CHAPTER 1 – Introduction, Literature Review and Thesis Objectives

1.1 Introduction

In this chapter a review of the research literature of the Slow Loris (*Lorisidae*) generally, and that of the Malaysian Slow Loris (*Nycticebus coucang coucang)* in particular is presented. Based on these reviews, the research objectives of this research project are developed, and described.

1.1.1 Literature Review

The Malaysian Slow Loris is a member of the *Lorisidae* family. The place of this family in the wider order of primates is now presented.

1.1.2. Natural History of the Family *Lorisidae*

1.1.3 Classification, Distribution, Evolution and Systematic

Kingdom Animalia, Phylum Chordata, Class Mammalia, Order Primates, Suborder *Prosimii/Strepsirhini*, Infraorder *Lorisiformes*, Family *Lorisidae* and *Galagidae* see the classification below (Figure 1.1).

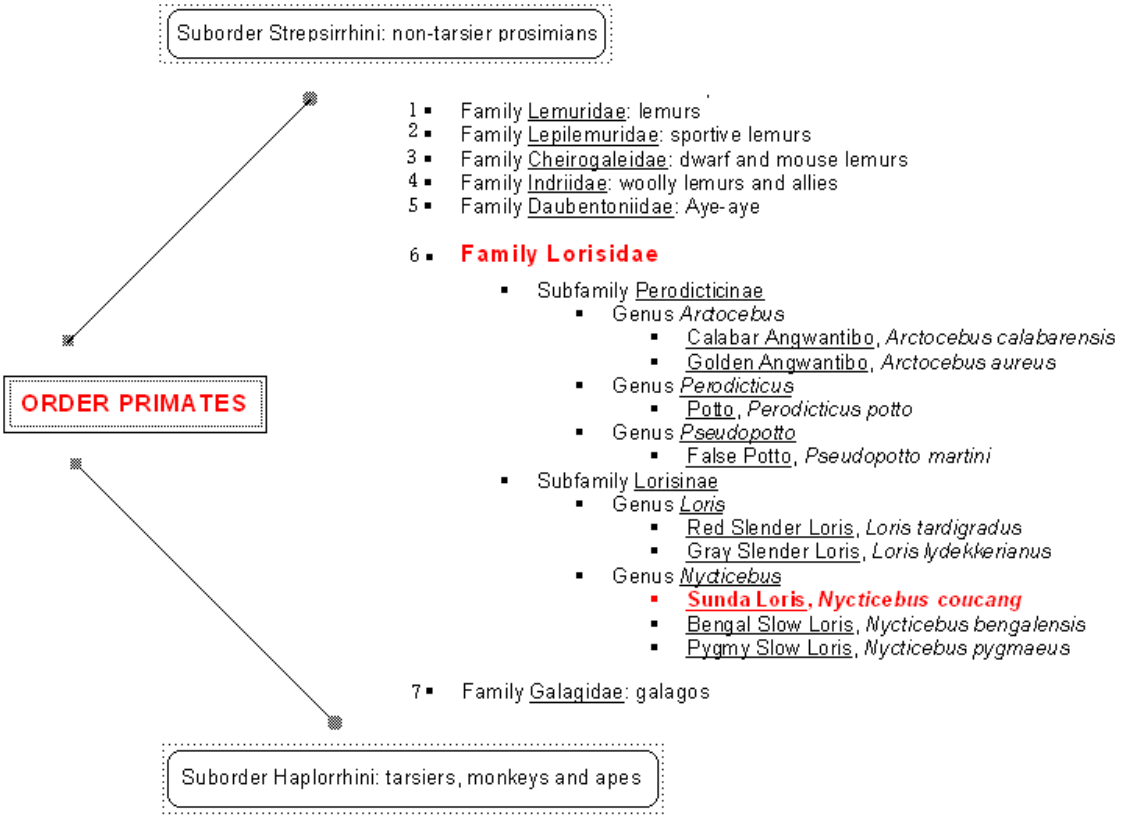

Figure 1.1 (Classification of Family *Lorisidae*, Groves, 2005)

The family *Lorisidae* consists of 6 genera and 11 species, divided into two subfamilies: *Galaginae* (1 genus and 6 species of African Galagoes) is specialized for vertical clinging and leaping; and *Lorisinae* (4 genera and 5 species of African Pottos and Asian Lorises) is specialized for slow climbing (McNeely, 1988). Asian Lorises are divided in two genuses, Slender Loris and Slow Loris (*Nyticebus*).

The family *Lorisidae* consists of two subfamilies *Lorisinae* (two genuses in Asia, *Loris* and *Nycticebus*) and *Perodicticinae* (three genuses in Africa, *Perodicticus*, *Arctocebus* and *Pseudopotto*) (see Figure 1.2. below)

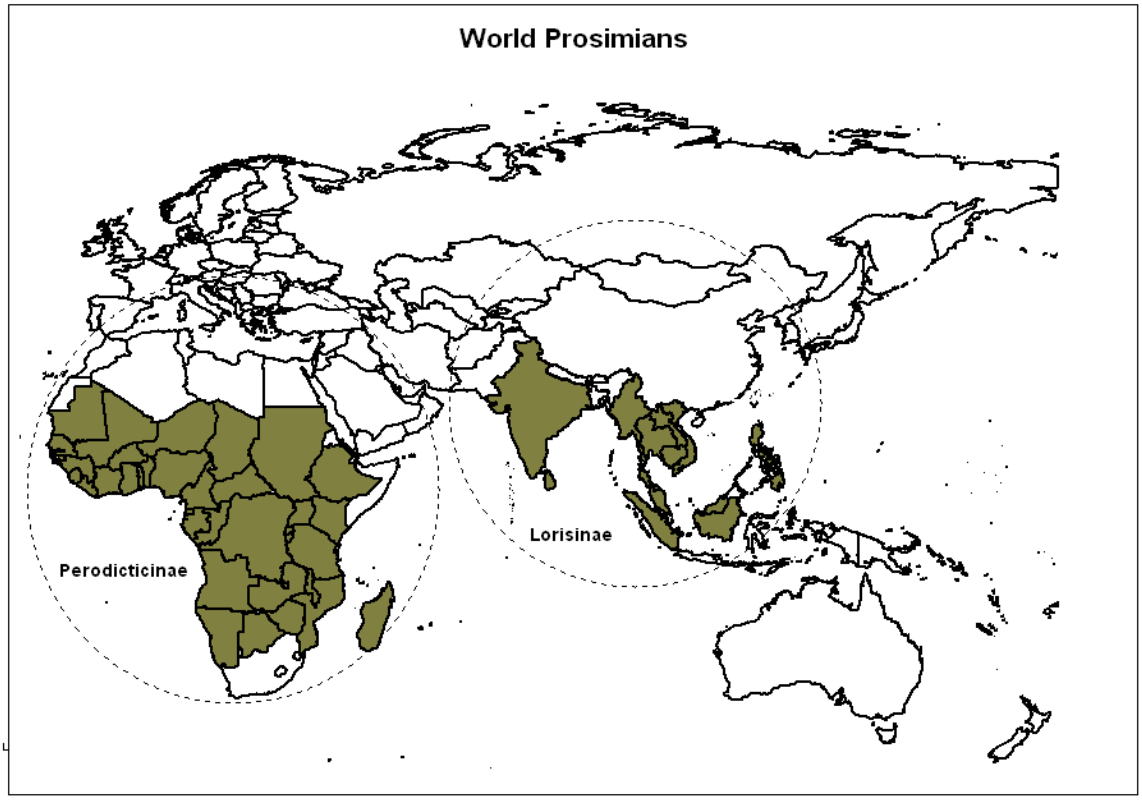

Figure 1.2 Family *Lorisidae* are two subfamilies (*Lorisinae* and *Perodicticinae*)

There is evidence of earliest fossil for the distinctive *strepsirrhine* toothcomb (Seiffert, 2003). These discoveries approximately double the previous temporal range of undoubted *lorisiforms* and lend the first strong palaeontological distribution of the family *Lorisidae*.

Studies of *Morphology, Molecular Biology* and *Biogeography* have shown that data bearing on early primate evolution, suggesting that the clade containing extant (or 'crown') *strepsirrhine* primates (*Lemurs, Lorises and Galagos*) arose in Afro-Arabia during the early Palaeogene (Yoder, 1996), but over a century of palaeontological exploration on that landmass has failed to uncover any

conclusive support for this hypothesis (Simons, 1997). It has been described by Seiffert (2003) that the first demonstrable crown S*trepsirrhin*es from the Afro-Arabian Palaeogene- where a *Galagids* and a possible *Lorisids* from the late middle Eocene of Egypt, the latter of which provides support to the hypothesis of an ancient Afro-Arabian origin for crown *Strepsirrhini* and an Eocene divergence of extant *lorisiform* families (Yoder,1996, 1997).

The primate clade *strepsirrhini*—now represented by the distinctive 'tooth combed' prosimians of the Old World tropics and Madagascar—is one of the three major extant primate groups alongside *Anthropoidea* (monkeys, apes and humans) and *Tarsiiformes* (tarsiers). Within *strepsirrhini*, it is clear that a major dichotomy exists between monophyletic *Lorisiformes* (containing African *Galagos* or 'bushbabies' and African and Asian *Lorises*) and monophyletic (and wholly Malagasy) *Lemursiformes* (Yoder, 1996; Charles-Dominique, 1970; Martin, 1990; Porter, 1997), but a poor *Strepsirrhine* fossil record has left the age and place of origin of their common ancestor open to debate (Marivaux, 2001; Martin, 2000).

Given the probable paraphyly of African *Lorisiforms* with respect to Asian *Lorises* (Yoder, 1996, 1997; Porter,1997), the proximity of Madagascar to the African mainland (McCall, 1997), and the distribution of more generalized primates in the Palaeogene fossil record of northern continents and Africa (Rose, K. 1994), it is now generally believed that extant *Strepsirrhines* shared a common Afro-Arabian ancestor (Yoder,1996,1997), but the earliest undoubted record of crown *Strepsirrhini* has long been to be the early Miocene (about 20 millions years old) *lorisids* (*Lorises*) and *galagids* (*Galagos*) from East Africa (Simpson,1967; Phillips, 2002). These Miocene *Lorisiforms* considerably postdate estimates of basal *Strepsirrhine* divergence times that have been reconstructed using local molecular clocks, which suggest a divergence of *Lorisiforms* and *Lemursiforms* 50–62 millions ago, and a much wider window of 23–55 million for the divergence of *lorisids* and *galagids* (Yoder, 1996, 1997; Porter, 1997).

Major aspects of the family *Lorisidae* phylogeny and systematics remain unresolved, despite several studies (involving morphology, histology, karyology, immunology, and DNA sequencing) aimed at elucidating them (Masters, 2005). Using molecular data and morphological data Masters (2005) investigated the evolution of the enigmatic family of *lorisidae* for all four well-established genera: *Arctocebus*, *Loris*, *Nycticebus*, and *Perodicticus*.

Data sets consisting of 386 bp of 12S rRNA, 535 bp of 16S rRNA, and 36 craniodental characters were analyzed separately and in combination, using maximum parsimony and maximum likelihood (Masters, 2005). The morphological data set yielded a paraphyletic lorisid clade with the robust *Nycticebus* and *Perodicticus* grouped as sister taxa, and the *Galagids* allied with *Arctocebus* (Masters, 2005). All molecular analyses maximum parsimony (MP) or maximum likelihood (ML) which included *Microcebus* as an out group rendered a paraphyletic *Lorisid* clade, with one exception: the 12S + 16S data set analyzed with ML (Masters, 2005). It was presented in the same study the information regarding a temporary land bridge that linked the two now widely separated regions inhabited by *Lorisids* that may explain their distribution (Masters, 2005)

1.1.4 General Anatomy of the Family *Lorisidae*

Lorisidae are characterized by a 2.1.3.3/2.1.3.3 dental formula. The lower incisors and canines projects forward (procumbent) to form the tooth comb. Females have 2 or 3 paired mammae (Hill, 1953). Males do not have a bacculum. Both sexes have equal length fore- and hind-limbs for slow quadrupedal locomotion. They possess a very strong grip with reduced 2nd digit in the hand and *Reta mirabilia* (blood storage channels) to allow the grip to be maintained for long periods. There appears to be fairly distinct African and Asian forms with gracile and robust species being found in both areas (Hill, 1953).

1.1.5 Genus within the Family *Lorisidae*

1.1.5.1 In Africa: *Arctocebus* and *Perodicticus*

These are found in the Angwantibo mountain ranges in Cameroon and Nigeria, between the Niger and Sanaga Rivers (Wilson & Reeder, 1993).

This species is primarily insectivorous, and eats mostly those insects that are unpalatable to other insectivores, but also eats fruit. Caterpillars *(Lepidoptera*) are the most common insects consumed with beetles (*Coleoptera*), ants (Hymenoptera), and crickets (*Orthoptera*) also eaten (Lee, 1988; Hladik, 1979; Charles-Dominique, 1977). This species consume insects rejected by other animals (Hladik, 1979). The *Calabar potto* tends to forage alone (Lee, 1988). Before eating a caterpillar, an individual wipes its hands along the caterpillar, removing most of the hairs that could cause irritation (Charles-Dominique, 1977). Gums constitute a scarce part of the diet of this species (Charles-Dominique, 1977). This species is found between at tree heights of 5 and 15 meters above the dense undergrowth preferring to spend most of the time on lianas and small branches (Lee, 1988). The *Calabar potto* sleeps in trees with dense foliage cover (Charles-Dominique, 1977). This is a nocturnal arboreal species.

The *Potto* has an unusual arrangement of spines on its cervical vertebrae that are supposed to be for defense (Charles-Dominique, 1977). In *Pseudopotto* (only skeletal material known) the vertebral spines are shorter (Schwartz, 1996).

Arctocebus sp. has a nictitating membrane, which is unique amongst primates (Montagna, 1966). This species has a pelage coloration that is orange to yellow brown on the dorsal side and white, light gray, or buff on the ventral side (Rowe, 1996). On the face, there is a white line from the brow to the nose (Nowak, 1999).

Altruistic behavior was noted in two *Perodicticus potto, an* alpha male and his mate, as the result of the death of the beta male. Even when the bulk of the food supply was limited, they persistently left a portion of food for the absent individual. Such behavior is unexpected in a prosimian and is of considerable evolutionary interest (CowGill, 1972).

The methods of home range analysis were compared for two species of nocturnal prosimians: Central Pottos (*Perodicticus potto edwardsi*) and Cross River Allen's Galagos (*Sciurocheirus cameronensis*). Radio-tracking studies of 10 Pottos and 8 Galagos from October 1999 – November 2000 was conducted in the montane rain forests of southwest Cameroon (Pimley, 2005.) Adult Potto home ranges averaged 145.2 ha (MCPs) versus only 28.4 ha via kernel analysis; the difference was statistically significant. The mean home range of Galagos was 18.3 ha via MCPs and 2.19 ha via kernel analysis; the difference was statistically significant (Pimley, 2005)

Studies on this species refer to *Artocebus calabarensis* only recently recognized as a fully separate species (Eisenberg et al., 1972; Bourlière, 1985; Charles-Dominique, 1971, 1977, 1979). Status, threats, and distribution are analyzed in other studies (Lee, 1988).

1.1.6 In Asia: *Loris* and *Nycticebus*

1.1.6.1 Description

The Lorises' name comes from the Dutch word "*loerus*" which translates as "clown" (Lydekker, 1901). The word *Nycticebus* means "night ape" (Pournelle, 1955).

Lorises are tailless, slow moving, cryptic, nocturnal primates. They are mainly insectivorous with adult body sizes from about 200g to 2kg. This primate has

very large eyes, which are directed forward. Their tightly clinging hands and feet have human-like nails. The nail on the second digit of the foot is elongated and rolled up to form a grooming claw (Nowak, 1999)

The Slow Loris (*Nycticebus coucang*) is squirrel-sized, slow climbing arboreal prosimians distributed in the tropical rain forest areas of South East Asia. Their nocturnal habits and their life in the dense upper canopy of their habitat make detailed field studies extremely difficult (Barrett, 1984)

Therefore, many aspects of their social system and their life style in nature are as yet unknown. Nevertheless, laboratory studies indicate they might be solitary or live in small family groups (Hill, 1933; Zimmermann, 1984; Rasmussen, 1986). Albeit often maintained in captivity, successful breeding seems to be rare as documented at the Duke Primate Center and a colony studied by Zimmermann (1977, 1979, 1980, 1981, 1983, and 1985).

This species can maintain a grip while remaining completely immobile for long periods due to a specialized network of blood vessels in the limbs (Hill, 1953). Like other prosimian, Lorises have closely spaced incisors on the lower jaw that form a dental comb, as well as a set of brachial glands that may exude a strong-smelling substance when the animals are under stress. This substance has been found to be toxic when combined with saliva (Alterman, 1995). Loris bites have been known to cause severe illness and even death in humans. In those who develop an allergy, an anaphylactic (allergic) shock within seconds to minutes after a bite is possible; symptoms may be red, itching skin, very low blood pressure, shock, convulsions of muscles (pain), respiratory and heart problems (Wilde, 1972; Pschyrembel, 1995).

It has been reported that the protein in the brachial organ secretions of *Nycticebus coucang* exhibits extensive amino-acid sequence similarity to Fel d1, the major allergen from the saliva and integument of the domestic cat*, Felis*

catus. Another study suggests that the chief protein in the brachial organ exudate is an allergen (Krane, 2003).

1.1.6.2 Classification and Systematics

The genus Slender Lorises, includes six subspecies under *Loris tardigradus*, and is found in India and Sri Lanka.

The genus Slow Loris includes four subspecies *Nycticebus pygmaeus*, the pygmy Slow Loris, is found in Vietnam, Cambodia, Laos and Southern China (Wolfheim 1983). The four subspecies which belong to *Nycticebus coucang* are: 1) *Nycticebus bengalensis,* the Bengal Slow Loris, distributed throughout North East India, Bangladesh, China and Thailand; 2) *Nycticebus coucang menagensis*, found in Borneo, Bangka and Belitung in Indonesia, and Tawitawi (Philippines); 3) *Nycticebus coucang javanicus* restricted to the island of Java, Indonesia; and 4) *Nycticebus coucang coucang* distributed across Peninsular Malaysia, Sumatra and the Natuna Islands (Groves 1998, 1971)(Figure.1.3).

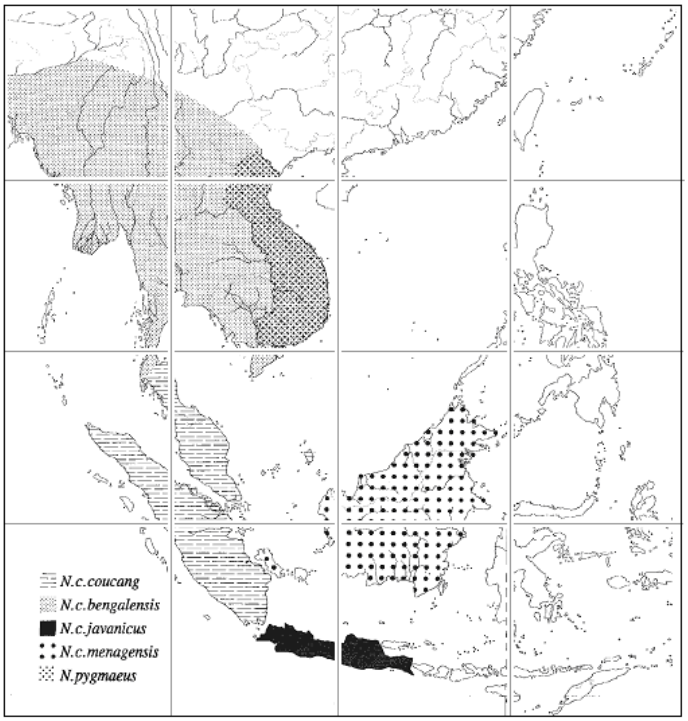

Figure 1.3 Map showing recent distribution of common and pygmy Slow Lorises in Southeast Asia (after Hill, 1953; Groves, 1971; Fooden, 1991).

The correct name for the Philippine Slow Loris has been confirmed as *Nycticebus coucang menagensis* (Lydekker, 1893); the designated the type locality is the vicinity of Tataan, Tawi-Tawi Island, the Philippines, and collected between 5 October and 5 November 1891: It was concluded that the specimen upon which the name *Menagensis* is based no longer exists. A series of 17 additional specimens from the vicinity of Tataan (topotypes) were collected in late October and early November 1892, which are deposited at the Bell Museum of Natural History in Minneapolis and at the Field Museum of Natural History in Chicago (Timm, 1992).

1.1.6.3 Ecology and Behavior of *Loris* and *Nycticebus*

This reclusive mammal looks like a cute, cuddly, little bear when in repose, but takes on distinctive primate characteristics as it moves high in the forest canopy, or, nearer ground, in the bamboo clumps on the jungle fringe. It is uniquely nocturnal. It is this latter characteristic that is the main reason why the Slow Loris is one of the least known, and little studied of Malaysia's primates. Due to a lack of information, its conservation status has been classified as "indeterminate" (Eudey, 1997; Wolfheim 1983).

Being cryptic and nocturnal, as well as rare, the Slow Loris, or as it is called in the Malay language, *Kong Kang,* is extremely difficult to observe in the wild. In order to preserve this unique primate and to correctly identify its conservation status, it is important to obtain comprehensive data for developing effective protection measures the mammal needs in view of the mounting risks it faces as its lowland forest habitats are threatened by hunting for food and capture for pets, logging, deforestation, and conversion of forest habitats to agriculture and human settlement.

Research on the Slender Loris has been conducted in the field in countries such as India and Sri Lanka. For instance, in India's Kalakad-Mundanthurai Tiger Reserve the distribution and habitat use of Slender Loris (*Loris tardigradus)* (Gupta, 1995) was investigated.

Johnson *et al* determined the diurnal activities of the Slender Loris (*Loris tardigradus)* in the Mundanthurai Sanctuary, Tamil Nadu (India). Johnson (1984) and Nekaris (2000) studied the Mysore Slender Loris *(Loris lydekkerianus)* in Madurai. The activity budget and positional behavior of the Mysore Slender Loris (*Loris tardigradus lydekkerianus*) in Tamil Nadu, India was studied by Nekaris

(2001). Vijayalakshmi (1981) determined the population distribution of the Slender Loris (*Loris tardigradus*) in India. This primate was found in the tropical evergreen, semi evergreen, mixed deciduous and subtropical broad-leaf type forests of India (Choudhury, 1992). The feeding ecology of the Mysore Slender Loris (*Loris lydekkerianus lydekkerianus*) was studied by Nekaris, (2001) for 10.5 months in a dry scrub forest at Ayyalur Interface Forestry Division, Tamil Nadu, and South India.

This researcher (Nekaris) recorded and analyzed 1240 feeding incidents, which indicate that the Lorises were almost exclusively faunivorous, with 96% of all feeding events representing animal prey. Of prey items that could be identified (n D 605), 62.9% were ants and termites (Nekaris, 2001). Lorises fed on 9 orders and 17 families of insects, plus spiders, mollusks, and small vertebrates. Lorises infrequently fed on gums and a legume pod. They usually grabbed prey with one hand, while other appendages firmly held the substrate (Nekaris, 2001). A high proportion of insects eaten by Slender Lorises (71%) occurred in patches or aggregations. The utilization of aggregated social insects may have implications for understanding the unusually high degree of gregarious behavior exhibited by the Lorises (Nekaris, 2001).

Observations under caged conditions were made on the behaviour of four mother Lorises (*Loris tardigradus lydekkerianus*) towards their own and alien infants (Kadam, 1980). There appears to be no mutual recognition between the mother and her infant, and the relationship appears to be less specific. The infants are accepted by and get settled with any lactating female. It was reported by Kadam (1980) that in the first few weeks after birth, there is an intense attachment exhibited by the mother Loris towards her baby. When the baby is separated, it exhibits a series of "fixed action patterns." As the infant grows older, maternal interest declines and is lost after about 15 - 20 weeks post partum. Vocalization

of the separated juveniles evokes greater maternal response than the visual cue (Kadam, 1980). Detailed observations have been made on the pre-parturitional and post-parturitional changes in the mother (Kadam, 1980).

The reproductive cycle of the Slender Loris *(Loris tardigradus lydekkerianus*) was described by Narayan (1927). A biannual breeding season was shown and these two seasons occurred during April—May and October-November (Narayan, 1927).

A survey on *Loris tardigradus lydekkerianus* was conducted in 6 forest divisions in the southern parts of the state of Andhra Pradesh, South India. Relatively high densities of Lorises occurred in mixed deciduous forests and in adjoining farm lands interspersed with trees. Three distinct populations inhabit the study area. Mewa (2000) recommend conservation measures for *Loris tardigradus.*

A population density study was carried on Slender Lorises (*Loris lydekkerianus*) in Karnataka, South India, intermittently during November 2001 – July 2004 and estimated their relative abundance via direct sightings (Kumara, 2006). Two subspecies, *Loris lydekkerianus lydekkerianus* and *Loris lydekkerianus malabaricus* with different morphological traits, occur respectively in the eastern drier region and the western wet region of the state (Kumara, 2006). Results observed on the distribution of *Loris lydekkerianus lydekkerianus* in a small region in the Southeast, contradicts earlier reports of its abundance throughout the state (Kumara, 2006).

Loris lydekkerianus malabaricus occurs throughout the Western Ghats as a contiguous population. The encounter rates of *Loris lydekkerianus lydekkerianus* and *Loris lydekkerianus malabaricus* are 0.41 individuals / km2 and 0.21 individuals / km2, respectively (Kumara, 2006). Whereas several forest tracts in the distributional range of *Loris lydekkerianus malabaricus* are protected areas,

no such area exists in the distributional range of *Loris lydekkerianus malabaricus*. It faces serious conservation challenges because it largely occurs in commercial plantations, which can be relatively unstable habitats as harvesting can take place at any time (Kumara, 2006)

Studying parasitic protozoa of some mammals, Raje (1961) found a trichomonad infected *Loris tardigradus lydekkerianus* which is found in the Eastern Ghats, westward of the cities of Mangalore and Mysore (Pocock, 1939). In Vietnam, studies on the interactions between mother and infant Slow Loris (*Nycticebus coucang*) and Pygmy Lorises (*Nycticebus pygmeus*) were conducted by Fitch (2001). Field sightings of the Pygmy Loris were done by Duckworth (1994).

Vocalization studies and physical growth were studied for on *Nycticebus coucang* by Zimmermann (1985, 1989). Whistling in connection with sexual interest or as a contact seeking call has been described by Rasmussen (1986), and Zimmermann (1985). Mothers use long distance whistle call as a response to their infants when they are calling and are separated (Zimmermann, 1985).

Lorises are known to consume invertebrate prey that has a repugnant smell and taste (Hladik, 1979).

An assessment of the distribution and conservation status of the Bengal Slow Lorises (*Nycticebus bengalensis*) has been done in Assam and Meghalaya in northeastern India forest reserves, plantations, tea estates, and areas bordering forests in 10 districts of the 2 states. Slow Lorises were observed in only 4 districts in Assam (Radakrishra et al, 2006). Disturbances caused by tree felling, road kills by speeding vehicles, and trapping and hunting are the chief survival threats to the species (Radakrishra et al, 2006). This study emphasized immediate implementation of conservation measures to ensure the future of the

species and recommended additional population surveys to define the distributional extent of the Bengal Slow Loris (Radakrishra et al, 2006).

Gestation length as determined from mid-estrus was 186 -187 days. Copulation takes place over two to five consecutive days during estrus. Litter size for each of the recorded births was one. Lactation lasts for five to seven months (Zimmermann, 1989). Sexual maturity is reached at about 1 1/2 to 2 years. Physical growth and the first appearance of main locomotor, behavioral and vocal patterns are described until nutritive weaning (Zimmermann, 1989).

The body temperature and oxygen consumption of freshly trapped Slow Loris *(Nycticebus coucang)* and Common Tree Shrews (*Tupaia glis)* were measured in Malaysia. The Slow Loris had low body temperature and oxygen consumption, while the values for the Common Tree Shrew were relatively high (Whittow, 1977).

One study of re-introduction of the *Nycticebus pygmeus* was done by Streicher (2003). The rain forest where it can be seen is rich in *Dipterocarpaceae* and may be subdivided into lowland (below 300 m) and hill (300m to 1,000m) areas (Fooden, 1991).

Here, using the Pygmy Loris (*Nycticebus pygmaeus)* as a model, Fisher (2003) presented a method for manipulating olfactory cues to influence mate choice decisions to promote conservation efforts (Fisher, 2003).

1.1.6.4 Social Grouping

Several factors are well known to influence social group size in primates (Caraco & Wolf, 1975; Pulliam & Caraco, 1984; Hass & Valenzuela, 2002). Among these, food distribution and predation pressure are the two best studied factors

(Chapman et al., 1995; Janson & Goldsmith, 1995; Hass & Valenzuela, 2002; Downes & Hoefer, 2004). Furthermore, the social brain hypothesis suggests that, in species that live in socially bonded groups (such as many primates and carnivores), group size can be constrained by cognitive abilities (Dunbar, 1992). On the other hand, the social systems of solitary mammals, and more specifically Slow Loris, have been described as being much more uniform and much less complex (Charles-Dominique & Martin 1970; Martin 1972; Alexander 1974; Eisenberg 1981; van Schaik & van Hooff 1983; Fleagle 1988). However, in various mammal species previously regarded as classic solitary species, higher rates of direct inter-individual encounters than expected have been found (Caro 1994; Waser et al.1994; Sterling & Richard 1995; Gehrt & Fritzell 1998a; Kays & Gittleman 2001).

1.1.6.5 Evolution and Phylogenetics

In order to study the evolutionary relationships among the species of three species of Slow Lorises (*Nycticebus coucang, Nycticebus intermedius, and Nycticebus pygmaeus)* mitochondrial DNA polymorphisms in 15 specimens were analyzed by Zhang (1991). Results suggest" that there are two valid species in the genus, *Nycticebus coucang* and *Nycticebus pygmaeus,* and that *Nycticebus intermedius* should be included within *Nycticebus pygmaeus*. Divergence between the two species may have begun 2. 7 million years ago. Evolution of gross morphology, chromosomes, and mitochondrial DNA in the Slow Lorises appears to be concordant (Zhang, 1991).

Even where some gene-flow can be demonstrated between *Nycticebus coucang* and *Nycticebus bengalensis,* this appears to be very limited and does not affect the essential homogeneity and diagnosticability of the two taxes (Groves, 1998). The biogeographic implications of the taxonomic findings of this study are

noteworthy. They confirm the distinctness of Sulawesi in contrast to a Sundaland / Southern Philippines link *(Tarsius);* the separation of the Indochinese and Sundaic faunal sub-regions *(Nycticebus);* and the uniqueness of the Sri Lankan "wet zone" *Loris* (Groves, 1998).

The molecular phylogeny covering all recognized taxa in *Nycticebus coucang* was established to provide information for further evaluation. This was done by standard sequencing of the partial D-loop (ca.390 bp) and cytochrome b (425 bp) from 22 specimens. (Zhang, 2006)

The chromosome complements of two male and two female adult Slow Lorises *(Nycticebus coucang)* have been studied in blood cultures cultivated *in vitro* for three days. Nine of the chromosome pairs were metacentric, the remaining 15 pairs, and sub-metacentric. The X chromosome is a long sub-metacentric, ranking 4 in order of decreasing size. The Y chromosome is a rather long metacentric and ranks 15 in the same order (Egozcu, 1966)

1.1.7 *Nycticebus c. coucang* in Peninsular Malaysia: Literature Review

There are two species of Slow Loris in Malaysia: *Nycticebus c. coucang* which is distributed in Peninsular Malaysia, Sumatra and on isolated South China Sea islands such as the Natuna Islands (Groves, 1998, 1971); and *Nycticebus c. menagensis*, which, on the other hand, is found only on the islands of Borneo, Bangka-Belitung, Indonesia, and Tawi-Tawi (Sulu Sea, Southern Philippines)

This reclusive mammal (Photo 1.1) takes on distinctive primate characteristics as it moves high in the forest canopy, or, nearer the ground, in the bamboo clumps on the jungle fringe. It is uniquely nocturnal. It is this latter characteristic that is the main reason why the Slow Loris is one of the least studied of Malaysia's

primates. Due to a lack of information, its conservation status has been classified as “indeterminate” (Eudey, 1997; Wolfheim, 1983).

Photo 1.1 showing *Nycticebus coucang coucang* [taken by Jaime Castillo]

Being cryptic and nocturnal, as well as rare, the Slow Loris, or as it is called in the Malay language, *Kong Kang,* is extremely difficult to observe in the wild. In order to preserve this unique primate and to correctly identify its conservation status, it is important to obtain comprehensive data for developing effective protection measures the mammal needs in view of the mounting risks it faces as its lowland forest habitats are threatened by hunting for food and capture for pets, logging, deforestation, and conversion of forest habitats to agriculture and human settlement

Fifty percent of Malaysia is still forested and some parts of it are considered to be among the oldest of the tropical rainforests in the world. There are 145.000 varieties of plant, 600 bird species, the giant Leatherback Turtle; over a 100 species of snakes, 80 species of lizards, a huge collection of insects and 200

mammal species. Within the biodiversity of primates in Malaysia, it is possible clearly to identify two species*: Nyticebus c. coucang* in Peninsular Malaysia and *Nycticebus menagensis* in Sabah (Malaysia) on the island of Borneo. This study will focus on *Nycticebus c. coucang* in Peninsular Malaysia. This species has a considerable distribution in different provinces and on some pristine islands (Jenkins, 1987; Fooden, 1991).

The map of the distribution of *Nycticebus coucang coucang* in Peninsular Malaysia according to researchers is as follow (Penang, Perak, Kepong (Selangor), specifically, Bukit Lanjan and the Sungai Buloh Forest Reserve, near Kuala Lumpur, and the District of Manjung. Perak (Whittow 1977; Hill, 1953; Groves, 1971; Wiens, 1995) (Figure 1.4).

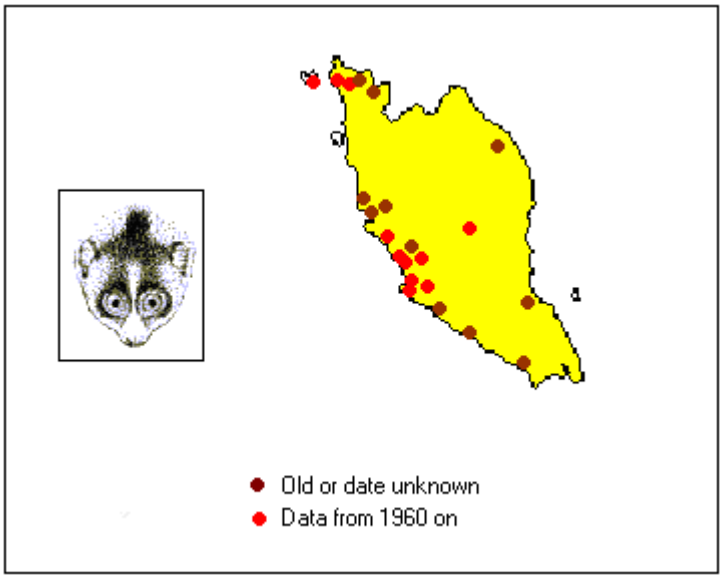

Figure 1.4 Distribution of (*Nycticebus coucang coucang)* in Peninsular Malaysia based on the distribution maps in Hill (1953), Groves (1971) and Jenkins (1969).

Nycticebus c. coucang is arboreal, seldom coming to the ground, and lives in the canopy of dense Malaysian rainforest below 1500 meters, but also in secondary forests and bamboo thickets, and is nocturnal (Elliot, 1967).

Nycticebus c. coucang was observed to feed upon a common giant snail, *Achatina fulica* (Elliot, 1967). There is one report of a group of six Slow Lorises found together; the group appeared to be five Slow Loris males following a female in estrus (Elliott, 1967). In 1981, Marsh conducted a survey of primates in Peninsular Malaysia including the Slow Loris. There are two quantitative studies on the natural behavior of Slow Lorises (*Nycticebus c. coucang*) in Malaysia conducted by Barret (1981) and Wiens (2002). For the *Nycticebus c. coucang* at Sungai Tekam, Peninsular Malaysia a lower density in disturbed habitats was mentioned (Barrett, 1981). The same author studied the ecology of some nocturnal, arboreal mammals in Peninsular Malaysia (Barret, 1984). A comprehensive behavioral field study on *Nycticebus coucang* was carried out between May 1995 and July 1999 in the Manjung District, Perak State in Peninsular Malaysia (Wiens, 2002). The area included parts of the Segari Melintang, Tanjung Hantu and Batu Undan Forest Reserves as well as the village Labuan Bilek and its surrounding area (Wiens, 2002).

One study of a Slow Loris home range was done in Manjung (coastal low lands) in the West Coast of Peninsular Malaysia by Wiens (2002). He proposed that none of the individuals radio-collared with a fixed home range used its home range exclusively. This was suggested by data on radio-collared Slow Lorises as well as by chance visual observations of un-collared Slow Lorises made during tracking of focal animals. However, he noted that he never positively identified same-sexed adult Slow Lorises sharing their home range with another. Besides that, he mentioned that he never positively identified adults sharing their home range with more than one adult of the opposite sex. It was concluded, according

to his data, that home ranges overlapped extensively among all dyadic combinations of individuals from the same spatial group for which data allowed such analysis.

Regarding activity patterns, individuals were exclusively nocturnal and became active soon after sunset (Wiens, 2002). According to his data, he concluded that resting only made up on average of 1.6% of active time. Time did not differ significantly between sexes, or between adults and sub-adults. It was reported that individuals spent an average of 12.1% of their active time feeding. There was no difference in the time spent feeding between the sexes (Wiens, 2002).

Wiens also suggested that Slow Lorises at Manjung live in extended family groups consisting of an adult pair and it's their offspring. It is therefore likely that the mating system is monogamy (Wiens, 2002). Vigilance is also unlikely to be increased. Slow Lorises did not use alarm calls in the wild; neither have alarm calls been reported from captive animals (Wiens, 2002). High fruit consumption in unlogged forest was observed. In logged-over forest, no foraging or feeding on fruit was recorded but instead, high insect consumption was observed. In logged forests, higher altitudes were preferred for foraging (Wiens, 2002). It was reported by the same author that the diet of infants in Manjung depends on information obtained from older Slow Loris. However, he mentioned that infants were never involved in direct interactions with adults Slow Loris other their parents (Wiens, 2003).

1.1.8 Discussion of the Present Status of Research on the Malaysian Slow Loris

Research on the Slow Loris in Malaysia has been based on behavioral observations, including those made in the field. The most advanced methods

used in the study of the Slow Loris have been the deployment of radio tracking technologies as used by Wiens in his dissertation completed in the year 2002, and the results of which were then used to process home ranges which he then analyzed for their geometry and their implications to the behavioural patterns of the Loris (Wiens, 2002).

The author of this current research project originally intended to use a similar radio tracking technologies as used by Wiens (2002), but enhanced by using modern hand-held Global Positioning Technologies (GPS) to study the Malaysian Slow Loris in a different natural setting from that used by earlier researchers in Peninsular Malaysia, specifically, in natural forest settings. However, preliminary research undertaken in the Krau Nature Reserve in the East Coast State pf Pahang in Peninsular Malaysia showed it was extremely difficult to find Slow Loris in the wild in mountainous forests areas, and so a decision was taken to study the Slow Loris in the Kampung Boloh area just south of the Krau Nature Reserve, in the same District of Temerloh, State of Pahang. The Kampung Boloh study area is a mixture of remnant natural forests interspersed with smallholding agriculture landholdings cultivated by rural Malay peoples.

With the purpose of making this research project more original in its use of technologies and methodologies when compared with earlier academic and published studies, this researcher undertook to review a wider range of modern technologies for application in his study of the Slow Loris, these ranging from more advanced locational and mapping technologies and methodologies, including Global Positioning Systems (GPS), Remote Sensing imagery, Geographical Information Systems (GIS), in combination with Molecular Biology techniques to give this research project a new and unique technological bases upon which to study the Slow Loris.

1.2 Review of Methodologies Used to Study the Slow Loris

1.2.1 (Radio-telemetry) Radio - Tracking Techniques Overview

Radio-tracking is a technique used for determining indirectly information about an animal through the use of radio signals from or to a device carried by the animal. “Telemetry” is the transmission of information through the atmosphere usually by radio waves, so radio-tracking involves telemetry (Mech, 2002). The first functional system was created by Cochran and Lord, (1963). It began to be used “in earnest in the early 1960’s when small transistors became readily available” (Kuechle, 1982). Radio tracking brought two new advantages to wildlife research; the ability to identify individual animals and the ability to locate each animal when desired. These advantages have led to the wide application of radio- tracking since the first complete workable system was designed (Cochran & Lord, 1963).

Radio-tracking has been used on animals of diverse body sizes inhabiting terrestrial, arboreal, and aquatic habitats. The technique has widely been used with a variety of vertebrates to: calculate home ranges, plot movements, record physiological data and assist in locating animals for direct observation (Lehner, 1979).

Kenward et al (1987) has provided a comprehensive account of how to radio-track animals. A number of outstanding reviews on radio-tracking also have previously been produced (Bearder, 1980; Cheeseman, 1982). This remarkable technique is used frequently by the researcher to accurately locate animals for further observation, to determine home range, movement, habitat use, migration routes, activity patterns, predator-prey relationships, survival, and to locate nests

and roots (Fuller, 1987). Radio-tracking was described by Kalpers (1988) as a wide technique application that transformed field studies and promises to provide answers to a host of biological questions. It was concluded by Bearder (1980) that radio-tracking was seldom exploited to its full potential in ecological research.

Radio-telemetry has been invaluable for studying animal behavior. It is often the only means of obtaining information. However, while technical improvements have been made, the basic technology has long remained the same (Mech, 1992). Wildlife-related telemetry is also known as radio tagging, radio-tracking or simply ‘tagging’ or ‘tracking’. Wildlife telemetry enables researches to monitors individual animals in many varied terrains, environments, and states (Long & Weeks, 1983). Studies of radio marked individuals has provided detailed information on movements and distribution on fast-moving or secretive species that are otherwise difficult to observe without such technology (Warnock, & Takekawa, 2003).

There is an abundance of literature on wildlife telemetry (for example, Kenward, 1987; Priede & Swift, 1992). Since the classic paper by (Cochran & Lord 1963), tracking and biotelemetry studies have been conducted and more than 500 species throughout the world have been studied. Tracking has to be considered a major ecological technique in studying animal behavior, especially as regards nocturnal, erratic, or migrating species which can’t be observed easily (Kalpers, et al., 1988).

The technique requires the live capture of animals and usually the attachment of a collar or other device to them. It then usually requires someone to listen for a signal from the device periodically. This means people in the field in vehicles, aircraft, and on foot (Mech, 2002)

Radio-tracking has increasingly been used in the study of wild primates, primarily with animals that fit the above descriptions (Kalpers, 1988). This includes nocturnal species (Charles-Dominique, 1977; Charles-Dominique & Bearder, 1980; Bearder & Martin, 1979; Harcourt & Nash, 1986; Crompton & Andau, 1987; Harcourt, 1991). Animals such as solitary males that are erratic in the movements and difficult to observe, have been studied (Kawai et al 1968; Jones & Bush, 1988). Other species living in difficult terrain and dense vegetation have been studied (Anderson & Moor, 1971; Tattersall & Sussman, 1985). Small animals living in thick vegetation (Terborgh & Goldizen, 1985; Peres, 1989; Buchanan, 1991) and terrestrial species which have large home ranges (Anderson, 1982) have been part of studies by researchers

By using this technique, primatologists have been able to gather data on several prosimians such as: *Cheirogaleus major, Microcebus murinus, Daubentonia madagascarensis (*Wright & Martin, 1995; Corbin & Schmid, 1995) respectively. The techniques to locate *Callitrichid* groups have evolved from a collar and bell system (Neyman, 1977) to radio-tracking systems using light-weight radio-collars (Garber, 1993; Baker, 1991; Buchanan, 1991). Radio-collars have successfully been used in some of the larger bodied *Callitrichids* (500 g or more).

Traditional telemetry technologies used to study wildlife include Very High Frequency (VHF), Global Positioning System (GPS), satellites, Global Location Sensor (GLS), and hyperbolic systems (Mech, 2002).The three most common technologies used by wildlife biologists are compared in table (Table 1.1).

The original, and still most widely used, wildlife telemetry method uses very high frequency telemetry. Low frequency radio waves tend to travel farther than do higher frequency waves and are less affected by reflection off vegetation and

topography, but they require longer antennas to accommodate the longer wavelengths (Mech, 2002; Kenward 2001).

Another derivation of this type of telemetry system is automated very high frequency radio tracking, which can be prone to problems (Kenward, 2001). Some automated very high frequency systems provide suitable area coverage and low positional error, but they tend to be very expensive (Samuel & Fuller, 1996). Others are suitable only for small areas and thus are limited to use in studies involving small animals (Briner, 2003). The advantages of Very High Frequency VHF tracking are relatively low cost, reasonable accuracy for most purposes, and long life; disadvantages are that it is labor intensive and can be weather-dependent if aircraft-based. Notwithstanding, VHF radio-tracking is by far the most useful and versatile type of radio-tracking, for not only does it yield location data , but it also allows researchers to gather a variety of other types of information (Mech 1974, 1980, 1983). Satellite tracking requires a much higher initial cost and is much less accurate (mean accuracy = 480 meters) and, for most species, is shorter-lived than VHF systems (Fancy, 1988).

The use of Global Positioning Systems (GPS) as the preferred method of tracking is becoming more common in wildlife studies. This technology uses a time-of-arrival algorithm to triangulate an animal's location from signals emitted by satellites orbiting the earth (Kenward, 2001). Depending on collar weight, some Global Positioning System (GPS) collars store data and drop off the animal when expires to allow data retrieval; others transmit the data to another set of satellites that relay it to the researchers. GPS tracking also has a high initial cost and at present is relatively short-lived and applicable to mammals the size of a wolf or larger, or to birds on which solar cells can be used (Mech, 2002). Today, Differential Global Positioning Systems (DGPS) have now replaced the original

GPS applications almost completely, even though the original acronym can be used interchangeably (Mech & Barber, 2002).

Table 1.1 Adapted from (Merrill, 2002); Collar weight varies by Species and Collar Manufacturer; Depending on Frequency of Data Downloading.

	Characteristics	**VHF**	**Satellite**	**GPS Collar**
1	Collar Weight[2]	560 g	520 g	830-920 g
2	Initial investment per collar	$300	$3,000	$3,000
3	Cost per 100 locations	High	Medium	Low
4	Data retrieval potential	High	High	Low to high depending on likelihood of dispersal
5	Accuracy	Medium to high depending on effort	(+/-500 m)	High; usually accurate to 20 meters
6	Longevity	< or = 6 years	1-12 months depending on cycling	3 weeks- 10 months depending on interval between location attempts
7	Interference from weather	High (aerial telemetry)	None	None
8	Interference from habitat	Low	High	High
9	Interference from topography	Medium	High	High
10	Intrusiveness after collaring	High	None	None to high[3]

Comparative Characteristics of Global Positioning System (GPS) Collar Telemetry Very High Frequency (VHF) and Satellite, ($ =USD)

There are three components to a radio tracking system - a transmitter a receiver and a power source. The transmitter is attached to a collar worn by the animal. The transmitters emit a signal on a specific frequency that can be monitored by a receiver. The receiver, carried by the handler, is a small radio-like device fitted with a special directional antenna. The directional antenna is utilized to receive the radio waves emitted by the transmitter and inputs this into the receiver. The receiver then interprets the signal strength and displays the data in the form of a needle, row of LEDs or LCD display often in conjunction with an audio speaker. By taking a reading (sweeping the antenna in a 360-degree arc) the direction of strongest signal can be determined thereby indicating the bearing to the animal tagged.

Commercially available transmitters range in weight from 350 milligrams (Kenward, 2001) to 400 grams and more (Samuel & Fuller, 1996). Global Positioning System (GPS) transmitters generally weigh 100 grams or less; some prototypes weigh as little as 33 grams (Kenward, 2001). Transmitter weight should not exceed 5% of the animal's body mass (Kenward, 2001), and researchers who abide by this protocol report few negative effects on their telemetered subjects (Agren, 2000; Fitzgerald, 2003; Whitaker & Shine, 2002).

Although, some researchers have exceeded this guideline due to special circumstances it has been advised that transmitter mass should be kept well below the recommended standard whenever possible (Andersen, 2000; Kenward, 2001). Andersen further suggests limiting transmitter weight to 2-3% of body mass in studies that involve bats and birds, both of which rely on lift for escape and survival.

The smallest of satellite transmitters may negatively affect the behavior of certain pelagic bird species. Additionally, they reported a very high mortality rate associated with use of implantable satellite transmitters has been reported (Philips, 2003; Krausman, 2004).

1.3 Mapping Techniques

1.3.1 Biotope Mapping and Cartalinx

A biotope is very broadly defined as biota interacting with the physical habitat: plants and animals found at a location together with their immediate physical surroundings. It is a term that stems from a practical community ecology in which ecologists have attempted to summaries what they see (and count or measure) in the field for the purpose of describing general ecological patterns (Yılmaz,1997).

The biotope has two main components:

(a) The plants and animals (biota)

(b) The physical habitat features.

The biotope concept stems from practical community ecology in which ecologists have attempted to summaries what they see (and count or measure) in the field for the purpose of describing general ecological patterns. Biotope mapping should be a reasonably rapid process and one that is not too susceptible to differences in interpretation between field recorders. Thus, biotope should be easy to recognize and identify with confidence (Freeman, 2003).

1.3.2 Cartalinx Overview

CartaLinx is a spatial database development and topological editing tool that can be used with various Geographic Information Systems (GIS) software products such as IDRISI®, ArcGIS® and MapInfo®. CartaLinx is used to create, manipulate and build topology of vector coverage and associated attribute values files. The data are then typically exported to a GIS either as entire coverage or as a series of map layers. CartaLinx flexibly allows for data entry from a variety of sources, including the use of a mouse as a coordinate input device, COGO (coordinate geometry) bearing and distance measurements, and any GPS device capable of outputting NMEA1 format data records over an RS-232 cable connection. Over 500 digitizing tablets are supported through the Virtual Tablet Interface™.

CartaLinx is a registered trademark of Clark University. ArcGIS is a registered trademark of ESRI, Inc. MapInfo is a registered trademark of MapInfo Corporation. Access and Windows are registered trademarks of Microsoft Corporation. VTI is a trademark of Digitizer Technology Company (taken from Clarklabs internet website).

1.3.3 Features

CartaLinx uses a full-topology editor/digitizing system with capabilities for automatically building vector topology (connectivity between nodes, arcs and polygons); automated generation of polygons and assignment of ID's by means of polygon locators (label points); insertion, deletion or movement of nodes, arcs or arc vertices; real-time projection/datum transformation of digitizer and GPS input data to meet mapping reference system specifications; and feature filtering

and extraction to new spatial databases based on feature attributes (filter) or location (clip).

CartaLinx also has significant analytical and mapping capabilities, including relational database query support, producing custom maps based on specific feature attributes; support for mathematical modeling, creating new derivative data fields using mathematical operations on existing fields; interactive feature inquiry, instantly displaying attribute data for any feature by simply clicking on that feature; real-time route monitoring using GPS with an image backdrop; and text placement with scale-sensitive sizing (taken from Clark Labs internet website) .

1.3.4 GPS and GIS

Recent technological advances in satellite global positioning systems (GPS) and Geographic Information Systems (GIS) have revolutionized the field of wildlife telemetry, enabling researchers to greatly improve the overall quality and quantity of movement data collection and analysis compared with results obtained with traditional observational or tracking techniques (Harris, 1990; Millspaugh & Marzluff, 2001).

Technological advance has led to the increased use of Global Positioning Systems (GPS) and Geographical Information Systems (GIS) in the study of ecological problems in time and space (Haslett, 1990; Green, 1993). The GPS technique obtains information with higher precision, especially at the spatial scale. GPS and GIS therefore constitute excellent tools for studying spatial and temporal aspects of wildlife (Slonecker & Carter, 1990). GIS is a system of hardware and software used for storage, retrieval, mapping, and analysis of geographic data GIS can also be described as including the operating personnel

and the data that go into the system. Spatial features are stored in a coordinate system (such as, Latitude / Longitude, State Plane, Universal Transverse Mercator (UTM), etc.), which references a particular place on the earth Descriptive attributes in tabular form are associated with spatial features. Spatial data and associated attributes in the same coordinate system can then be layered together for mapping and analysis.

Components of GIS might be the followingVector Data, Raster Data, Metadata, PC, Software, and Database. GIS can be used for scientific investigations, resource management, and development planning; Data from various sources can be brought together into a common database and integrated using GIS software.

Data types should be defined as the following:

1. Vector Data Points, lines & polygons
2. Raster Data Arial photos, Sat images, sonar images, radar data
3. Metadata Data about data

The application of GIS may be many but to mention few:

1. Mapping where things are (Data from GPS)
2. Overlay (Overlaying different data using maps to look for relationships)
3. Change analysis (Overlaying time series data; Quantifying change, i.e. area of habitat recovery)
4. Data integration (Using data from a range of sources on the same computer)

5. Spatial Analysis (Distance measurements; Neighbourhood relationships)

1.4 Molecular Techniques

1.4.1 Integration of Field Methods and Molecular Biology

Scientists have generally inferred spacing patterns of mammals and social structure from behavioral observations, mark-recapture data, or radio-telemetry locations. Obtaining such data in the field is challenging and resulting datasets often suffer from small sample sizes or lack of data for 1 or more study animals (Aubry, 2004).

Logistical constraints on the geographic extent of most radio-telemetry studies may prevent long-distance movements or dispersal events from being detected and such movements can also be confused with transmitter failure (Avise 1994). Furthermore, unsuccessful copulations, cuckoldry, or multi-paternity litters can be impossible to detect with traditional research approaches, and familial relationships can be inferred incorrectly when adults who are not the biological parents are behaviorally or spatially associated with young (Avise, 1994)

Studies of the ecology, behavior and social organization of primates in the wild have contributed to the understanding of mammalian social systems and their evolution (DiFiore, 2003). Nevertheless, even in the most complete long-term studies of wild primate populations, it is difficult to fully elucidate certain features of social systems such as dispersal patterns, patterns of within-group, relatedness, and the effective genetic mating system (DiFiore, 2003).

It is impossible through observational studies alone to fully evaluate the effect of kinship on shaping patterns of social behavior or to examine the link between individual behavior (dominance interactions, alternative mating tactics) and reproductive success (DiFiore, 2003).

Literature review has shown that fields such as ecology and genetic information could combine and explain remarkable scientific questions (Hughes, 1998). It has proven essential the development of genetic analysis of animals living in social groups in the wild, for uncovering patterns of paternity and relatedness among individuals, and understanding male and female reproductive strategies (Coltman et al, 1999; Worthington Wilmer et al, 1999; Di Fiore, 2003; Griffith, 2002; Vigilant, 2001). Scientists are able to infer several aspects of behavior, sociology, and spacing in animals from the genetic patterns uncovered by relevant markers. Molecular ecology (a field of evolutionary biology which is concerned with the application of molecular genetics) has been contributing to answer several aspect in the animal kingdom such as reproductive success (Stockley, 1994), individual identification (Roques, 1999), the mating system (Zenuto, 1999), kinship (Queller & Goodnight 1989; Queller et al, 1993), and dispersal (Favre et al, 1997; Mossman & Waser, 1999).

The development of molecular techniques over the last decade has provided evolutionary and conservation biologists with new tools to address fundamental and applied research issues (Parker, 1998; Haig, 1998). There has been an intensive growth of research on molecular genetics from the past two decades to investigate relatedness between individuals in a population, reproductive success, and mating systems, providing a wealth of new information about the biology of many species, including genetic structure, dispersal, social structure and other behavioural parameters (Birkhead, 1990; Gilbert, 1991; Pemberton 1992; Amos, 1993; Morin, 1994; Webb, 1995; Burke & Bruford, 1987).

Application of such analyses in the study of endangered species should thus provide vital information regarding aspects of their ecology. A number of informative review papers describing types of genetics markers, their analysis and their uses in behavioral and ecological field studies can be consulted in (Hillis, 1996; Avise, 1994; Ferraris & Palumpi, 1996; Smith & Wayne; 1996; Baker, 2000).

1.4.2 Molecular Markers in Behavioral, Social Structure and Genetic Structure in Non-human Primates

Like most techniques, genetic methods have strengths, weaknesses, and limitations (Hedrick, 1999). Nevertheless, when used properly they can become significant tools to answer questions which may not be evident from observations alone (Avise, 1994, 2000); especially in circumstances where more traditional methods are inadequate to address key issues or evaluate hypothesis, (Ferraris & Palumbi, 1996; Goldstein & Schlötterer, 1999). There are several molecular techniques (Table 1.2) to determine familial relationships and, on a smaller scale, relationships among lineages and social groups, and the redefinition of the interface between social behavior, social structure and population genetics (De Ruiter, 2004).

Table 1.2 Appropriate molecular techniques for studies involving relationships (maternal and paternity) in primates (De Ruiter, 2004)

Molecular technique	Sample material required	Research areas addressed	Associated drawbacks
Protein analysis	Blood or other tissues	Unravelling family or social group relatedness	Not all variability in genes leads to protein differences
Restriction fragment analysis	Fresh tissue with sufficient quality and quantity of DNA	Mainly paternity determinations based on the absence of alleles in know possible fathers	DNA fragment patterns do not relate directly to specific genes
PCR analysis plus genotyping	Small quantities of DNA from, for example, faeces or hair roots	1. Microsatellites Paternity analysis based on the similarity and prevalence of alleles between the individuals concerned	Some heterozygote individuals may appear homozygote because of the non-amplification of null alleles with poor quality DNA or low copy numbers
PCR analysis plus sequencing		2. mtDNA Investigating family relationships and female dispersal	
		3. Y-chromosome analysis Investigating family relationships and male dispersal	Only a few microsatellites and little sequence variation
		4. Various genes of interest such as the MHC	

In the 1970s, mitochondrial DNA or mtDNA was recognized as a powerful genetic marker. Before that time, cytoplasmic genome was thought to be an interesting evolutionary relict from a time when eukaryotic cells were first invaded by the bacteria-like ancestor of modern mitochondria (Margulis, 1981). For many reasons, mtDNA is a valuable molecular marker; most notably, it is usually maternally inherited without recombination, and thus the molecular signals of genetic drift are particularly robust (Avise, 2004). The mtDNA is advantageous

for scientist addressing lineage-specific issues (McLuckie, 1999) and has been used widely for the study of population structure due to the high evolutionary footprint of differential dispersal it shows between the sexes (Prugnolle & de Meeus, 2002).

Microsatellite loci are short tandem repeats of DNA sequences (2-5 base-pairs) that occur throughout the genomes of most eukaryotes (Amos, 1999; Litt & Luty, 1989; Weber & May, 1989; Tautz, 1989). High mutation rate at microsatellite loci results in high levels of polymorphism, making this marker ideal for studies of population genetic structure, genetic relatedness, patterns of gene flow, genetic drift, and population size changes (Beaumont & Bruford 1999 ; Chambers & MacAvoy, 2000).

1.4.3 Mitochondria

The mtDNA is situated in mitochondria and the nuclear DNA is situated in the nucleus (Figure 1.5). It has been established that the two types of DNA have a different evolution origin. The endosymbiotic theory explains that MtDNA derives from the bacterial hereditary material, as a consequence of the incorporation processes of these microorganisms in the precursor eukaryotic cells, without being digested after that (Margulis, 1970). Mitochondria are now generally believed to have originated by a symbiotic relationship between two types of bacteria: a protoeukaryote without mitochondria and a proteobacterium with respiration but a much simpler internal cellular organization.

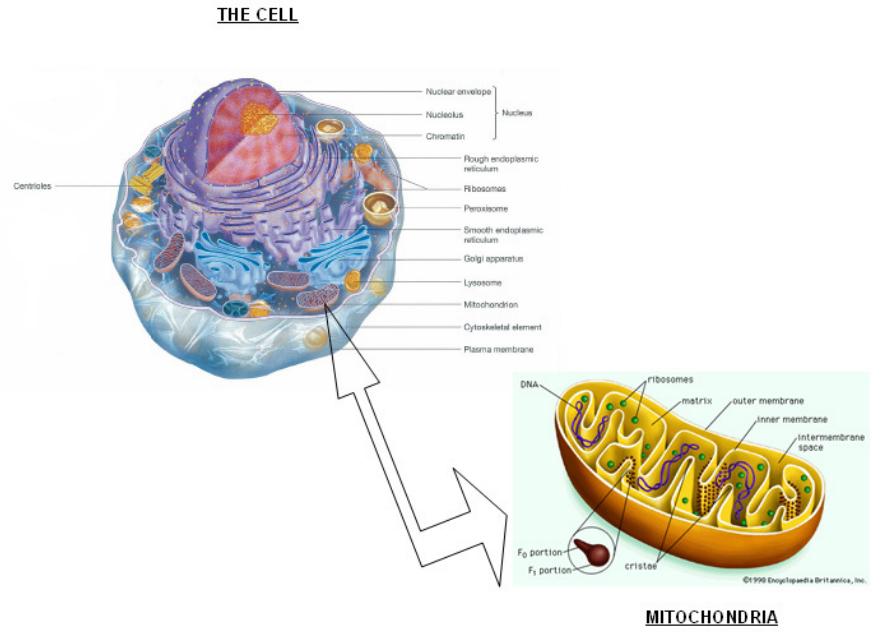

Figure 1.5 Showing parts of the Cell and Location of the Mitochondria within it (Encyclopedia Britannica, Inc. 1998)

This engulfed the other, which eventually became mitochondria. The full sequence of events proposed by this theory has been discussed by Margullis (1981) and further described by others (Gray, 1999, Lang, 1999; Brown & Doolittle 1997). Mitochondrial DNA is a closed circular molecule of 16, 500 to 17,200 bp in most mammals (Arnason, 2002), (Figure 1.6). The human mitochondrial DNA size is 16.6 kb and it was the first human chromosome to be completely sequenced, in 1981 (Anderson, 1981). The mitochondrial genome lacks introns, has small intergenic spacers where the reading frames even sometimes overlap (Skelly & Maleszka, 1991). It is composed of about 37 genes coding for 22 tRNAs, two rRNAs and 13 mRNAs, the latter coding for proteins mainly involved in the electron transport and oxidative phosphorylation of the mitochondria (Rubio, 2000). The control region (Figure 5) is very compact little non-coding sequences, essentially just the 1 kb D-loop region, and has even some overlapping genes (Clayton, 1982). The D-loop is located between two

tRNA genes, F and T. It is the primary non-coding region, and is responsible for the regulation of heavy OH and light OL strand transcription and of H-strand replication (Clayton, 1991).

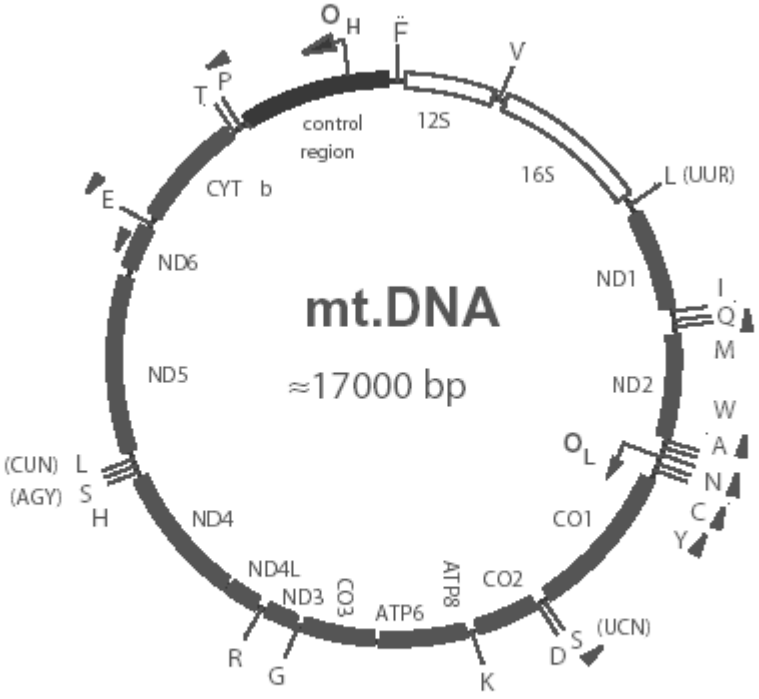

Figure 1.6 Showing Mammalian Mitochondrial Genome (Arnason, 2002)

mtDNA codes for 13 subunits of the respiratory chain. Subunits I, II and III of cytochrome *c* oxidase are encoded by *COXI, COXII* and *COXIII*. One subunit of cytochrome b is encoded by the *CYTB* gene. Seven subunits of NADH dehydrogenase are encoded by *ND1-6* and *ND4L* (Attardi, 1986). The FO portion of the ATP synthase has two mitochondrial encoded subunits, ATP6 and ATP8 also called A6 and A8 (Mariottini, 1983).

The RNAs needed for mitochondrial translation are encoded by mtDNA in humans and other metazoans. Ribosomal RNAs needed in ribosomes are encoded by 12S and 16S rRNA genes. A set of genes for 22 transfer RNAs is found in human mtDNA (Barrell, 1980). No other rRNA genes are known to be imported into mitochondria. Leucine and serine each require two tRNAs, dedicated to different codon groups: Leu (UUR) and Leu (CUN), and Ser (UCN) and Ser (AGY) respectively, whereas all other amino acids each require only one tRNA to read their codons. (Barrell, 1980) Cells contain a high copy number of mitochondrial genomes, the number varying from one cell type to another, between 1,000 and 10,000 copies per cell (Lightowlers, 1997; Larsson & Clayton 1995). Normally short-lived human cells (sperm leukocytes) have a low mtDNA copy number, whereas long-lived cells (skeletal muscle, brain cells, oocytes) tend to have a high copy number of mtDNA (Chynnery & Samuels 1999).

Mammalian mitochondrial DNA is believed to be organized in structures called nucleoids. This is a protein-DNA complex having 4-5 copies of mtDNA in yeast (Miyakawa, 1987, 1995) and probably around 5 to tens of copies. The number of nucleoids varies from one organism to another and from once cell type to another (Spelbrink, 2001).

Cells are homoplasmic for a give mtDNA, despite the fact that mtDNA sequence variation between individuals in the population is high, due to a higher frequency of mutations in mtDNA, 5 to 10 times higher than in the nuclear genome (Brown, 1979, 1982). Two models of mitochondrial DNA replication are well documented by (Clayton, 1982; Holt, 2000). In the first model, there are two replication origins, one for each strand. Synthesis of the leading (H-) strand stars at the heavy-strand origin (OH) adjacent to the D-loop and continues by strand displacement around two-thirds of the genome (Clayton, 1982). The model which proposes

that replication begins from a single origin and that the leading and lagging strands are synthesized simultaneously (Holt, 2000)

1.4.4 D-Loop - mtDNA Control Region (CR)

Non-coding mtDNA control region (CR) or D-Loop is a non-coding sequence of variable length containing the promoters for the transcription (LSP, HSP), heavy-strand replication origin (O_{II}), and the displacement loop (D-loop) in vertebrates (Chang & Clayton, 1986; Clayton, 1982). The control region, also called D-loop region in vertebrates and hyper variable region in humans, evolves usually more rapidly than the other mitochondrial protein coding regions (Brown, 1986). The CR has been extensively employed in population genetic surveys in a broad variety of organisms including primates (Clifford, 2004, Warren 2001; Jensen, 2001).

D-loop mitochondrial DNA is maternally inherited in most species (Gyllesten, 1991; Zouros, 1992). Sequence data from the Control Region are more effective than cyt b and other mitochondrial coding regions for the identification of closely related species (Brown, 1986).

Control Region or D-Loop sequences are widely used in animal phylogeographic studies due in part to the mitochondrial genome (mtDNA) characteristics including maternal inheritance, the absence of recombination and the evolutionary information that can be drawn from sequence data (Bowling, 2000; Kavar, 1999; Kim, 1999; Oakenfull, 2000; Vilà, 2001; Oakenfull & Ryder, 1998; Avise, 1994, 2000).

Studies carried out by using allozymic and chromosomal polymorphisms have failed to reveal geographic patterns of variation. However, DNA sequencing

provides a more sensitive and powerful tool to address questions related to social and population genetic structure, phylogeography and phylogeny (Mudry, 1981; Szapkievich, 2003). Moreover, some regions of mtDNA, the non-coding control region (CR) or 'D-loop', have a high substitution rate, which makes them very useful in the reconstruction of recent demographic events (Hewitt, 2000).

The non-coding control region of mitochondrial DNA has become one of most commonly used markers for analyzing patterns of intra-specific geographic variation, demographic past of (populations and species) and population structure due to its maternal transmission, rapid evolution, lack of recombination, and the possibility of ordering multiple haplotypes by phylogenetic reconstructions (Brumfield, 2003; Anderson , 1981; Avise, 1987, 1993; Wallace, 1995 ; Harrison, 1989 ; Sunnucks, 2000; Ankel ,1996)

This tool has proven invaluable for the new fields of molecular ecology and phylogeography (Ballard, 2004). Control Region or D-Loop sequences provide valuable information when assessing phylogenetic relationships and phylogeography in monkeys (Hayasaka, 1988; Tosi, 2003; Smith & McDonough, 2005; Kawamoto, 2006).

There are several reasons why undertaking sequencing of Mitochondrial DNA-D-loop can be more successful than genomic DNA. One of those is much higher copy number of mitochondrial DNA (Robin, 1998). Furthermore, mitochondrial DNA is strictly maternally inherited and thus is not subject to recombination in meiosis (Giles, 1980). Hence an individual usually has a uniform mitochondrial DNA population (Brown, 1982). Consequently, the mitochondrial sequences of relatives within a population or group for sample individuals related within the same group are identical.

D-Loop Mitochondrial DNA sequences is simple and straightforward and therefore provides a useful tool for the individual identification (Bowling, 2000). The variability generation of mitochondrial DNA can only occur through new mutations. The control region or D-Loop region is the most polymorphic region of the human mtDNA (Cann, 1984; Aquadro, 1983). The steps involved in performing mitochondrial sequence comparisons are illustrated in the (Figure 1.7)

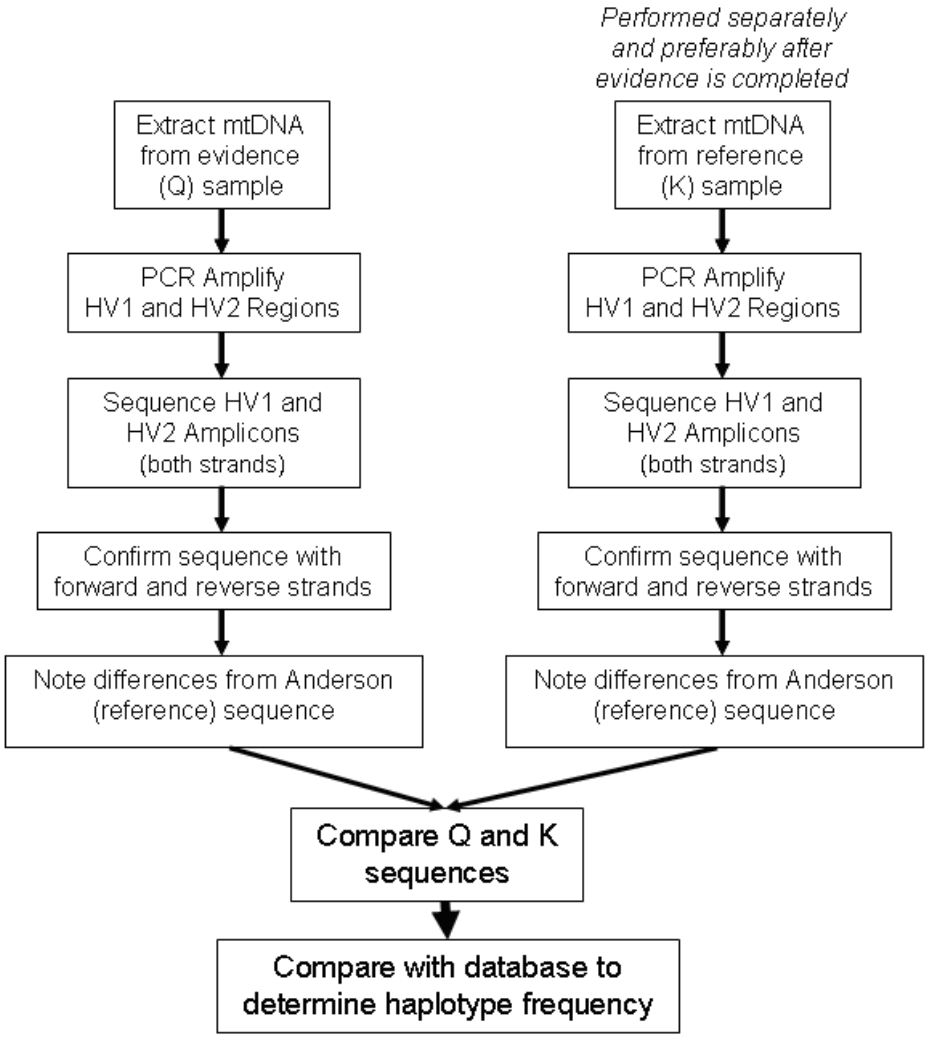

Figure 1.7 Steps involved in performing Mitochondrial Sequence Comparisons (Butler, 2005)

1.4.5 Microsatellites

A decade after the development of mtDNA as a molecular marker, geneticists recognized that locus (chromosomal) repetitive elements in the nuclear genome could be used as genetic markers (Avise, 2001) (Figure 1.8).

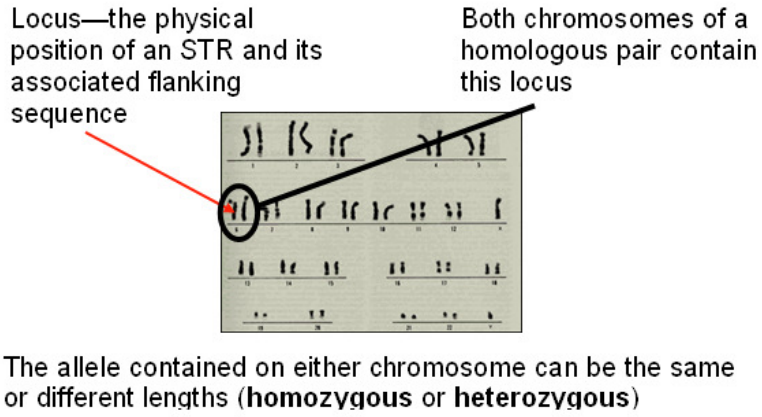

Figure 1.8 Showing Chromosomes Karyotype Analysis

Repetitive DNA consists of tandem repeat sequences and interspersed repeats. Tandem repeated sequences account for 10% of the genome. These regions consist of sequences which are a repeat of anywhere from 3-250 bp in length. These are typically referred to as satellite DNA because of the tendency of the sequences to form “satellite” bands in equilibrium density gradients. This simplifies the separation of these from other chromosomal DNA.

- Types of repeated DNA
    - Tandomly repeated
        - Telomeres
        - Satellite (VNTRs)
        - Minisatellite (STRs)

The existence of two or more discontinuous, segregating phenotypes in a population is called 'polymorphism'. Polymorphic microsatellites genetic markers are widely used in several fields such in ecological studies of wild populations by establishing familial relationships amongst sampled individuals by genetic typing, and by examining genetic structuring with respect to the spatial distribution of animals in a population (Dayanandan, 1998). Aspects of the dispersal and mating system might result in spatial structuring of genetic relatedness (Morin 1994; de Jong 1994; Pope, 1992). These markers are very useful in paternity analysis, having data on single loci has the additional advantages that paternity estimates can be made not via exclusion process, where in the potential fathers must be known, but via inclusion analysis (De Ruiter, 2004).

Microsatellite loci are DNA sequences that contain short, repetitive elements whose copy number varies among alleles. They are found thousands of times in vertebrate genomes and are not expressed, microsatellites generally are considered to be neutral markers, barring tight linkage to a functional gene (Bruford & Wayne, 1993). Microsatellite sequences can be replicated in vitro by means of the polymerase chain reaction (Erlich & Arnheim, 1992).

The genomes of most eukaryotes contain thousands of loci containing short nucleotide sequence motifs tandemly repeated many times (Tautz, 1986), such as (CT/GA) n. These tandem repeat loci are categorized rather arbitrarily into groups based on the size of the repeat unit. Loci containing shorter repeat units are called microsatellites, also referred to as simple sequence length polymorphisms (SSLP), simple sequence repeats (SSR), or short tandem repeats (STR) (Bruford, 1993). Loci with larger repeat units are called minisatellites (the loci used in the original form of DNA fingerprinting).

STR loci are part of a larger class of polymorphic loci, which are based on length polymorphisms arising from the presence of alleles having varying numbers of tandem repeats (Debrauwere, 1997; Schlotterer, 1994). They often occur in the un-translated parts of known genes and consist of short, repetitive sequence elements of 2 to 7 base pairs in length (Tetranucleotide: AAAG AAAG AAAG AAAG; Trinucleotide: CTT CTT CTT CTT CTT; Dinucleotide: GAG AG AG AG AG. Typically tetranucleotide and pentanucleotide repeats are used because they have a high degree of variability between individuals and are easily interpreted on sequencing gels and capillary electrophoresis instruments.

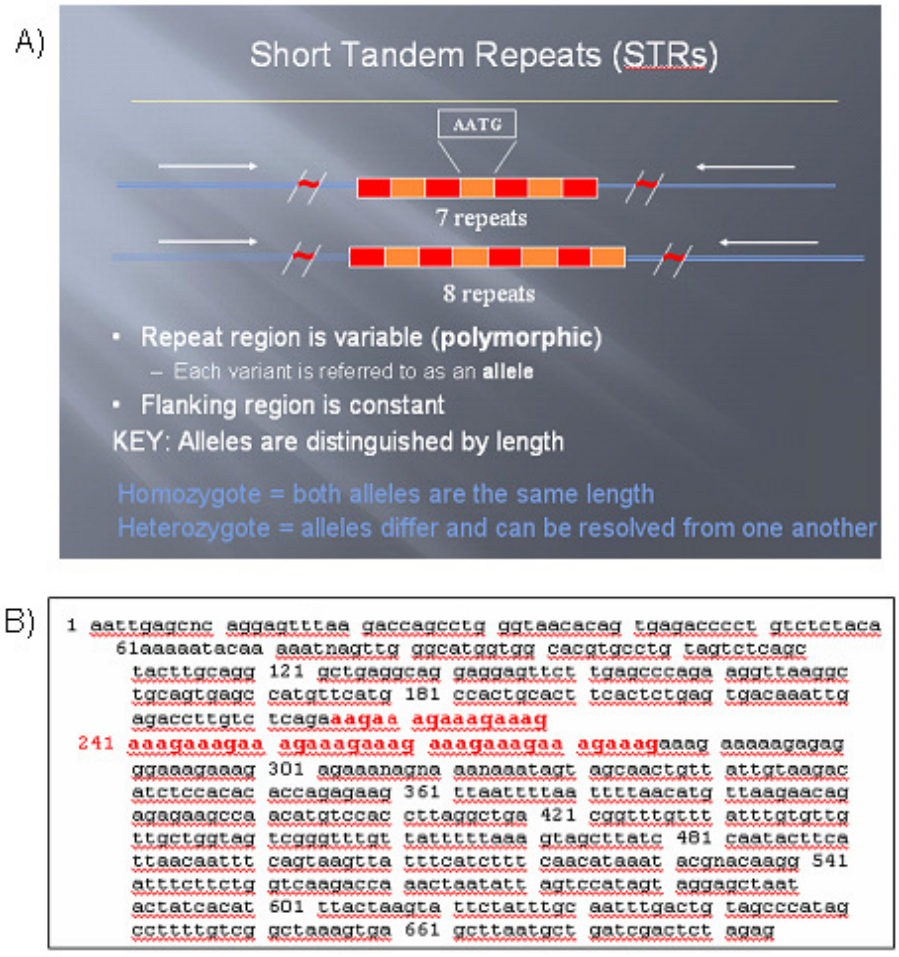

Figure1.9 (A) Showing the Short Tandem Repeat AATG (B). The Repeat Sequence is “aaga” – This particular individual has 14 repeats

The exact number of repeats varies in each organism and therefore, PCR amplification will result in PCR products of different lengths depending on the number of tandem repeats (Lawler 1991; Hammond, 1994; Crouse, 1995) (Figure 1.9 A and B).

Variation at microsatellite loci is thought to result from slip-strand mispairing, a process that adds or removes repeat units during DNA replication, resulting in a greatly elevated rate of mutation (approximately 10^{-3} to 10^{-5})per generation) compared to other neutral loci (Jeffreys 1985, Levinson & Gutman 1987; Schlötterer & Tautz, 1992; Ellegren, 2000).

The eukaryote genome is highly interspersed with microsatellites. Poly (G) and polu (A) are the simplest of the microsatellites (AC) is by far the most frequent, appearing as 5 10X10^4 in the individual mammalian “islest” genome (Hamada, 1992). Many other microsatellites have been reported, among them: polu 9TC, poly (CAC), and poly (GATA) (Tautz & Renz, 1984)

These abundant microsatellite repeats are well distributed throughout the organism genome and are a rich source of highly polymorphic markers, which often may be detected using PCR. Even though DNA sequences vary from individual to individual, the same STR sequences are found in similar locations in almost every individual genome. The numbers of repeats of these short sequences are unique to each individual (Strand, 1993). By mapping multiple STR locations, a unique fingerprint for any individual can be created. Alleles of these loci are distinguished from one another using radioactive, silver stain or fluorescent detection following electrophoretic separation, the microsatellite protocol is simple, once primers for SSRs have been designed. The first stage is a PCR, depending upon the method of detection one of the primers is fluorescently or radioactively labeled. The PCR products are separated on high

resolution polyacrylamide gels, and the products detected with a fluorescence detector (e.g. automated sequencer) or an X-ray film. The investigator can determine the size of the PCR product and thus how many times the short nucleotide was repeated for each allele (Figure 1.10), where repeat lengths migrate different distances according to their sizes.

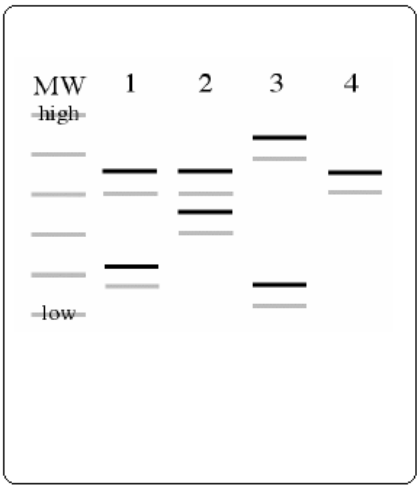

Figure 1.10 Polymorphism in Microsatellites

The amplification products of STR are less than 400bp long, much smaller than VNTR that usually have core repeats of 9 to 80 bp and expansion size of 100 bp to 20 kb (Schumm, 1996; Schichman, 2002). The smaller size means that the PCR can be used to amplify very small amounts, less than 1 ng of DNA and it also permits analysis of degraded DNA (Promega, 2001). The STR DNA sequences also show sufficient variability among individuals in a population that they have become important in several fields including genetic mapping, linkage analysis and forensic studies (Jarne & Lagoda, 1996).

1.4.6 How to Obtain a Microsatellite Marker?

1.4.6.1 Isolation and Development of Microsatellite DNA

Polymorphic microsatellites loci have been isolated from partial genomic libraries (selected from small insert size) of the target species by using the traditional method. It requires screening the library for clones bearing one or more tandem repeats, sequencing the clones and developing PCR primers to amplify the tandem repeat (Tautz 1989; Asley and Dow 1994; Queller, 1993) (Figure 1.11).

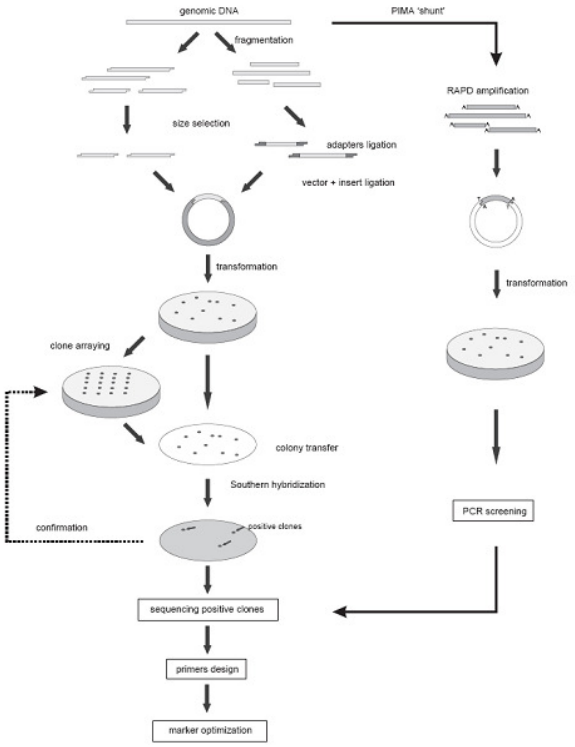

Figure 1.11 Schematic representation of Microsatellite Isolation (Zane, 2002)

However this traditional approach of isolating microsatellites markers is tedious and inefficient for species with low microsatellite frequencies. Therefore, several protocols have been devised in order to reduce the time invested in microsatellite isolation and to significantly increase yield (Zane, 2002), (Table 1.3).

Table 1.3 Library cost, time investment, and yield compared among the different protocols of microsatellite isolation (Zane, L. 2002)

Protocol	Protocol set up (US dollars)	Library (US dollars)	Time†	Yield
Traditional	2000–4000	< 400	1 month	Low
RAPD based	1000	< 100	1 week	Variable
Primer extension	1000–4000	< 400	2 weeks	Medium/High
Selective hybridization	1000–4000	< 400	1–2 weeks	Medium/High
Private companies	None	5000–10 000	None	High

1.4.6.2 The Application of Microsatellites Isolated From Various Species in Order to Amplify the Corresponding Polymorphic Loci in Related Species

The fact that novel markers often have to be isolated, when analyses of new species start, acts as hindrance for the widespread utilization of microsatellites among ecologist lacking facilities for cloning and sequencing (Primmer, 1996). Microsatellite markers that are sufficiently polymorphic in a previously unexamined species of interest present a challenge. The identification of these novel markers requires some technical expertise and an average of several months of work (Zane, 2002). Moreover, because of the costs associated with cloning species-specific markers, human microsatellites markers are increasingly being used to amplify simple tandem repeat polymorphisms (STRPs) in nonhuman primates. Some microsatellites are highly conserved between humans and non-human primate species (Witte & Rogers, 1999; Coote & Bruford, 1996; Gerloff, 1995; Morin & Woodruff, 1992; Constable, 1995; Kayser, 1996; Launhardt, 1998; Washio, 1992; Rogers, 1992).

There are several publications describing the application of microsatellites isolated from various species to amplify the corresponding and polymorphic loci in closely related species but not in more distant species. Together, these studies suggest that it may be possible to establish different sets of markers usable over a broad range of species (Moore, 1991; Pepin, 199; Deka, 1994; Schlotterer, 1991; Roy, 1994; FredhoLm & Winter 1995; Breen, 1994; Estoup, 1993).

One drawback of cross-species amplification is that success rates decline with evolutionary distance between the target species and source species. Cross-species amplification success varies not only between taxonomic groups, but also among microsatellite loci. Although many markers fail to amplify even in closely related species, some markers have higher utility than others (Primer, 1995, 1996 and 2005; Galbusera, 2000; Dawson 2000).

1.4.6.3 Multiplex STR System Information

The STR loci or DNA microsatellite may be amplified using a defined set of co-amplifiable PCR primers for two or more STR loci combined in one mixture. The desire to gain more information from a sample, coupled with the need to limit consumption of a DNA sample where its availability may be limited has led to the co-amplification and typing of multiple STR systems. Multiplex PCR, which involves adding more than one set of STR primers to the reaction in order to target multiple locations throughout the genome, is an ideal technique for DNA typing (Sprecher, 1996). This multiplex amplification strategy can be applied to the CSF1PO, TPOX, TH01 triplex loci and F13A01, FESFPS, vWA triplex.

By using 3 STR loci, which are TH01, TPOX and CSF1PO the chance for two different individual sharing the same allele for all three loci, would be

approximately 1 in 800. However by combining this multiplex with another multiplex containing F13A01, FESFPS and vWA markers, that is by using 6 different STR scattered at different chromosomes, the power of discrimination increases to approximately 1 in 1.4 x 10^6 (Promega, 2001). Allelic ladders also have been developed for these STR loci to simplify allele's determinations.

1.4.6.4 Allelic Ladder

Allelic ladders are composed of a collection of most or all of the amplified alleles found for the genetic loci of interest in the general population (Duewer, 2004). This has become an ideal size marker in STR typing, because the size markers and the amplified unknown alleles will contain not only the same size fragments, but also the same sequence fragments or repeat units. The assignment of alleles using a well-defined allelic ladder is a precise, reliable, simple and easy method, which is independent of the separation and detection methods used with it. Allelic ladder components and samples co-migrate in gel electrophoresis regardless of the gel matrix or running buffer selected (Schumm, 1997).

Furthermore, the allelic ladder for STR loci could be used in combination, and they separate well following electrophoresis. Sequence analysis of each ladder component indicates that fragments differ by integral multiples of the core repeat sequence of each locus and all alleles included in the allelic ladders have been sequenced to confirm the DNA sequence and number of repeat units contained within each allele (Schumm, 1997; Lins, 1998). By using this discrete ladder, two different alleles of a heterozygote, which differ by only 4 bp, resolved by a highly discriminating technique such as the denaturing polyacrylamide gel electrophoresis, can be precisely assigning. Whereas for VNTR, ideally the size difference between two bands should be at least 50 bp (Wang, 2002).

1.4.6.5 Cross-species Microsatellites

The identification of novel microsatellite markers that are sufficiently polymorphic in a previously unexamined species of interest presents a challenge. The identification of novel microsatellite markers requires some technical expertise and an average of several months of work (Zane, 2002). Moreover, the costs associated with cloning species-specific primers have brought scientist to use primers already developed to amplify microsatellites markers, or simple tandem repeat polymorphisms (STRPs) in nonhuman primate species. Some STRP priming sites are highly conserved between humans and many non-human primates' species (Washio, 1992; Rogers, 1992; Constable, 1995; Witte & Rogers, 1999).

Therefore the strategy of cross-species amplification (ie., using loci characterized in one species to analyze representatives of another usually closely-related species) has been widely used in primates (Bradley, 2000; Coote & Bruford, 1996; Kayser 1996; Moore 1991; Morin & Woodruff, 1992; Perelygin, 1996; Rogers 2000; Smith, 2000).

1.5 The Polymerase Chain Reaction PCR

1.5.1 The Principle of PCR

The principal of the Polymerase Chain Reaction (PCR) was first reported in 1971 (Kleppe, 1971), but it was only after the discovery of the thermostable Taq DNA polymerase that this technology became easy to use (Saiki, 1988; Lawyer 1989). PCR is an enzymatic process in which a specific region of DNA is replicated over and over again to yield many copies of a particular sequence (Saiki, 1988; Reynolds, 1991). This molecular process involved three main steps that are

commonly called as denaturation (heating), annealing (cooling) and elongation (extension) which occured in a precise thermal cycling pattern over a minimum of 30 cycles. The two DNA template strands are first separated or denatured by heat. The sample is then cooled to an appropriate temperature to bind (anneal) the oligonucleotide primers. Finally the temperature of the sample is raised to the optimal temperature for the DNA polymerase and it extends the primers to produce a copy of each DNA template strand. For each cycle, the number of DNA molecules (with the sequence between the two PCR primers) doubles (Saiki & Gelfand 1988) (Figure 1.12)

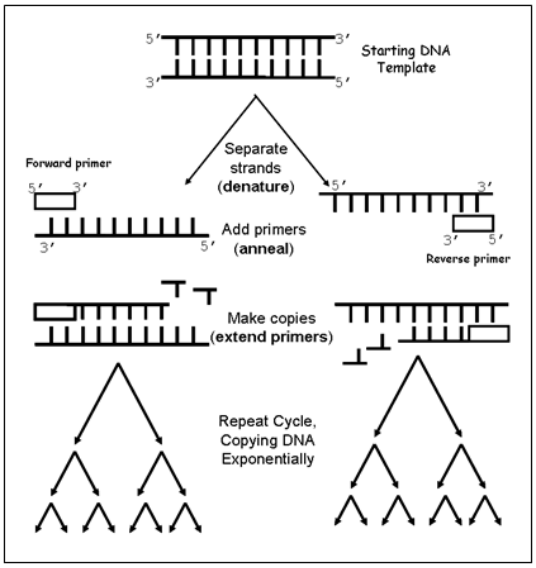

Figure 1.12 DNA amplification process with the Polymerase Chain Reaction (Butler, 2005)

The idea of for the Polymerase Chain Reaction started 1983 with Kary Mullis while working in a biotechnology firm located near Berkeley, California (Mullis, 1990). In 1993 Mullis was awarded the Nobel Prize for the discovery of PCR. The description of PCR was published for the first time in the paper about detection of the mutation causing sickle cell anemia in whole genomic DNA (Saiki, 1985). The

details of the PCR method and its uses were published in articles in the next two years (Mullis, 1986, 1987).

1.5.2 The Standard Thermal Cycling Parameters for PCR

The PCR Thermal cycling typically involves 3 different temperatures that are repeated over and over again between 25 to 35 times. At 94 °C, the DNA strands separate, or “denature”. At 60 °C, primers bind or “anneal” to the DNA template and target the region to be amplified. At 72 °C, the DNA polymerase extends the primers by copying the target region using the deoxynucleotide triphosphate building blocks. The typical PCR parameter is shown below:-

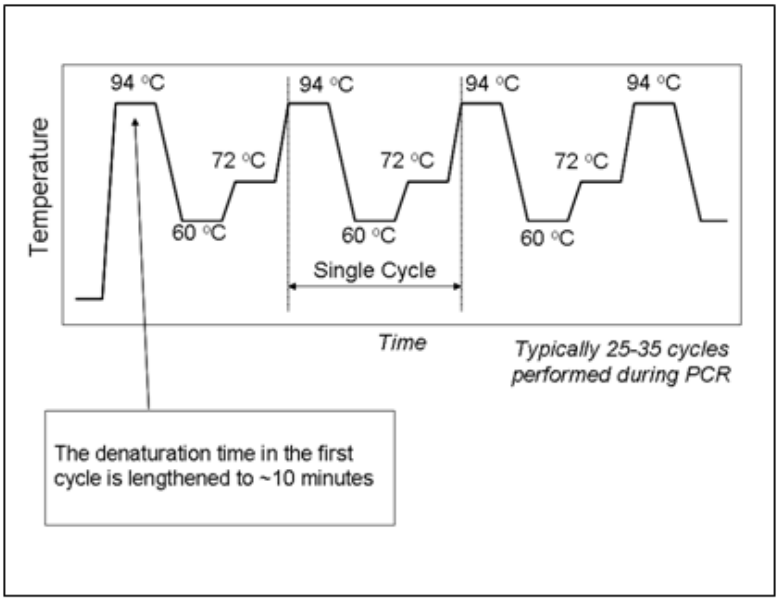

Figure 1.13 Thermal Cycling Temperature Profile for PCR (Butler, 2005)

The entire PCR process is about 3 hours in duration with each cycle taking approximately 5 minutes on conventional thermal cycles: 1 minute each at 94 °C, 60 °C, and 72 °C and about 2 minutes ramping between the 3 temperatures (Innis, 1988) (Figure 1.13). In practice, at least 10,000 DNA template molecules

which correspond to 300 ng genomic DNA are needed to perform an amplification without any problems. Upon completion of the 40 cycles, from 1 copy the multiplication will reach a total of 10^{12} copies of the same DNA template at the end of cycle. Polymerase chain reaction enables researchers to produce millions of copies of a specific DNA sequence in approximately two hours. This automated process bypasses the need to use bacteria for amplifying DNA.

1.5.3 The Important Components of PCR

The requirements for a standard PCR are a thermostable DNA polymerase, a small amount of input DNA as a template and two specific oligonucleotide primers which has a complementary sequence of the DNA template. These components used in a PCR are mixed and sterile deionized water was added to the mixture to achieve the desire volume and concentration of each of the components. The most important component of a PCR reaction is the two primers or the oligonucleotides, which are short DNA sequences that precede the region to be copied. A primer acts to identify or target the portion of the DNA template to be copied (Knoth, 1988). The other components of a PCR reaction (Table 1.4) consist of template DNA that will be copied. Building blocks made up of each of the four nucleotides, and a DNA polymerase that adds the building blocks in the proper order based on the temperature DNA sequence.

Table 1.4 Typical Components for PCR Amplification

Reagents of a PCR	Recommended Concentrations
Tris-HCL, pH 8.3 (25 ^{o}C)	10-50mM
Magnesium chloride	1.2-2.5mM
Potassium chloride	50mM
Deoxynucleotide triphosphates (dNTPs)	50-200µM each dATP, dTTP,dCTP,dGTP
DNA polymerase, thermal stable	0.5-5U
Bovine serum albumin (BSA)	100µg/mL
Primers	0.1-1.0µM
Template DNA	1-10ng genomic DNA

The DNA polymerase originally used for the PCR was extracted from the bacterium *E. coli*. However, heating also irreversibly inactivated this polymerase, so new enzyme had to be added at the start of each cycle after each cycle of DNA synthesis. Furthermore, this polymerase could not copy with high fidelity as well as yielding nonspecific products. The next polymerase to be used for PCR "The bacterium *Thermostable. Aquaticus"* was purified and subsequently cloned (Gelfand, 1993). *T. aquaticus* lives in hot springs, and produces a DNA polymerase which is not irreversibility inactivated at high temperature. This enzyme allowed a complete PCR amplification to be done without opening the reaction tube. The DNA synthesis step could now be done at a higher temperature than was possible with the *E. coli* enzyme. PCR is so sensitive that a single DNA molecule has been amplified, and single-copy genes are routinely extracted out of complex mixtures of genomic sequences and visualized as distinct band on agarose gels.

1.5.4 Analysis of PCR Products: Gel Electrophoresis as a Tool to View DNA Results Obtained From the PCR.

Agarose gel electrophoresis is the easiest and the most common way of separating and analyzing the amplified DNA or the PCR products. This method has great resolving power, yet is relatively simple and straightforward to perform. It is a widely used method that separates molecules based upon charge, size and shape. It is particularly useful in separating charged biomolecules such as DNA, RNA and proteins (Robyt, 1990). The purpose of the gel might be to look at the DNA, to quantify it or to isolate a particular band (Milan, 1959). The DNA is visualized inside the gel matrix by addition of the ethidium bromide dye. This dye binds strongly to DNA by intercalating between the DNA bases and it produces a fluorescent by absorbing invisible UV light and transmits the energy as visible orange light (Berg, 2002; Lodish, 2004).

The gel is made by dissolving agarose powder in boiling buffer solution. The solution is then cooled to approximately 55 ^{o}C and poured into a rack containing a comb. Samples are prepared for electrophoresis by mixing them with components that will give the mixture density, such as glycerol or sucrose (Berg, 2002). This makes the samples denser than the electrophoresis buffer. These samples are loaded with a micropipette or transfer pipette into wells that were created inside the gel by a template during casting. The dense samples sink through the buffer and remain in the wells.

A direct current power supply is connected to the electrophoresis apparatus and current is applied. Charge molecules in the sample enter the gel through the walls of the wells. Molecules having a net negative charge migrate towards the positive electrode (anode) while net positively charged molecules migrate towards the negative electrode (cathode). Within a range, the higher the applied

voltage, the faster the samples migrate (Robyt, 1990). The buffer serves as a conductor of electricity and to control pH. The pH is important to the charge and stability of biological molecules (Robyt, 1990). The DNA molecules are negatively charged and they move towards the positive electrodes in the electrophoresis chamber.

Agarose is a polysaccharide derivative of agar. The gel contains microscopic pores which act as a sieve. The sieving properties of the gel influences the rate at which a molecule migrates. Smaller molecules move through the pores more easily than larger ones. Molecules can have the same molecular weight and charge but different shapes. Molecules having a more compact shape can move more easily through the pores.

Factors such as charge, size and shape, together with buffer conditions, gel concentrations and voltage, affects the mobility of molecules in gels (Anonymous, 1997). Given two molecules of the same molecular weight and shape, the one with the greater amount of charge will migrate faster. In addition, different molecules can interact with agarose to varying degrees. Molecules that bind more strongly to the agarose will migrate more slowly. (Figure 1.14).

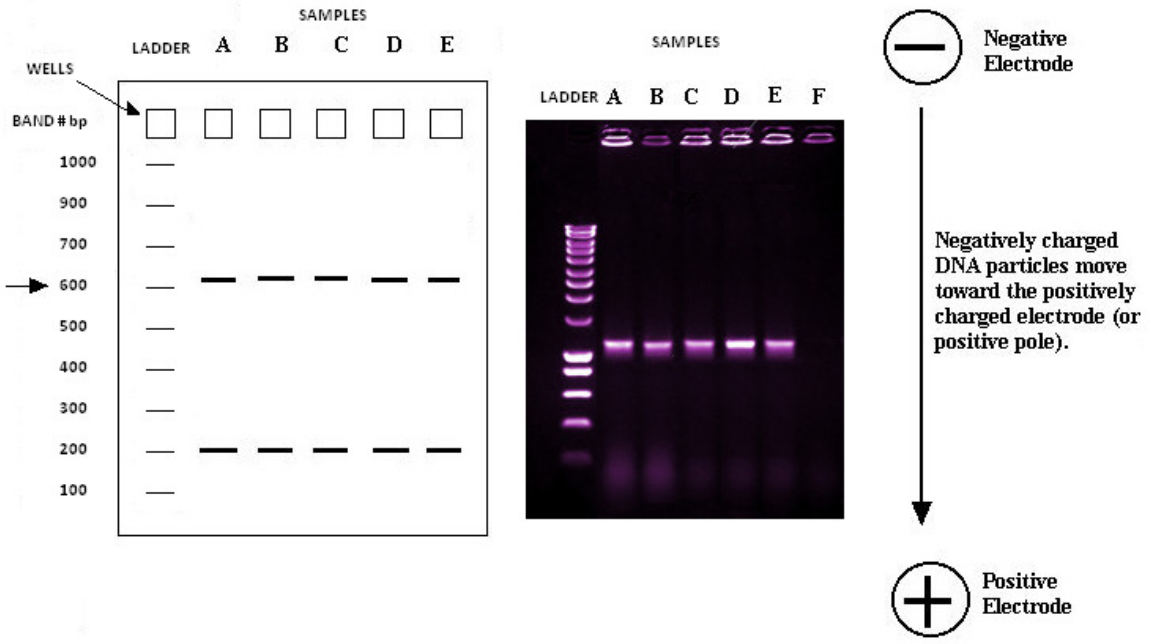

Figure 1.14 Diagram and digital image of agarose gel stained with ethidium bromide: The ladder is a commercially obtained DNA size marker, number of samples loaded and band estimating different number of base pair. The position of the wells and direction of DNA migration is noted.

1.6 DNA Sequencing

The main researchers who developed the bases of DNA sequencing during the 1970s were Gilbert (1973, 1980), Maxan (1977) and Sanger (1977, 1980). It was later in the 80s when Hood (1986) based on "Sanger-Chain-Termination Sequencing" brought this method further to a more efficient and high-throughput technology; leading the path to a Dye-Fluorescene and Capillary array electrophoresis (Figure 1.15).

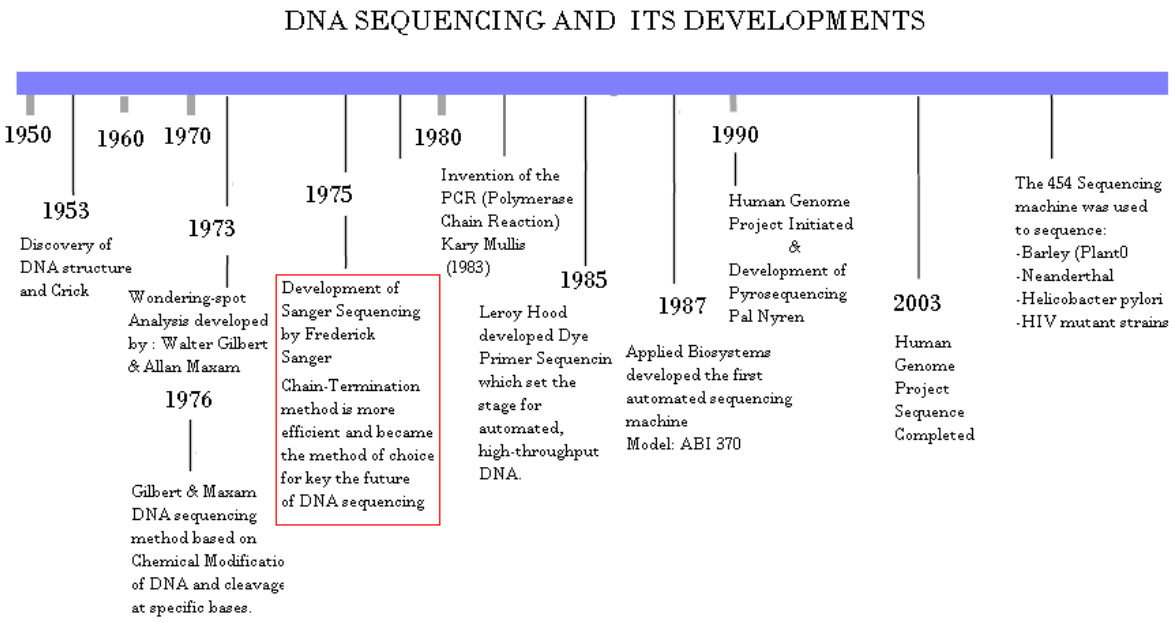

Figure 1.15 Showing DNA and its Developments

The procedure of Maxam-Gilbert sequencing consisted of determining the nucleotide sequence of a terminally labeled DNA molecule by breaking it at adenine, guanine, cytosine, or thymine with chemical agents. Partial cleavage at each base produces a nested set of radioactive fragments extending from the labeled end to each of the positions of the base. Polyacrylamide gel electrophoresis resolves these single-stranded fragments; their sizes reveal in order the points of breakage. The autoradiograph of a gel produced from four different chemical cleavages, each specific for a base in a sense, shows a pattern of bands from which the sequence can be read directly (Maxam & Gilbert, 1977). This sequencing method become obsolete due to its technical complexity prohibiting its use in standard molecular biology kits, extensive use of hazardous chemicals, and difficulties with scale-up.

Simultaneously, while Maxam and Gilbert from Harvard working on their DNA sequencing approach, there was a scientist from Cambridge (Sanger) on the

same endervour who developed sequencing techniques, which built the bridge to a revolutionary path of a discovery of living organism genomes.

Sanger's sequencing is based on the DNA from the host genome to be used as primers for the PCR amplification of the entire genome. The amplified portions of DNA are then assembled by their overlapping regions to form contiguous transcripts. The final step involved the utilization of custom primers to elucidate the gaps between the "contigs" thus giving the completely sequenced genome. This technique is very similar to standard PCR, except that the dNTPs are labelled with either a radioactive or fluorescent tag. Fluorescent technique has proved to be easily automated and does not rely on the preparation of an autoradiograph (Wu, 1993).

1.6.1 Radioactivity

For labeling with radioactivity, four reaction tubes are prepared, each of which contains template, primer, Taq polymerase, all four dNTPs (one of which is labeled), and one of four dideoxy (dd) NTPs (a different one in each tube). The various lengths of DNA are then separated by size using polyacrylamide gel electrophoresis (PAGE) and detected, by autoradiography. The autoradiograph can be read in an automated fashion and stored on a computer for manipulation (Howe, 1995).

1.6.2 Fluorescence

Fluorescent labeling can be carried out in a single tube because each of the four dyes fluoresces at a different wavelength. Two alternative methods can be used in fluorescent sequencing whereby each nucleotide in the DNA sequence is labeled with a different dye color and a chromatogram is produced, with each

color representing a different letter in the DNA code – A, T, C, or G. (Howe, 1995).

- Dye termination: Any primer can be used together with four fluorescently labeled ddNTPs, it is able to cope with single or double stranded DNA. Nevertheless, the specificity of the Taq polymerase results in uneven incorporation of the labeled bases (Sanger, 1997).

- Dye primer: the primer used for chain extension is pre-labeled with the fluorescent dyes. Before sequencing, the template has to be cloned into one of these cloning vectors. The method does overcome the uneven incorporation of labeled bases by Taq polymerase. Irrespective of the labeling method used, PCR sequencing begins when the DNA template strands are separated and a single primer is allowed to anneal. The extension reaction is undertaken in the presence of the dNTPs and either one or all of the ddNTPs. The ddNTPs act as extension terminators. The DNA sequences produced by the PCR reaction will share a common 5' end but have different 3' ends, depending upon which ddNTP halted extension (Smith, 1985).

1.7 DNA (Collection, Extraction)

DNA-based techniques are frequently used in biological research and the facilities and expertise required to conduct sophisticated analysis are widely available. There are several routine processes/protocols done in the laboratory that enables scientists to mass-produce specific segments of DNA in a test tube after it has been collected in the field (Figure 1.16).

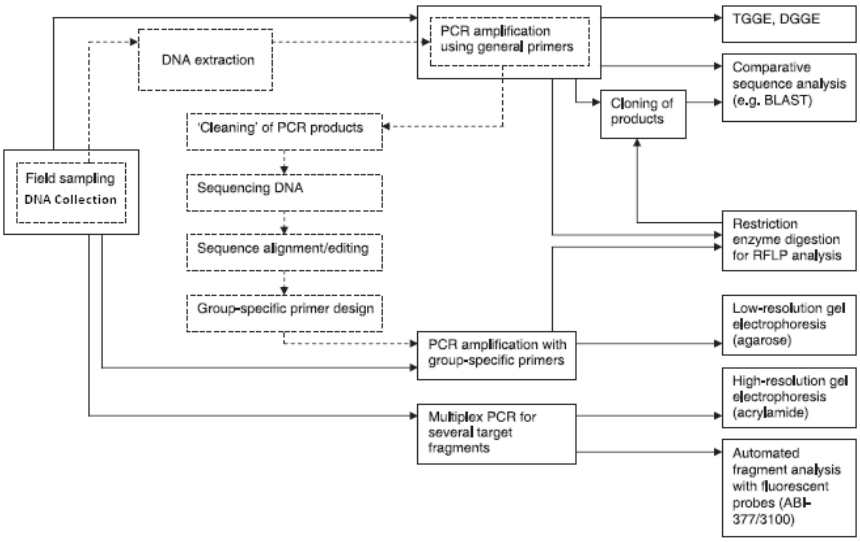

Figure 1.16 Broken lines describing the use of DNA-based techniques after the DNA sample has been collected in the field.

1.7.1 DNA Collection and Preservation

EDTA tubes, a colorless compound used to keep blood samples from clotting before tests are run. Its chemical name is ethylene-diamine-tetra-acetic acid (Munz, 1935). FTA^{TM} paper was developed as a method for storage of DNA (Burgoyne, 1994).

It is an absorbent cellulose-based paper that contains four chemical substances to protect DNA molecules from nuclease degradation and preserve the paper from bacterial growth (Burgoyne, 1996; Eguchi, 2000). Buccal DNA collector may be used for direct collection of buccal cell samples (Fox, 2002; Schumm, 2004). A disposable toothbrush can be used for collecting bucall cell in a non-threatening manner (Burgoyne, 1997; Tanaka, 2000). Inside the laboratory, DNA samples are either stored in a refrigerator at 4°C or a freezer at -20°C. For long periods of time, extracted DNA samples may even be stored at – 70° C (Hochmeister, 1998)

1.7.2 DNA Sample Sources

The genetic information stored in cells in DNA is present in every nucleated cell and is therefore present in biological materials collected. The introduction of PCR has extended the range of possible DNA samples that can be successfully analyzed because many copies are made of the DNA markers to be examined. DNA has been isolated and analyzed from a variety of biological materials. A wide range of biological material has been tested with PCR-based DNA typing method DNA:

Blood and Blood stains	Budowle et al. (1995) Budowle, B, Baechtel, F.S. Comey
Seman and semen stains	Budowle et al. (1995)
Bones	Gill et al. (1994)
Teeth	Alvarez Garcia et al. (1996)
Hair with root	Higuchi et al. (1998)
Hair shaft	Wilson et al. (1995)
Saliva (with nucleated cells)	Sweet et al. (1997)
Urine	Benecke et al (1996)

Feces	Hopwood et al. (1996)
Debris from fingernails	Wiegand et al. (1993)
Muscle tissue	Hochmeister (1998)
Cigarrete butts	Hochmeister et al. (1991)
Postage stamps	Hopkins et al. (1994)
Envelope sealing flaps	Word and Gregory (1997)
Dandruff	Herber and Herold (1998)
Fingerprints	Van Oorschot and Jones (1997)
Personal Items: razor blade, Chewing gum, wrist watch, Ear wax, toothbrush	Tahir et al. (1996)

1.7.3 DNA Extraction Protocols:

1.7.3.1 Organic (Phenol-Chloroform) Extraction

This involves the serial addition of several chemicals:

- Cell Lysis Buffer – lyse cell membrane, nuclei are intact, pellet nuclei.
- Resuspend nuclei, add Sodium Dodecly Sulfate (SDS), Proteinase K. Lyse nuclear membrane and digest protein.
- DNA released into solution is extracted with phenol-chloroform to remove proteinaceous material.
- DNA is precipitated from the aqueous layer by the additional of ice cold 95% ethanol and salt
- Precipitated DNA is washed with 70% ethanol, dried under vacuum and re-suspended in TE buffer (Figure 1.17).

Some protocols involve a Centricon 100 (Millipore, Billerica, MA) dialysis and concentration step in place of the ethanol precipitation to remove heme inhibitators (Comey, 1994).

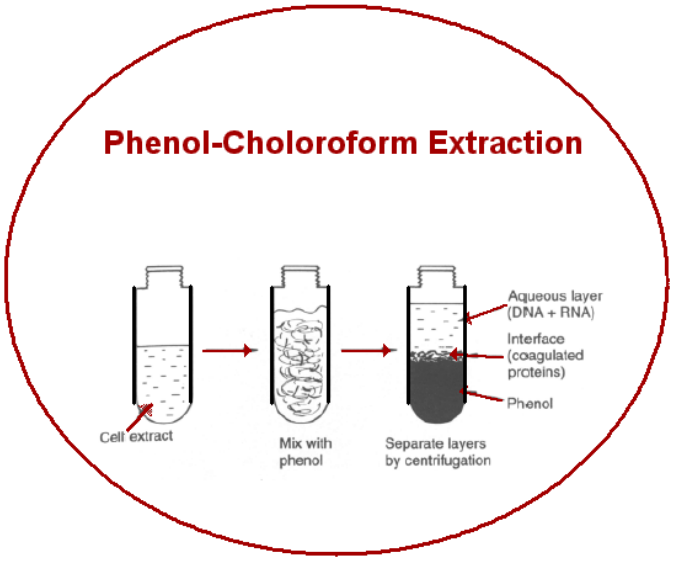

Figure 1.17 Schematic representation of Phenol-Chloroform Extraction

1.7.3.2 Non-Organic (PROTEINASE K AND SALTING OUT)

- Cell Lysis Buffer - lyse cell membrane, nuclei are intact, pellet nuclei.
- Re-suspend nuclei in Protein Lysis Buffer containing a high concentration of Proteinase K. Lyse nuclear membrane and digest protein at 65oC for 2 hours. Temperature helps denature proteins, and Proteinase K auto digests itself
- To remove proteinaceous material, LiCl is added to a final concentration of 2.5 M, and incubated on ice. Proteins precipitate out and are pelleted by centrifugation.
- DNA remains in solution. Transfer supernatant to a new tube, care must be taken not to take any of protein pellets.

- DNA is precipitated by the addition of room temperature isopropanol. LiCl will not precipitate with DNA.
- Precipitated DNA is washed with 70% ethanol, dried under vacuum and re-suspended in TE buffer.

1.7.3.3 CHELEX (Ion Exchange Resin) EXTRACTION

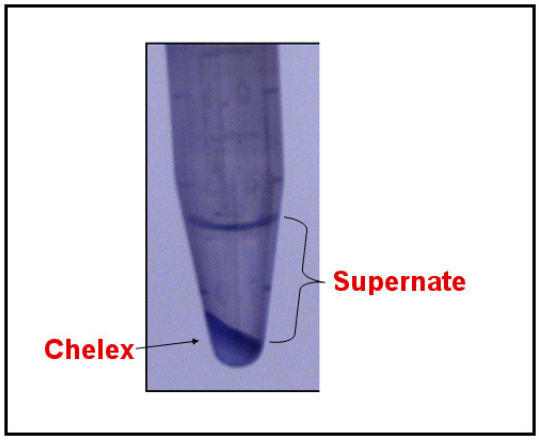

Figure 1.18 Schematic representation of Chelex DNA Extraction.

It is an ion-exchange resin that is added as suspension to the samples. Chelating-resin suspension can be added directly to the sample. It was introduced in 1991, (Chelex 100 from Bio-Rad Laboratories, Hercules, CA); and has been used successfully to extract DNA from forensic specimens, formalin fixed, paraffin wax embedded tissue, whole blood of mice, dried blood spots on filter paper discs, and cultures or clinical samples (Walsh, 1991; Uda, 1994; Wingberg, 1991; Lanar DE, 1991; Vignoly, 1992) (Figure 1.18).

1.7.3.4 FTA™ Paper

This paper was designed for room temperature collection, shipment, archiving, and purification of nucleic acids from a wide variety of biological samples for PCR analysis (Figure. 1.19). These include blood, buccal, cells, tissue cells, cultured

cells, microorganisms, and plant tissue. FTA cards are impregnated with a patented chemical formula that lyses cell membranes and denatures proteins upon contact. Nucleic acids are immobilized and protected from UV damage and microbial and fungal attack. Infectious pathogens in samples (Watman Ltda, 2008)

Figure 1.19 Image shows FTA Cards for DNA Collection and Shipment and Storage.

FTA cards are the Rapid Preparation and Ambient Storage of DNA from Whole Blood and Other Biological Samples:

- It's a unique mixture of strong buffers, protein denaturants, chelating agents, and a UV absorbing, and free radical trap.
- The reagents are impregnated into a cellulose-based filter matrix such as Whatman BFC180 or 31ET paper
- Kills blood borne pathogens on contact
- Immobilizes DNA within the matrix
- Protects DNA from degradation
- Allows for long-term storage at room temperature (Watman, 2009).

1.8 Thesis Research Objectives

Based on the above review of the literature of the Slow Loris (*Nycticebus c. coucang*), and methods and techniques used in its study, this research project intends to study the Malaysian Slow Loris in the wild combining the radio telemetry methods supported by GPS, GIS, Remote Sensing and other mapping techniques, and combined these with molecular biology methods which, this writer hopes, will send a new bench mark in primate studies in Malaysia.

The research objectives of this thesis project are:

(1) To develop a detailed Biotope Map of the Studies Areas to provide the physical biogeographical setting in which the Slow Loris will be tracked and its resulting spatial patterns analyzed;

(2) To evaluate the Ranging Patterns and Line Corridors used by the Slow Loris (*Nycticebus c. coucang*) in the wild, as revealed by radiotelemetry, and as plotted back into the biotope map of the study area.;

(3) To identify paternal and maternal genetically inheritance of those Slow Loris (*Nycticebus c. coucang*) the researcher tracked in the field, as well as in combination with those DNA samples obtained in other several

localities within Peninsular Malaysia by using cross-species microsatellites makers and D-loop mtDNA sequences; and finally,

(4) To use these findings and models derived from these, to advance conclusions that are hopefully new to the study of the Slow Loris, and to deepen the scientific community's understanding of this rare and difficult to study animal.

1.9 Conclusion:

The research report that now follows shall comprise the following chapters:

In **Chapter 2**, the specific materials and methods used by this research project shall be described in some detail for the purpose of enabling other researchers to fully understand and, if they wish, to replicate these methods in their own research projects.

In **Chapter 3**, the research site in Bukit Boloh, Temerloh District, the State of Pahang, is described to provide the setting in which the research project was undertaken.

Chapter 4 presents and analyses the original Biotope Map of the study site which was undertaken by the researcher in the course of this project.

In **Chapter 5**, the Ranging Patterns of the Slow Loris are mapped, and analyzed, and the spatial behaviour of the Loris is modeled.

In **Chapter 6**, molecular biology techniques are applied, and a genetic analysis of the relationships between the individual Slow Loris identified, tagged, and tracked in the field, is presented.

The final chapter, **Chapter 7**, presents a discussion of the diverse and wide ranging results of this study, and seeks to draw conclusions, and to make recommendations to guide future researchers interested in studying the Malaysian Slow Loris.