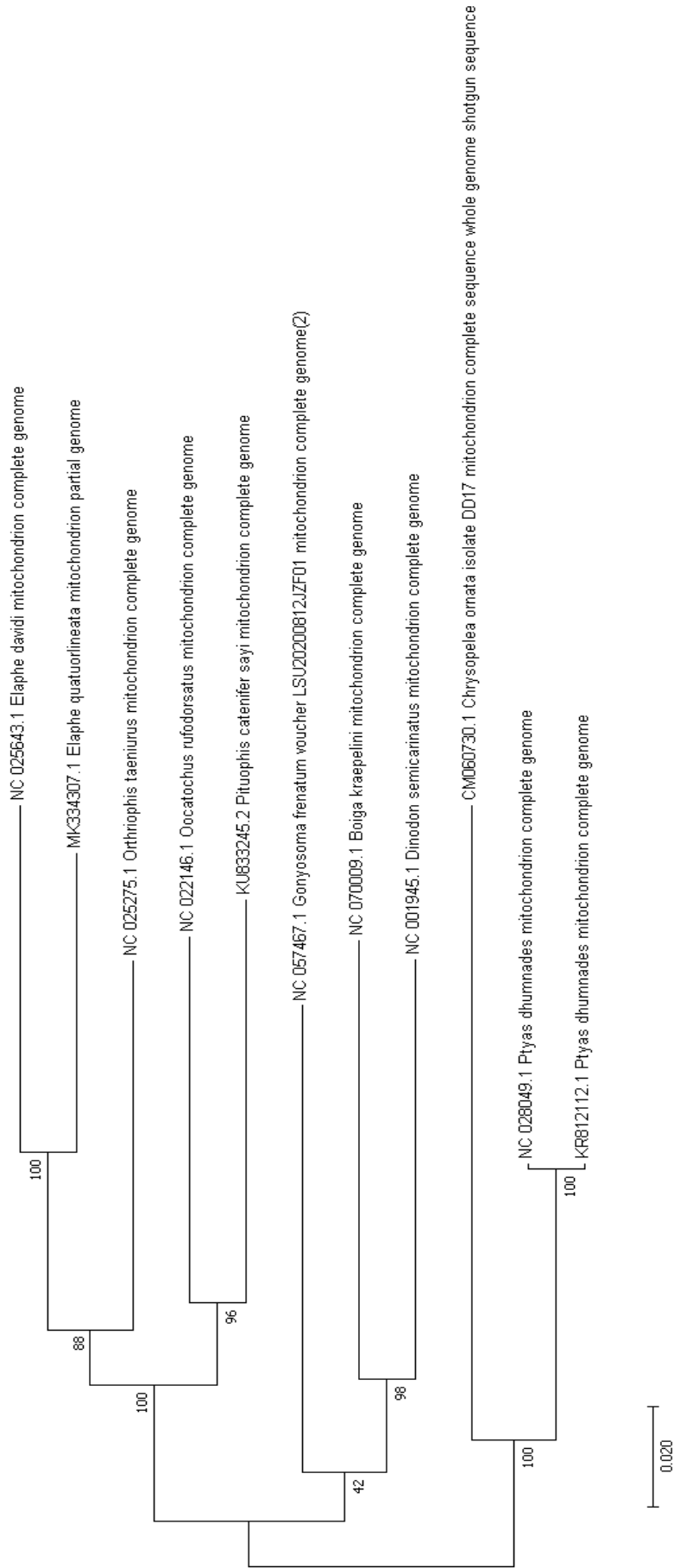


Figure 4.4 Phylogenetic tree was constructed through MEGA 7.0 (Kumar et al. 2016) using the Maximum Likelihood method based on the Tamura-Nei model involving 10 complete mitochondrial genome sequences of Colubridae along with their accession numbers. All positions, containing gaps and missing data were eliminated. Bootstrap values are shown for each nodes based on 1000 replicates

4.2.4 Results:

The complete mitochondrial genome sequencing of *Chrysopelea ornata* is 17,252 base pairs long. The base composition of this genome is as follows: 36% adenine (A), 27% thymine (T), 12% guanine (G), and 25% cytosine (C). This genome has a GC content of 38%. The mitogenome of *Chrysopelea ornata* contains 37 genes, including 13 protein-coding genes, 22 tRNA genes, two rRNA genes, and two non-coding regions. We identified nine genes, including eight transfer RNA (tRNA) genes and NAD6. The phylogenetic results, based on the complete mitochondrial genome of *Chrysopelea ornata*, show a strong relationship within the Colubridae family.

4.2.5 Conclusion

The complete mitochondrial genome analysis of *Chrysopelea ornata* sheds lights on the evolutionary biology, genetic diversity, and conservation of this unique gliding snake species. Through analysis of the mitogenome, researchers are able to provide insights into the fossil record of phylogenetic relationships (or lack thereof) between *C. ornata* and its closely related snake counterparts, identifying their familial differences, divergence, and pattern of evolution. The high mutation rate and maternal inheritance of the mitochondrial genome provide an effective system for tracing lineage and population structure that are necessary to identify distinct populations and assess their genetic health. Data on population structure, reproductive status, and not least, the role of genetic variation are especially important against the backdrop of habitat fragmentation and environmental changes that puts, at least some, species under impending threat. In addition, mitogenomic datasets can provide important insights into forensic studies, including in the context of regulating the illegal wildlife trade, as well as identifying seized material. The findings contribute to our understanding of the biology and evolutionary pathways of *Chrysopelea ornata* and have implications for conservation as well as shedding light on the diversity present within its habitats.

4.2.6 References

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CHAPTER V

ORAL MICROBIOTA OF VARANUS LIZARDS

Forensic science is continually diminishing because to the ongoing integration of novel biological and microbiological methodologies. The examination of oral microbiota from nonhuman species has developed into a field of forensic microbiology, particularly focussing on reptiles like *Varanus* lizards, which are of significant importance. Monitor lizards inhabit various ecosystems and are distinguished by their formidable bite force and opportunistic feeding habits. The unique microbiome of *Varanus* species can be utilised forensically in bite mark analysis, postmortem scavenging investigations, and bacterial source tracking. Analysing the microbial communities in the oral cavities of these reptiles can function as a forensic instrument to identify bite marks and ascertain if an animal was killed or scavenged, hence inferring geographic location through microbial markers.

Varanus oral microbiota contains potentially pathogenic bacteria, which make it a useful forensic marker in the diagnosis of bite-related injuries and deaths. Identification of certain strains of bacteria, traced from bite wounds to or suspected inhabitation of a particular reptile, can act as forensic evidence for animal attack cases or illegal wildlife trade, while their relative stability and resilience, will allow for a more reliable indicator of the oral microbiota of reptiles under a range of environmental conditions.

Because of the species-specificity of reptiles as well as the sequencing of next-generation sequencing, forensic scientists can create microbial signatures that will assist traditional bite mark analysis or be species-specific or even individual-specific. In forensic investigations of wildlife-human interactions, or postmortem scavenging, the timing of injuries, whether they were inflicted ante- or post-mortem, is critical information. All *Varanus* lizards are well-known scavengers, eating carcasses in the wild and in urban settings. Scavenging lizards do not have similar predatory bite wound specializations to oral microbiota, and they are significantly exposed to both decomposing tissue as well as environmental contaminants. Profiling microbial communities from bite wounds allows forensic scientists to determine when a person interacted with a *Varanus* lizard; antemortem (before death) or postmortem (after death) interactions can be distinguished. A high relative abundance of putrefactive bacteria (*Clostridium* and *Bacteroidetes*) in a bite wound indicates that the lizard ate the corpse postmortem. By contrast, the finding of common oral commensal bacteria with absence of putrefactive species suggests

antemortem biting. *Varanus* lizards across habitats, diets, and geographic locations have unique microbial compositions. Such variability enables forensic scientists to exploit oral microbiota for microbial source tracking (MST), which links the source of biological evidence to the specific microbial community it harbors. In illegal reptile trade, animal cruelty, or poaching cases, forensic scientists can analyze oral microbiota from confiscated lizards or bite wounds and use it to trace the origin of the lizard.” This ultimately becomes a method of strengthening wildlife forensic investigations and supporting conservation efforts that explores illegal trafficked animals back to its origin of habitat. An additional forensic feature of *Varanus* oral microbiota is its zoonotic disease transmission potential. Several *Varanus* species are carriers of pathogenic bacteria responsible for serious infections in animals and humans. Microbial profiling can be used in forensic investigations where reptiles play a role in the transmission of infection, tracing the source of the infection to the index lizard or environment.

"Oral Microbiota of *Varanus* Lizards: Diversity, and Forensic Applications"

5.1 Introduction

More than 50-year-old law the Indian Wildlife (Protection) Act of 1972 provides for an explicit ban on trade of monitor lizard species as these reptiles fall under Schedule-I category of the law. Additionally, the Bengal monitor lizard and the Yellow monitor lizard (genus: *Varanus*) are listed on Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). are heavily trafficked, mainly for their meat, which is viewed as a delicacy and medicinal property. Research has shown products derived from monitor lizards have been used to treat a variety of health complaints, including asthma, hemorrhoids, rheumatism and arthritis. Several pathogenic and virulent species have been detected in monitor lizard saliva with injuries inflicted by this species often associated with sepsis and eventual bacteremia. However, no fatalities have been documented in the USA, where monitor lizards are kept as pets, due to these reptiles; the recent discovery of venom in monitor lizards may be the exception. The intricate network of tissues and organs that make up the mouth cavity offers a very diverse home for microorganisms, the most common of which are bacteria. (Kilian, 2018; M. Lu et al., 2019). Among terrestrial vertebrates, reptiles are a quite significant group that although their oral and intestinal flora is still mostly unknown, particularly significantly when weighed against other taxa of vertebrates (Colston & Jackson, 2016) Many gram-positive, gram-negative and anaerobic bacterial species could result in a secondary infection and anaerobic bacteria. Anaerobic bacteria can also produce gas gangrene following infection of the bite wound; gram-positive bacteria can cause symptoms infection, including an abscess, cellulitis, meningitis, sepsis, and urinary tract infections; and Gram-negative bacteria can cause gastroenteritis, sepsis, respiratory infection, meningitis, several infection symptoms, diarrhea, fever and soft tissue infection (Artavia-León et al., 2017; Chen et al., 2011; Gilmore & Ferretti, 2003; Goldstein et al., 1981; Jho et al., 2011; Lam et al., 2011; P.-Y. Liu et al., 2017; Shaikh et al., 2017; Theakston et al., 1990)

5.2 Sample Collection

The biological samples of *Varanus bengalensis* and *varanus flavescens* tagged as 17D and 18D respectively are obtained from Jungle Mahal Zoological Park Jhargram , West Bengal.

5.2.1 Extraction of Genomic DNA

Mitochondrial DNA was extracted using the Qiagen QIAamp DNA Mini Kit, following the manufacturer's protocol.

5.2.2 Library Preparation

The paired-end sequencing library was generated using QIAseq FX DNA Library Kit beginning with 100 ng of DNA. The DNA was subsequently enzyme digested into smaller fragments, then end-repaired and 'A' added to the 3' ends in preparation for adapter ligation. Then platform-specific adapters were ligated onto both ends of the DNA fragments. These adapters include sequences required for binding the dual-barcoded libraries to a flow cell during sequencing, and allow for the PCR amplification of reasoned adapter-ligated fragments and the use of typical Illumina sequencing primers. A high-fidelity amplification was performed with HiFi PCR Master Mix to maximize Yields from the small amount of starting material.

5.2.3 Quantity and quality check (QC) of library on Bioanalyzer:

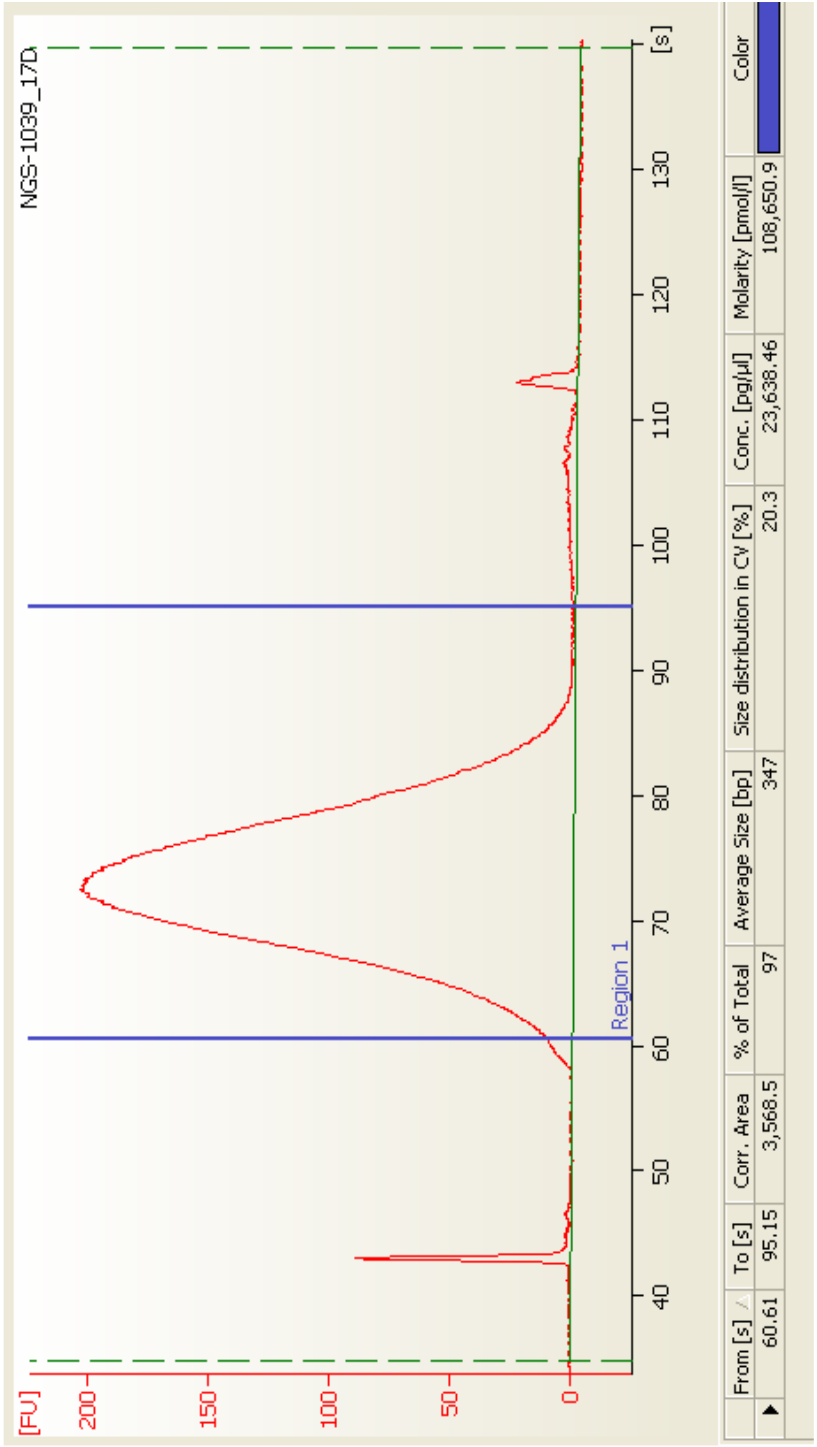
The amplified library was analyzed in Bioanalyzer 2100 (Agilent Technologies) using High Sensitivity (HS) DNA chip as per manufacturer's instructions.

5.2.4 Cluster Generation and Sequencing:

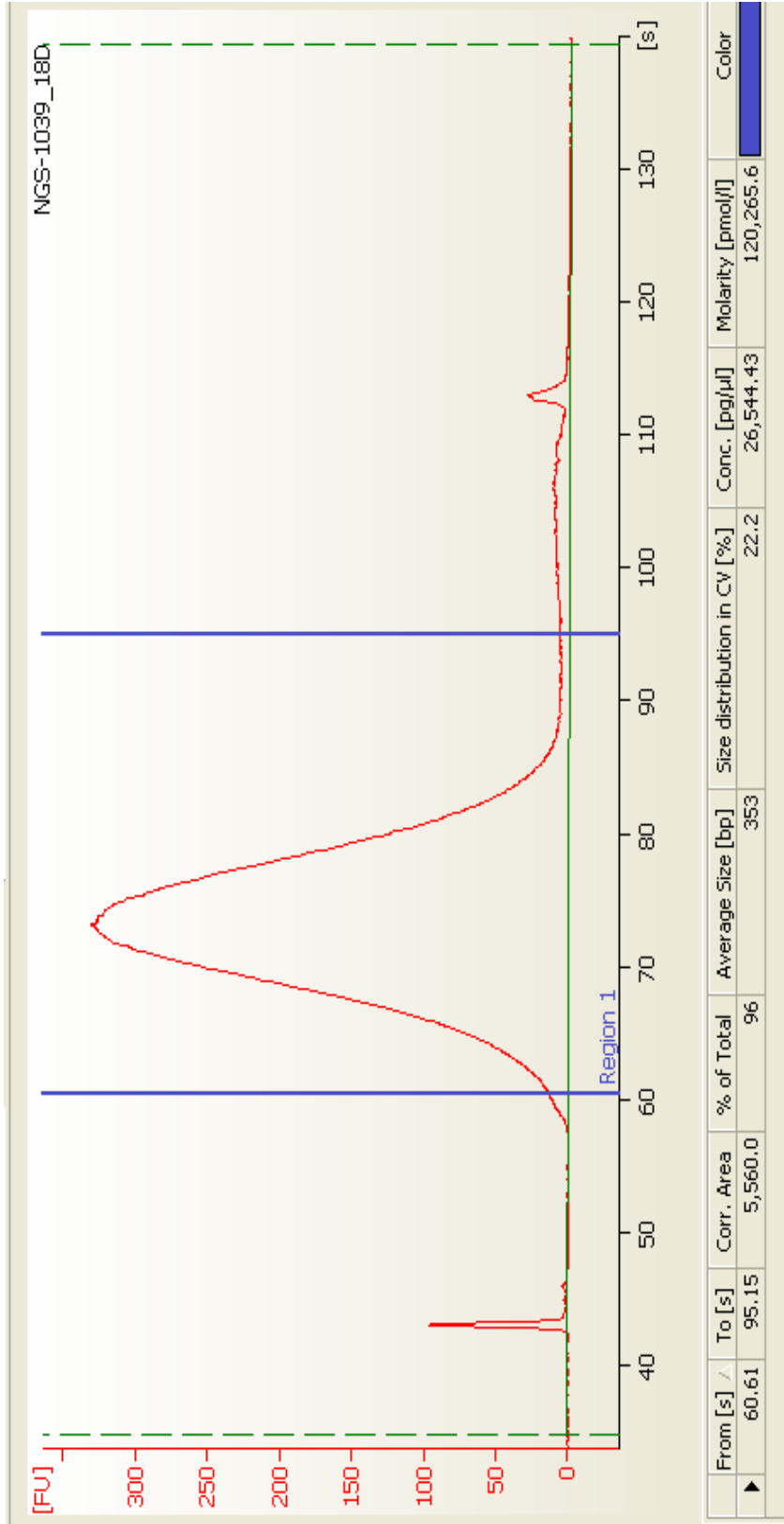
The library will be loaded onto the Illumina platform for cluster generation and sequencing. With paired-end sequencing, both the forward and reverse strands of the identity template

fragments can be sequenced. The library molecules will hybridize to complementary adapter oligonucleotides on the paired-end flow cell.

Specific adapters are incorporated for selective cleavage of the forward strands after re-synthesis of reverse strands during sequencing. This reverse strand is copied and sequenced from the other end of the fragment.



5.3 Bioanalyzer profile of sample 17D



5.4 Bioanalyzer profile of sample 18D

5.2.5 Conclusion:

The library was prepared from mitochondrial DNA samples 17D and 18D by *QIAseq FX DNA Library kit*. The average size of libraries is in 347bp and 353 bp for sample 17D and 18D respectively. The libraries will be sequenced on Illumina platform (2x150 bp chemistry) to generate ~1.5-2 GB data per sample.

5.2.6 Reads data statistics

The next generation sequencing for the 17D and 18D samples were performed on the Illumina platform. The statistics of data generated are shown in the table given below.

Table 5.5 Table High quality reads statistics

<i>Sample Name</i>	<i>Reads (R1)</i>	<i>Reads (R2)</i>	<i>Reads (R1+R2)</i>	<i>Total Bases</i>	<i>Total Data(Gb)</i>
<i>17D</i>	<i>17339853</i>	<i>17339853</i>	<i>34679706</i>	<i>4871545396</i>	<i>4.87</i>
<i>18D</i>	<i>12331783</i>	<i>12331783</i>	<i>24663566</i>	<i>3424689583</i>	<i>3.42</i>

5.2.7 Bioinformatics Analysis

High quality paired end data of 17D and 18D samples were assembled with CLC genomics workbench-6. The scaffolds obtained from assembly were searched for similarity against NCBI's Nucleotide (NT) database using BLASTN algorithm of 17D and 18D respectively

5.6 Results

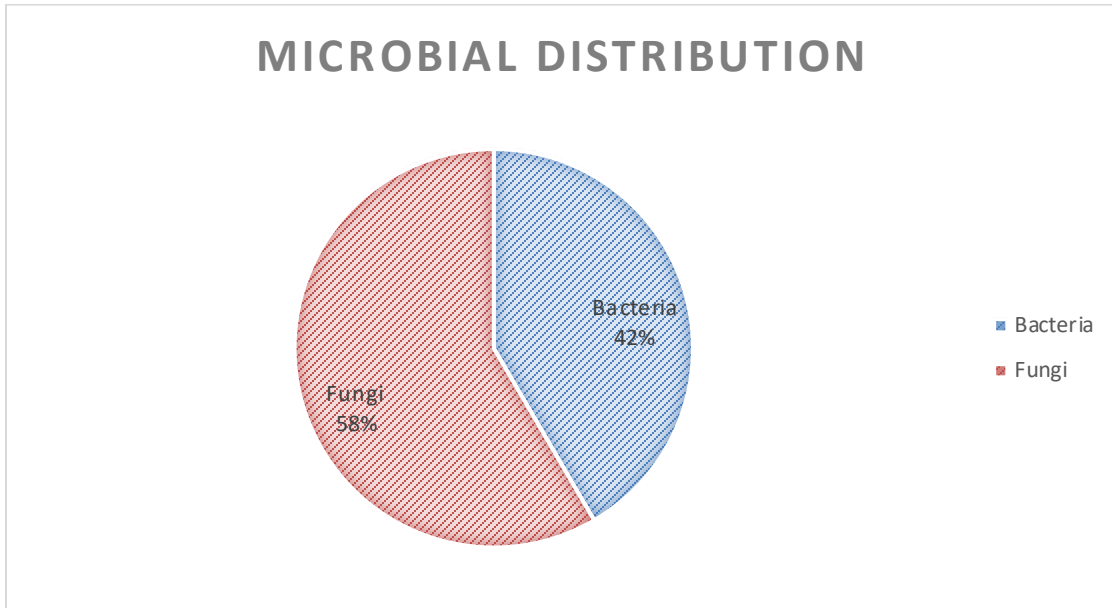


Figure 5.7 Microbial distribution among the oral swab of *Varanus Bengalensis* (17D)

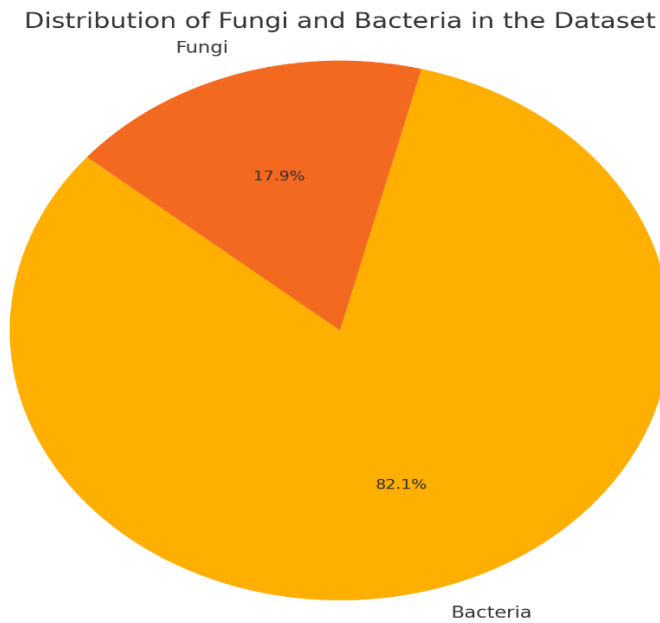


Figure 5.8 Microbial distribution among the oral swab of *Varanus Bengalensis* (18D)

Table 5.9 List of harmful gram-positive bacteria known to cause diseases to human present in the datasets of 17D, 18D

Severe Gram-Positive Bacteria	Details
<i>Staphylococcus aureus</i>	Particularly Methicillin-resistant <i>Staphylococcus aureus</i> (MRSA). Causes skin infections, pneumonia, sepsis, toxic shock syndrome.
<i>Streptococcus pyogenes</i>	Group A Streptococcus (<i>GAS</i>). Causes strep throat, necrotizing fasciitis (flesh-eating disease), toxic shock syndrome.
<i>Streptococcus pneumoniae</i>	Causes pneumonia, meningitis, sepsis, and otitis media.
<i>Clostridium difficile</i>	Causes severe colitis, often after antibiotic use.
<i>Clostridium botulinum</i>	Causes botulism, a life-threatening paralytic illness.
<i>Clostridium tetani</i>	Causes tetanus, leading to severe muscle spasms.
<i>Clostridium perfringens</i>	Causes gas gangrene and food poisoning.
Enterococcus faecalis and faecium	Particularly Vancomycin-resistant enterococci (VRE). Causes bloodstream infections, urinary tract infections, and endocarditis.
<i>Bacillus anthracis</i>	Causes anthrax, which can be fatal if untreated.
<i>Listeria monocytogenes</i>	Causes listeriosis, particularly dangerous in pregnant women, newborns, and immunocompromised individuals.

Table 5.10 List of harmful gram-negative bacteria known to cause diseases to human present in the datasets of 17D, 18D

Severe Gram-Negative Bacteria	Details
Escherichia coli	Especially Shiga toxin-producing <i>E. coli</i> (STEC), e.g., O157:H7. Causes severe foodborne illness and hemolytic uremic syndrome (HUS).
Klebsiella pneumoniae	Causes pneumonia, bloodstream infections, and urinary tract infections. Carbapenem-resistant strains (CRE) are highly dangerous.
Pseudomonas aeruginosa	Causes hospital-acquired infections, pneumonia, sepsis, and is highly resistant to antibiotics.
Acinetobacter baumannii	Causes ventilator-associated pneumonia, bloodstream infections, and is often multidrug-resistant.
Neisseria meningitidis	Causes meningitis and septicemia, both of which can progress rapidly and be fatal.
Neisseria gonorrhoeae	Causes gonorrhea, with antibiotic-resistant strains becoming increasingly prevalent.
Salmonella enterica	Causes typhoid fever (<i>Salmonella Typhi</i>) and non-typhoidal salmonellosis, leading to severe gastroenteritis.
Shigella species	Causes shigellosis, leading to severe dysentery.
Vibrio cholerae	Causes cholera, leading to life-threatening dehydration from profuse diarrhea.
Yersinia pestis	Causes plague (bubonic, septicemic, pneumonic), historically responsible for pandemics.
Legionella pneumophila	Causes Legionnaires' disease, a severe form of pneumonia.
Haemophilus influenzae type b (Hib)	Causes meningitis, pneumonia, and epiglottitis, especially in children.

Table 5.11 List of harmful Fungi known to cause diseases to human present in the datasets of 17D, 18D

Harmful Fungi	Details
Candida auris	Causes invasive candidiasis, often resistant to multiple antifungal treatments.
Candida albicans	Common cause of oral thrush, vaginal yeast infections, and invasive candidiasis.
Candida glabrata	Causes bloodstream infections, often resistant to azole antifungal treatments.
Candida tropicalis	Associated with bloodstream infections, particularly in immunocompromised patients.
Aspergillus fumigatus	Causes aspergillosis, which can affect lungs and spread to other organs in immunocompromised people.
Cryptococcus neoformans	Causes cryptococcal meningitis, especially in people with weakened immune systems.
Histoplasma capsulatum	Causes histoplasmosis, primarily affecting the lungs but can become disseminated.
Coccidioides immitis	Causes Valley Fever (coccidioidomycosis), leading to flu-like symptoms and severe lung infections.
Blastomyces dermatitidis	Causes blastomycosis, leading to lung infections and skin lesions.
Fusarium	Causes fusariosis, affecting skin, nails, and can disseminate in immunocompromised individuals.
Penicillium marneffeii	Causes penicilliosis, particularly in HIV-infected individuals in Southeast Asia.
Paracoccidioides brasiliensis	Causes paracoccidioidomycosis, a systemic fungal infection prevalent in Latin America.

5.12 Conclusion

And we also have in-depth comprehensive genomic information on many bacteria and fungi, including virulent pathogens (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Listeria monocytogenes* and *Fusarium* species) depict the subsequent distribution of the microorganisms present, which demonstrates the predominance of Gram-positive and Gram-negative bacteria interspersed with pathogenic fungal species, in the samples collected. From a forensic viewpoint such a set of data is extremely valuable from many perspectives. First, in microbial forensics, the detection of pathogenic microorganisms such as *Bacillus anthracis*(anthrax) and *Clostridium botulinum* (botulism) highlight the possibility of identification of biological threats or tracking bioterrorism agents. Forensic scientists can compare microbes on crime scenes with known strains of pathogens. Moreover, microbial succession patterns following death can be helpful to estimating the post-mortem interval (PMI). Identifying the bacteria and fungi involved in decomposition (*Aspergillus*, *Candida*, *Clostridium*, *Pseudomonas*) allows for more accurate estimations of PMI. Ultimately, the data enables source tracking in outbreaks, specifically examining pathogenic strains of *Salmonella enterica*, *Escherichia coli*, and *Listeria monocytogenes*, a key component to tracing where contaminated food sources may have originated. Moreover, the dataset contains strains with demonstrated sensitivity such as MRSA (Methicillin-resistant *Staphylococcus aureus*) and VRE (Vancomycin-resistant *Enterococci*) that allows forensic microbiology to follow the dispersal of antibiotic-resistant strains in the healthcare and community settings. Finally, novel forensic methods exploit distinct microbial fingerprints for human identification, and the dataset's granular bacterial and fungal profiles could aid in establishing such microbial fingerprints, given that traditional DNA material is often compromised. The genomic data offered holds immense potential for forensic applications, including bioterrorism detection, post-mortem analysis, outbreak tracking, and antimicrobial resistance monitoring, thereby bolstering forensic investigations and aiding in the broader domains of justice and public health safety.

5.13 References

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CHAPTER VI

SUMMARY

&

SCOPE of FUTURE WORK

The genomic research frontiers are wide open for *Chrysopelea ornata* (ornate flying snake) and *Varanus bengalensis* (Bengal monitor), and the prospective studies for its relatives (e.g., *Varanus flavescens*) have implications across evolutionary biology, forensic science, and conservation genetics. Whole genome sequencing (WGS) of *Chrysopelea ornata* can reveal the genetic mechanisms underlying its distinctive gliding ability, adaptive evolution, and ecological interactions, providing insights into the genes responsible for musculoskeletal adaptations, skin elasticity, and neural coordination. Genomic sequencing of microsatellites (short tandem repeats) can also be beneficial for population genetics studies to understand genetic diversity, population structure, and gene flow that is important for conservation purposes in degraded and fragmented habitats. Megenta also listed microsatellite markers, which could be utilized to characterize illegally-traded specimens for forensic purposes, as well as protein annotation from WGS, which could inform about physiological traits such as venom, metabolism, and immune response that may lead to pharmacological applications. However, complete mitochondrial genome of *Chrysopelea ornata* would provide more information on its phylogenetic relatedness and demographic activities, and mitochondrial markers such as mitochondrial cytochrome c oxidase (COI), mitochondrial 12S rRNA, mitochondrial 16S rRNA, mitochondrial NADH dehydrogenase, and mitochondrial COX2, could be used for species identification in forensic settings.

Likewise, research on the oral microbiota of *Varanus bengalensis* and *Varanus flavescens* can provide valuable insights into health, ecology, and evolutionary adaptations, since these microorganisms are essential for digestion, immune response, and resistance to diseases. By surveying the sequencing output, metagenomic can help understand how environmental stressors such as habitat degradation has affected microbial communities and overall health. Comparative genomics using whole genome sequences from *Varanus bengalensis* will enable the understanding of its genetic diversity, population structure, and evolutionary history, contributing in informing effective conservation strategies against the present and near future threats such as habitat loss, hunting, and illegal wildlife trade to *Varanus bengalensis*. Forensically informative nucleotide sequences (FINS) like COI, 12S rRNA, 16S rRNA, NADH dehydrogenase, and COX2 have shown to

significantly assist law enforcement entities in their investigations to combat wildlife crime. By applying FINS to *Chrysopelea ornata* and *Varanus bengalensis*, it is possible to improve the identification of species and track the origin of specimens traded illegally, and the mitochondrial markers provide a solid basis for a standardized approach to DNA barcoding and forensic identification. Combining multi-omics approaches (genomics, transcriptomics, proteomics, and metagenomics) will reveal an unprecedented understanding of the biology and ecology of these species, including insight into adaptive responses to environmental stressors necessary for making sure these organisms thrive in the face of a challenging future and to guide conservation efforts.

With extensive and well-constructed databases available, further development on microsatellite markers and mitochondrial sequences should also improve species and population identification and support the investigation of wildlife crimes in forensic science. Future work will further integrate metagenomics assays of microbial communities with host genomic information to understand host-microbial interactions and their implications for health and evolution. Ultimately, genomic analysis of *Chrysopelea ornata*, *Varanus bengalensis*, and *Varanus flavescens* holds great promise for contributing to the fields of evolutionary biology, conservation genetics, and forensic science, multifaceted approaches such as whole genome sequencing, microsatellite analysis, protein annotation, mitochondrial genome sequencing and studies on oral microbiota may provide answers to pressing questions related to challenges in conservation of biodiversity and wildlife crime.

CHAPTER VII

LIST OF PUBLICATIONS

LIST OF ORIGINAL PUBLICATIONS

Dinesh, Didla, Ishani Mitra, and Soma Roy. 2023. “The Complete Genome Sequence of *Chrysopelea Ornata*, Ornate Flying Snake.” *Biodiversity Genomes*, May.
<https://doi.org/10.56179/001c.75385>.

Dinesh D, Ishani Mitra and Soma Roy. 2022. “Forensic Identification of ornate flying snake (*Chrysopelea ornata*) through COX2” *International Journal of Creative Research Thoughts (IJCRT)*

Mitra, I., **D, D.**, Roy, S., & Haque, I. (2022). The complete mitochondrial genome study and phylogenetic analysis of Asiatic Water Snake (*Fowlea piscator*) using 'Next-Generation Sequencing' technology. *Mitochondrial DNA. Part B, Resources*, 7(11), 1939–1941. <https://doi.org/10.1080/23802359.2022.2139157>

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.

D Dinesh; Ishani Mitra; Soma Roy Title: Analysed data sets of Next generation sequenced whole genome of *chrysopelea ornata*: Microsatellites, gene prediction and proteomics for forensic applications. *Data in Brief* (Under review)

Dinesh D, Soma Roy and Ishani M “The first complete mitochondrial genome and phylogenetic analysis of the ornate flying snake *Chrysopelea ornata* (Shaw 1802) Colubridae” *Scientific Reports* (under Review)

D Dinesh; Ishani Mitra; Soma Roy "Oral Microbiota of *Varanus* Lizards: Diversity and Forensic Applications" *Conservation* (Under review)

ABSTRACTS/MANUSCRIPTS SUBMITTED IN CONFERENCES

D Dinesh Molecular identification of *chrysopelea ornata* (ornate flying snake) using COI gene 1st international conference on Forensic Science organized by Department of Forensic Science, School of sciences ,Jain (Deemed to be University), Bengaluru [oral presentation]

NADH Dehydrogenase subunit 2 (Nd2) Gene as a Forensically informative nucleotide sequence in identifying *chrysopelea ornata* at the international conference on Trends in Forensics, Law and Criminology: Empowering Criminal Justice in 21st Century and Beyond organized by The West Bengal National University of Juridical sciences, Kolkata

APPENDIX I

DNA SEQUENCES GENERATED IN THIS STUDY

WHOLE MITOCHONDRIAL DNA
SEQUENCE OF CHRYSOPELEA
ORNATA

GTTATCATAGCTTAAAATAAAGCATAGCACTGAAAATGCTAAGATGGTCATACCTGATC
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