PHYLOGENETIC ANALYSES AND THE REPETITIVE PATTERNS OF *TOMISTOMA SCHLEGELII* BASED ON MITOCHONDRIA DNA GENE REGIONS

By

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ABSTRACT

PHYLOGENETIC ANALYSES AND THE REPETITIVE PATTERNS OF *TOMISTOMA SCHLEGELII* BASED ON MITOCHONDRIA DNA GENE REGIONS

Taranjeet Kaur

The endangered *Tomistoma schlegelii*, a freshwater crocodilian endemic to Malaysia and Indonesia, is currently affected with severe habitat loss as their numbers in the wild have been decreasing. Phylogenetic inferences for this species which was carried out using newly designed primers for the protein coding ND 6 – $tRNA^{glu}$ – cyt *b* and the tRNA Thr – control region revealed five haplotypes each. The repetitive regions in the mitochondria DNA (mtDNA) control region (CR) not previously described were also amplified and sequenced. A high overall haplotype diversity of 0.78 was reported with evidence of population structure separating East Kalimantan, Sarawak, Peninsular Malaysia and Sumatra. Except for the H2 haplotype that occurred in both Peninsular Malaysia and Sarawak, haplotypes were generally geographically distinct. These findings allowed individuals of unknown origins in our data to be assigned into their respective geographical origins. Haplotype H4, occurring in Peninsular Malaysia, had the highest genetic distance and always formed a basal and reciprocally monophyletic clade in all the NJ, MP and ML trees. This lineage was not connected at the 95% connection limit in the parsimony haplotype network while for the minimum spanning network was placed on a separate evolutionary branch. These analyses suggest that H4 is a highly divergent lineage. Length variation ranging from 50 to 600 bp with intervals of ~ 50 and ~ 100bp were detected in

the mtDNA repetitive region among individuals and only one individual showed a heteroplasmic band. Sequencing the region after CSB III revealed a motif region and a variable number of tandem repeats (VNTRs) region which were stably inherited from the maternal parent to all offspring. A VNTR core unit of 104 bp is implicated for the cause of the length variation seen among individuals of *Tomistoma* and the repetitive patterns resembled those specific to other crocodilians.

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APPROVAL SHEET

This dissertation/thesis entitled "**PHYLOGENETIC ANALYSES AND THE REPETITIVE PATTERNS OF *TOMISTOMA SCHLEGELII* BASED ON MITOCHONDRIA DNA GENE REGIONS"** was prepared by TARANJEET KAUR and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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I hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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CHAPTER 1

INTRODUCTION

The endangered *Tomistoma schlegelii*, also known as the false gharial, is endemic to Malaysia and Indonesia (Auliya *et al*., 2006; Bezuijen *et al*., 2001) and is currently under Appendix I of CITES (IUCN 2009) with an estimated population of less than 2500 individuals left in the wild (Bezuijen *et al*., 2010). Previous census studies have all indicated a gradual decline of *Tomistoma* sightings in Kalimantan and Sumatra from the 1990s to 2000s (Auliya *et al*., 2006; Bezuijen *et al*., 1998; Bezuijen *et al*., 2001) while in Malaysia sightings have been rare since the late 1980s (Cox and Gombek, 1985; Sebastian, 1993; Simpson *et al*., 1998). The main threat of this freshwater crocodilian is habitat loss due to legal and illegal logging, urbanization, fishing activities and forest fires at peat swamps resulting in loss of suitable nesting sites and habitat for *Tomistoma* (Auliya *et al*., 2006; Bezuijen *et al*., 1998; Bezuijen *et al*., 2001).

Currently, most of these wild specimens are found in captivity in zoos and farms and even by individuals who acquired it illegally. Zoos and farms in Malaysia have approximately 77 specimens (Stuebing *et al*., 2004) while Singapore, Thailand and Indonesia have collectively 88 specimens (http://tomistoma.org).

Zoos and farms in Europe and the United States of America hold approximately 57 specimens (http://tomistoma.org).

Thus far, studies in both Malaysia and Indonesia have concentrated on surveys of *Tomistoma* in natural habitats and related issues involving conservation of habitat for the false gharials (Auliya *et al*., 2006; Bezuijen *et al*., 1998; Bezuijen *et al*., 2001; Bezuijen *et al*., 2002; Bezuijen *et al*., 2004; Stuebing *et al*., 2006). Information on the genetic diversity of this species is currently lacking. The available genetic information to date on *Tomistoma schlegelii* is on its classification within Crocodylia (Gatesy *et al*., 2003; Harshman *et al*., 2003; Janke *et al*., 2007; McAliley *et al*., 2006; Roos *et al*., 2009). Due to the ongoing habitat destruction of *Tomistoma* which is forcing this crocodilian to survive in the limited available habitat and a gradual decrease of numbers and density of this species is likely to result in a loss of genetic diversity due to genetic drift or inbreeding.

Without genetic information of the available wild caught individuals in captivity, breeding programs at such localities could result in inbreeding, if closely related individuals are bred, or outbreeding, if mating is carried out using two divergent lineages. Similarly, genetic information of potential wild populations is essential for a successful breeding and reintroduction program. Reintroduction of divergent lineages into the existing wild population could prove to be detrimental to the survival of this little known species in the wild. Apart from reintroduction

programs, accurate identification of lineage or populations would help in enforcement of wildlife law efforts concerning this species. A study at the intraspecific level is therefore important in order to make appropriate conservation management decision for *Tomistoma*. Hence, this study was carried out to document the genetic diversity and population structure of this sole member of *Tomistoma* using wild caught individuals held in captivity in the occurrence range of this crocodilian.

A total of 56 samples collected from Malaysia, Indonesia, Hong Kong and Singapore which were a mixture of wild caught specimens and farmed specimens with known and unknown origins (confiscated specimens) were used in this study. The 3' cytochrome *b* to the third domain of the mtDNA control region (cyt *b* – CR) and the 3' ND 6 – tRNA-Glu – 5' cytochrome *b* (ND 6 – $tRNA^{glu}$ – cyt *b*) were utilised to investigate the genetic diversity of *Tomistoma schlegelii* and to facilitate comparisons with other crocodilians for the same gene region.

Since the control region was used as one of the markers, and is known to have repetitive regions containing variable number tandem repeats (VNTRs) that were described in other crocodilians but failed to be amplified in *Tomistoma* and *Gavialis* (Ray and Densmore, 2002), new primers were developed to identify and characterise the repetitive region in *Tomistoma*. Investigation of parental inheritance was carried out as a 'family unit' was included in the sampling process.

Therefore, the objectives of this study are to:

- document the genetic diversity of the species in the occurrence range of this species and within sampling localities individually,
- identify if there is population structure over its occurrence range,
- identify if haplotypes are geographically associated, and therefore to assign the individuals with unknown origin to their respective geographical region,
- detect if species level divergence exist within *Tomistoma*,
- detect if VNTRs are present in the mtDNA control region of *Tomistoma*, and to characterize them accordingly.

CHAPTER 2

LITERATURE REVIEW

2.1 *Tomistoma schlegelii*

2.1.1 Description

Tomistoma schlegelii is one of the largest crocodilian with adult males ranging from 4.5 – 5.0 m while adults females are about 2.5 – 3.0 m long (Bezuijen *et al*., 1998; Bezuijen *et al*., 2010). The number of eggs in a clutch is up to 35 eggs (Bezuijen *et al*., 1998; Bezuijen *et al*., 2001). The snout of this species resembles the slender snout of the Indian gharials, *Gavialis gangeticus* but slightly wider in *Tomistoma schlegelii* and therefore is commonly known as the false gharials (Figure 2.1).

The genus name means 'cutting mouth' in Greek while the species is named after its Dutch discoverer, H. Schlegel. Juveniles are dark chocolate brown in colour with black banding on the tail, body and dark blotches at the jaw area (Figure 2.2). Their underbelly is dark, turns lighter and finally white as they grow older. Adults retain most of their juvenile pattern with the dorsal colouration turning brown to olive-green with dark cross bands (Bezuijen *et al*., 2001).

The total of 76 to 84 teeth are sharper and narrower than that of the other crocodilians that occur in its distribution range, the saltwater crocodiles *Crocodylus porosus* and *C. siamensis* (Figure 2.3). Its diet is reported to include a variety of invertebrates like shrimps to small vertebrates such as fish, monkeys and small deers (Bezuijen *et al*., 2001).

Figure 2.1: The slender snout of *Tomistoma*. An adult male basking in Zoo Negara, Malaysia.

Figure 2.2: Hatchlings of *Tomistoma*. One month old hatchlings in Jong's Crocodile Farm, Sarawak.

Figure 2.3: The sharp teeth of an adult male *Tomistoma*.

2.1.2 Classification Based On Fossils, Morphology and Molecular Data

Members of Tomistominae amongst others, based on morphological and fossil data, are *Tomistoma schlegelii*, *T. cariense*, *T. lusitania*, *T. petrolica*, *Toyotamaphimeia machikanensis, Crocodylus spenceri*, *Gavialosuchus eggenburgensis*, *Gavialosuchus americanus, Megadontosuchus arduini*, *Dollosuchus dixoni*, *Dollosuchoides densmorei*, *Kentisuchus spenceri* and *Paratomistoma courti* of which only *T. schlegelii* survives till date (Brochu, 1997; Piras *et al*., 2007; Brochu, 2007).

Based on the morphological data, outward similarities are found of the rostrum and jaws of the *Tomistoma schlegelii* and *Gavialis gangeticus* but when more and older fossils were considered, the rostrum, jaw and dental similarities are highly similar to *Crocodylus* and therefore morphological data places *Tomistoma* closer to *Crocodylus* while *Gavialis* forms a basal taxon (Brochu, 2003). The phylogenetic relationship within Tomistominae has differed due to the discovery of newer fossils. Previously, *T. schlegelii* was placed together with *T. lusitanica* on the same clade and they formed a sister taxa to *T. cairense* but later *Gavialosuchus eggenburgensis* was found to be closer to *T. schlegelii* than *T. lusitanica* (Brochu, 2003). More recently, *Toyotamaphimeia machikanensis* found in Japan is now considered the closest to the sole surviving Tomistominae. (Kobayashi *et al*., 2005; Piras *et al*., 2007).

Molecular data based on mtDNA (Janke *et al*., 2005; Roos *et al*., 2007) and nuclear genes or concatenate data (Gatesy *et al*., 2003; Harshman *et al*., 2003; McAliley *et al*., 2006) have placed *Gavialis* and *Tomistoma* as sister taxon. Restriction fragment analysis of nuclear ribosomal DNA and mtDNA showed that the living *Gavialis* and *Tomistoma* are closely related and form a sister taxa (Densmore and White, 1991). However, the nuclear ribosomal DNA placed these two closer to *Crocodylus* while mtDNA findings placed the two gharials closer to the *Alligator* (Densmore and White, 1991). However, all subsequent molecular data suggested the two gharials to be closer to Crocodylidae. Using the nuclear gene c – *myc*, *Gavialis* and *Tomistoma* were shown to be sister taxon and closer to *Crocodylus* (Harshman *et al*., 2003). The phylogenetic tree based on combined data of morphological traits and c – *myc* gene was congruent with the molecular data tree while the morphological data tree topology was rejected due to conflicting signals (Harshman *et al*., 2003). Similarly, Gatesy *et al*. (2003) also found that the mtDNA and nuclear gene trees were congruent but phylogenetic relationships within Crocodylia using concatenation with morphological data was incongruent with the molecular tree due to extensive character conflicts. McAliley *et al*. (2006) found that the nuclear and mitochondria markers separately and as concatenate data showed a closer relationship between the two gharials with *Crocodylus* than with *Alligator*.

The most conclusive phylogenetic relationship within this order was provided by Janke *et al*. (2005) and Roos *et al*. (2007) using the entire mtDNA genome of all

the crocodilians which showed strong statistical support for the traditional molecular data tree. Both studies showed closer association of *Gavialis* and *Tomistoma* to *Crocodylus*. Due to larger samples of crocodilian species and calibration points within the crocodilian tree as well as non – crocodilian calibration, the study by Roos *et al*. (2007) showed better consistency between molecular and fossil divergence estimates than the study by Janke *et al*. (2005). Using only nuclear genes, *Tomistoma* was placed closer to *Gavialis* and provided evidence that they should be included into Gavialidae instead of Crocodylidae (Willis *et al*., 2007).

2.1.3 Ecology and Habitat

2.1.3.1 Historical Distribution

Historical distribution range of Tomistominae covered northern Africa, Europe, mainland Asia and Japan during the early Eocene of which some were distributed at estuarine and coastal regions (Brochu, 1997). *Tomistoma schlegelii* was found in freshwater or peat swamps that ranged across a land mass formerly known as Sundaland (Auliya *et al*., 2006; Bezuijen *et al*., 1998; 2002; Brochu, 2003; Stuebing *et al*., 2006).

At 17000 years before present (and at 120 meters below present level of shorelines), Sumatra, Jawa, Peninsular Malaysia and Borneo were thought to be connected by the exposed Sunda Shelf (Voris, 2000) with four major river systems, the Malacca Straits River System, the Siam River System, North and

East Sunda River System. These systems contained peat deposits indicating the occurrence of Pleistocene peat swamps and fresh water lakes especially off the east coast of the Malay Peninsular (Voris, 2000). Stuebing *et al*. (2006) suggested that it had a wider distribution before the rising sea levels at the late Pleistocene altered land mass configuration of this region limiting the *Tomistoma* habitat to West Malaysia (Peninsular Malaysia), Borneo and south-central Sumatra. The destruction of habitat at present time (since the species was discovered in 1838) is also forcing this species to move farther inland from coastal areas (Stuebing, *et al*., 2006).

2.1.3.2 Current Distribution

Field surveys within South East Asia so far had located *Tomistoma schlegelii* in the peat swamps of Indonesia and Malaysia (Auliya *et al*., 2006; Bezuijen *et al*., 2002; Cox and Gombek, 1985; Sebastian, 1993; Simpson, 1998) (Figure 2.4). This crocodilian is known to be associated with peat swamps and rivers in Sumatra especially the South Sumatra and Jambi Provinces (Bezuijen *et al*., 1998; 2002), Central Kalimantan in the Tanjung Putting National Park rivers (Auliya *et al*., 2006) and Danau Sentarum in West Kalimantan (Bezuijen *et al*., 2004). Overall, the census studies showed a declining population of this species over the years.

The densitiy of *Tomistoma* in Leboyan river, west Kalimantan was reported to be 0.21 crocodiles / km in 1994 but reduced to 0.08 crocodiles/km in 2004 (Bezuijen

Figure 2.4: Distribution of *Tomistoma schlegelii*. Image from Bezuijen *et al*. (2010).

et al., 2004). Similarly, in South Sumatra Province, the upper Merang River showed a decline of 0.34 crocodiles / km to 0.16 crocodiles / km while the lower section of this river showed a decline from 0.16 crocodiles / km to 0.02 crocodiles / km from 1996 to 2002 (Bezuijen *et al*., 2002). The Air Hitam Laut River and Simpang Melaka Creek both in Jambi Province, Sumatra showed similar trends

within this time frame (Bezuijen *et al*., 2002). Bezuijen *et al*. (2002; 2004) attributed the decline due to habitat destruction and fishing activities. In contrast, Auliya *et al*. (2006) found that apart from sighting of varying sizes of *Tomistoma,* the density was increasing in the Sekonyer and Sekonyer Kanan rivers in Tanjung Putting National Park. However, no sightings were reported in the Buluh Kecil River also in Tanjung Putting National Park indicating that the stable breeding populations in the Sekonyer and Sekonyer Kanan rivers could be due to the conservation efforts on orangutans around that area since the 1970s (Auliya *et al*., 2006)

All previous wild caught *Tomistoma* of Malaysia were caught within peat swamps restricted to Sarawak and Peninsular Malaysia with sightings being rare since the early 1990s (Sebastian, 1993; Simpson *et al*., 1998). Three individuals were sighted by Cox and Gombek (1985) in the Ensengai River in Sarawak during one survey night while none were spotted at the Bera Lake, Pahang despite historical reports of the species as common in the area (Simpson, 1998). Other studies have been on compilation of distributional records for the false gharial in Malaysia (Sebastian, 1993). The occurrence of *Tomistoma* in Thailand is unconfirmed. Stuebing *et al*. (2006) reported that a lack of supporting data either suggests this species did not occur in Thailand or occurred in the southern region now under Malaysian sovereignty. There is no supporting data for *Tomistoma* occurrence in Vietnam and Singapore (Sebastian, 1993).

2.2 Mitochondria Genes

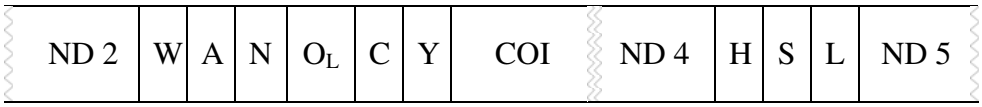
2.2.1 Mitochondrial Genome

Mitochondria is a circular organelle DNA and is found in cells of higher organisms. It is responsible for oxidative phosphorylation and production of adenosine triphosphate, ATP (McBride *et al*., 2006). It contains DNA which is separated from the chromosomal or nuclear DNA and is maternally transmitted with low or no recombination (Avise, 2000). Generally the mitochondria DNA (mtDNA) has two rRNA genes, two ATPase genes, four cytochrome genes, six NADH genes, 22 tRNAs which are found in between the genes and acts by providing the amino acids needed during translation, and a large non-coding region resulting in a genome size of 16 kilo bases (kb) (Moritz *et al*., 1987). Larger mtDNA genome sizes are usually due to the length variation of the non-coding region of the mtDNA (Rand and Harrison, 1989). There are other smaller non-coding regions at variable positions along the mtDNA genome for some groups of animals (Boore, 1999). These mtDNA genes are also seen in crocodilian together with the smaller non-coding region (Janke *et al*., 2005). The arrangements of these genes however vary among major taxonomic groups in vertebrates, with most conforming to one of the 'common' vertebrate tRNAs arrangements (Boore, 1999). The arrangement of the WANCY region (tRNA - Trp, - Ala, - Asn, - Cys, - Try genes, abbreviated by single letters of amino acids decoded) for instance has been found to differ within reptilians (Boore, 1999; Kumazawa and Nishida, 1995). The WANCY region differs from crocodilian and tuatara to squamate with the latter having a non-coding region within the

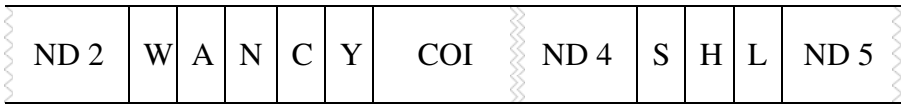
WANCY region, which is also the 'common' vertebrate arrangement described by Boore (1999) (Figure 2.5A and Figure 2.5B). In crocodilian, the tRNA – His, - Ser and - Leu (HSL) between the ND 4 and ND 5 genes and the tRNAs flanking the control region (tRNA – Phe) differ from the 'common' tRNA orientation (Boore, 1999; Kumazawa and Nishida, 1995) (Figure 2.5A and Figure 2.5B). The 'common' vertebrate arrangement for tRNA – Phe is between the control region and the 12S rDNA gene but in crocodilians the control region is flanked by the tRNA – Phe at its 5' end and the 12S rDNA gene flanks the control region at the 3' end (Boore, 1999). Other reptilian, such as the tuatara, conform to the 'common' vertebrate arrangement for this region (Quinn and Mindell, 1996) (Figure 2.6A and Figure 2.6B).

2.2.2 mtDNA Gene as a Genetic Marker

Apart from the abundance of these organelle DNA and a faster evolution rate compared to nuclear genes (Avise, 2000, Hwang and Kim, 2000), mtDNA genes are also a popular marker for evolutionary and phylogeography studies due to maternal inheritance with minimal or no recombination, (Avise, 2000; 2004; Moore, 1997) and neutral evolution (Vawter and Brown, 1986). However, recent reviews on mtDNA have indicated that it is not free of recombination and is not exclusively maternally inherited (Galtier *et al*., 2009). Recombination was suggested in human mitochondria when a high homoplasmy within species was observed and divergent lineages sharing point mutation (Eyre-Walker *et al*., 1999; Hagelberg *et al*., 1999), which was later found to be due to mutation hotspots and

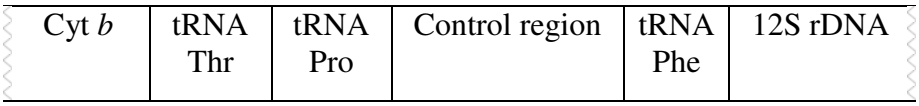


(A)

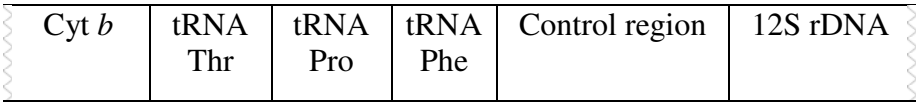


(B)

Figure 2.5: Schematic diagram of mtDNA WANCY and HSL region. Gene arrangements from 5' to 3' adapted from Boore (1999). Jagged lines indicate partial genes. (A) The 'common' vertebrate arrangement of the WANCY and HSL region. (B) The tRNA orientations in crocodilian.



(A)



(B)

Figure 2.6: Schematic diagram of mtDNA tRNA – Phe gene. Gene arrangements from 5' to 3' adapted from Boore (1999). Jagged lines indicate partial genes. (A) The 'common' vertebrate arrangement of tRNA – Phe. (B) The tRNA – Phe arrangement in crocodilian.

alignment error (Galtier *et al*., 2006; Hagelberg *et al*., 2000). Because methods for accurate detection of recombination is lacking, it should be considered when high amount of within species homoplasmy is seen using mtDNA gene, though it could also be due to mutation- induced convergences (Galtier *et al*., 2009).

Apart from recombination, biparental inheritance is found in marine mussels (Zouros *et al*., 1992) while paternal leakage has been reported in mice and sheep (Gyllesten *et al*., 1991). In the marine mussel *Mytilus trossulus*, the biparental mtDNA genomes had the paternally inherited control region (Burzynski *et al*., 2003).

Zhao *et al*. (2004) suggested that paternal mtDNA could be inherited either by paternal leakage in which the mtDNA from spermatids enter the eggs and are maintained at low levels in the fertilized eggs. The other possibility suggested was by recombination of the paternal mtDNA genome into the nucleus to form nuclear copies of mitochondria genes (Numt DNA), which interacted with the nuclear and mtDNA, and then transferred back into the mtDNA (Zhao *et al*., 2004) especially in cases of hybrids (Fontaine *et al*., 2007). Though there are exceptions to the clonal inheritance with no recombination of mtDNA, generally most taxa conform to the maternal inheritance with negligible recombination events (Avise, 2009).

The neutral theory suggests that most molecular variations are caused by random drift and selectively neutral mutations (Kimura, 1968). This theory has been used to infer population demography using molecular markers especially using mtDNA that were thought to be neutral. The neutrality of mitochondria gene has also been questioned (Galtier *et al*., 2009). Recurrent sweeps in large population and adaptation leading to reduced diversity of the mtDNA genome was suggested by Bazin *et al*. (2006) when mtDNA gene diversity was similar across animal taxa. The lack of recombination of the mitochondria makes genetic hitchhiking more likely when there is selection for a particular gene (Ballard and Kreitman, 1995).

There are several neutrality test to indicate if indeed the marker is neutral for demographic purposes, like the Tajima's D (1989), Fu and Li's D test (1993), the Fu's F_S test (1997) and the Ramos and Rozas's R2 (2002). The null hypothesis in these tests is neutral mutation with no recombination and deviations from neutral mutations are either purifying (a negative value) or balancing selection (a positive value). In the case of the Tajima's D and Fu and Li's D test, the presence of natural selection is only one of the possibilities when the null hypothesis is rejected. In contrast, the Fu's F_S can be used to identify the evolutionary forces in a population which is either undergoing subdivision, population reduction and over – dominance selection (Fu, 1996). Old mutations are seen in generations closest to the common ancestor while young mutations are seen in the most recent descendents, and an excess of the former would indicate population reduction or bottleneck while an excess of the latter would indicate population expansion (Fu,

1996). The Ramos and Rozas's R2 has been found to be more sensitive than Fu's F_S when the sample size is small (Ramos and Rozas, 2002). The more recent test, Fu's F_S and Ramos and Rozas's R2 tests are therefore most sensitive to detect population demography compared to the earlier test (Ramos and Rozas, 2002). Therefore, inferences at the population demography can be made with a better level of confidence.

Finally, though the mutation of mtDNA is generally higher than nuclear DNA (nuDNA) by ten folds (Clayton, 1984; DeSalle *et al*., 1987), the mutation is not constant across the genome. The mtDNA 12S rDNA is highly conserved and used in higher taxonomic level similar to the nuclear small subunit (SSU) rDNA, while the mtDNA non – coding region or the cytochrome c oxidase I (COI), have a high mutation rate suitable for population and species level comparisons (Hwang and Kim, 1999). Apart from mutation variation within the mtDNA genes themselves, the mtDNA mutation rate which was thought not to deviate too much within vertebrates (Moritz *et al*., 1987) is now reported to vary across taxa (Gatlier *et al*., 2009). The mutation rates of nuDNA in mammal lineages were reported to be more constant than mtDNA and therefore when using the mtDNA genome as a molecular dating tool, mutation rate heterogeneity should be taken into consideration (Galtier *et al*., 2009).

2.2.3. mtDNA Genes in Phylogenetic Studies

Interspecific and higher level comparisons are possible with mtDNA due to the conservations of the number of genes (most groups have 37 genes) and its arrangements (Boore, 1999; Moritz *et al*., 1987) while amino acids analysis is suitable for higher taxonomic level, such as at the family and even phylum (Hwang and Kim, 1999). Nucleotide sequences of the protein coding genes are used in cases of saturation of the third codon due to high substitution at this position usually at lower taxonomic level, such as the species and population level (Hwang and Kim, 1999).

The nucleotide analysis of its protein coding genes is suitable for species or population level analysis and are commonly used in crocodilian population studies (Cedeno – Vasquez *et al*., 2008; Farais *et al*., 2004; Glenn *et al*., 2002; Ray *et al*., 2000; Ray *et al*., 2004; Rodriguez *et al*., 2008; Vasconcelos *et al*., 2006; 2008; Venegas – Anaya *et al*., 2008, Weaver *et al*., 2008).

Generally, mtDNA cytochrome *b* gene (cyt *b*) was found to be more conserved compared to cytochrome c oxidase I (COI) (Hwang and Kim, 1999). Cyt *b* however is a common marker at the species and population level of crocodilian (Farais *et al*., 2004; Vasconcelos *et al*., 2006; Weaver *et al*., 2008) and was found to be as sensitive as the COI and the control region in caiman and crocodiles (Venegas – Anaya *et al*., 2008; Weaver *et al*., 2008). In the black caiman, the cyt *b* gene revealed a population expansion and population differentiation according

to the two main drainage type (Farais *et al*., 2004; Vasconcelos *et al*., 2008). The latter study found that the haplotype diversity, *h* was high at 0.91 compared to 0.69 by Farais *et al*. (2004). Similar findings of high haplotype diversity and population differentiation using mtDNA were seen in *Caiman crocodilus* (Vasconcelos *et al*., 2006; Venegas – Anaya *et al*., 2008).

The COI gene is probably the most common mtDNA used for species level comparisons as its efficiency as a barcoding tool is well documented in animals (Apakupakul *et al*., 1999; Herbert *et al*., 2004; Costa *et al*., 2007) due to the high diversity levels seen between species over a relatively short length and is flanked by conserved region where universal primers can easily work to amplify the region (Kress and Ericksoon, 2008).

Concatenation of data from mtDNA cyt b and cytochrome oxidase I (COI) in the caiman, revealed 3 evolutionary significant units (ESU) with a divergence of 1.6 to 6 million years ago (Venegas – Anaya *et al*., 2008). Apart from identifying management units, COI gene was also useful in classifying *Osteolaemus* species (Eaton *et al*., 2009). Concatenate data of three different mtDNA genes (12S rDNA, COI and cyt *b* – control region) of *Osteolaemus* revealed three different species within this genus which were also associated with geographical regions and was supported with two different nuclear genes and subsequently by combining all the five genes (Eaton *et al*., 2009).

Hybridization has been suggested using mtDNA data when the phylogenetic analyses showed incongruence with morphological data (Ray *et al*., 2004, Weaver *et al*., 2008). In Morelet's crocodile, Ray *et al*. (2004) found that some populations with C. *moreletti* morphology showed a *C. acutus* haplotype, while Weaver *et al*. (2008) found that *C. rhombifer* with *C. acutus* morphology formed a separate clade from those with the Cuban crocodile morphology.

The control region, an unassigned region and does not code for any proteins (Hwang and Kim, 2000), is a hypervariable region used as a molecular marker at species and population level. Although it is a hypervariable region, it is believed to have functional constraints as certain conserved sequences found across taxa are involved in the regulation of replication and transcription of the mitochondrial genome (Broughton and Dowling, 1994; Buroker *et al*., 1990; Chang and Clayton, 1985; Levinson and Gutman, 1987; Mundy *et al*., 1996; Wenick *et al*., 1994).

2.2.3.1 ND 6 – $tRNA^{glu}$ – cyt *b* Marker

The 3' region of NADH 6 to 5' region of cytochrome *b* (ND 6 – $tRNA^{glu}$ – cyt *b* marker) has been used as a marker for interspecific and intraspecific variation in crocodilians (McAliley *et al*., 2006; Ray and Densmore, 2003). In a preliminary genetic study of *Osteolaemus tetraspis*, this marker revealed high levels of variations comparable to that of between species when individual from Gabon in Africa were included (Ray *et al*., 2000). The Gabon population, using

microsatellite and morphology, revealed substantial evidence to be considered a different taxonomic unit (Eaton *et al*., 2009). However, McAliley *et al*. (2006) found low variation within *Crocodylus* in this 347 bp region but comparison between genus (*Crocodylus*, *Gavialis*, *Tomistoma* and *Mecistops*) was similar or higher than that seen by using the control region.

2.2.3.2 mtDNA Control Region

The control region is the only major non – coding segment in the vertebrate mitochondrial genome. The non – coding region known to be the most hypervariable region, evolving three to five times more rapidly compared with the rest of the mitochondrial genome and is not subjected to any selection making it suitable for population and species level analysis (Avise, 2000; Hwang and Kim, 2000).

However nucleotide diversity by this marker has been reported to be low with 0.07% to 1.3% in *Crocodylus moreletti* (Ray *et al*., 2004). Genetic divergence using this marker also revealed low values from 0 % to 1.6 % (Eaton *et al*., 2009; Weaver *et al*., 2008). Ray *et al*. (2004) suggested that this could be due to a low rate of substitution in the crocodilian mtDNA control region. Despite this, the divergence of the mtDNA cyt *b* – CR sequences and cyt *b* was useful in revealing a separate lineage in *Crocodylus rhombifer* which was confirmed with microsatellite studies (Weaver *et al*., 2008). The cyt *b* – CR marker was able to suggest hybridization between *C. acutus* and *C. moreletti* when eight individuals

showing *C. acutus* morphology had *C. moreletti* haplotype while another eight with *C. moreletti* morphology had *C. acutus* haplotype (Cedeno – Vasquez *et al*., 2008). The microsatellite findings confirmed the cyt *b* – CR marker findings (Cedeno – Vasquez *et al*., 2008). This study also found one individual with the American crocodile morphology showing a *C. rhombifer* haplotype (Cedeno – Vasquez *et al*., 2008). In the American alligator, the mtDNA d loop revealed a single population with very low diversity due to a past population bottleneck (Glenn *et al*., 2002).

2.2.4 Structure of the Control Region in General and in Crocodilians.

The non-coding region of most taxa consists of three parts known as Domain I, II and III with Domain I and III as the hypervariable domains with highly repetitive sequences (Sbisa *et al*., 1997). Although it is a hypervariable region, there are conserved sequences within this region found across taxa suggesting functional constraint in this region of the mtDNA genome.

Domain I contains 'termination associated sequences' or TAS/ ETAS while Domain III contains short conserved sequence blocks (CSBs) and promoters for H- and L- strand transcription. Domain III is where the replication of the H-strand begins and ends at the TAS in Domian I (Sbisa *et al*., 1997). Domain II is generally more conserved compared to the other two domains and contains CSB like sequences known as F, E, D, C and B boxes which are thought to be associated with regulating H- strand synthesis. (Xiao *et al*., 2006).

The mtDNA control region (CR) structure of Crocodylia and the mtDNA VNTRs patterns for Alligatoridae and Crocodylidae have been previously described (Ray and Densmore 2002; 2003) (Figure 2.7). Within Crocodylia, there was 57% variation among all genera of the mtDNA CR (Ray and Densmore 2002). In Domain I, there was length variation while Domain II showed high conservation of F –, E – and B – boxes within Crocodylia and birds. The D – box was found to be highly conserved within Crocodylia but the C – box was less conserved. In Domain III of the mtDNA CR, a six base sequence in the conserved sequences block (CSB) I was conserved when compared to other vertebrates while CSB II and III were conserved within Crocodylia (Ray and Densmore 2002). However, the VNTRs patterns after CSB III in *Alligator*, *Caiman* and *Crocodylus* were distinct (Ray and Densmore 2003).

In *Alligators*, there is a presence of a motif region consisting two motifs and a tandem repeat region (Ray and Densmore 2003). The tandem repeats in alligators showed chimeric aspects in which the second repeat (ATAATT*TTATATTA*<u>*TAGGGCC*</u>) had 5' portion of the first motif as its 5' region (underlined) while its 3' region consists of a 15 bp nucleotide sequence which is found before the first motif (in italic). The VNTR patterns in *Caiman* were repeated three times and had a truncated repeat that was similar to its 12S rRNA

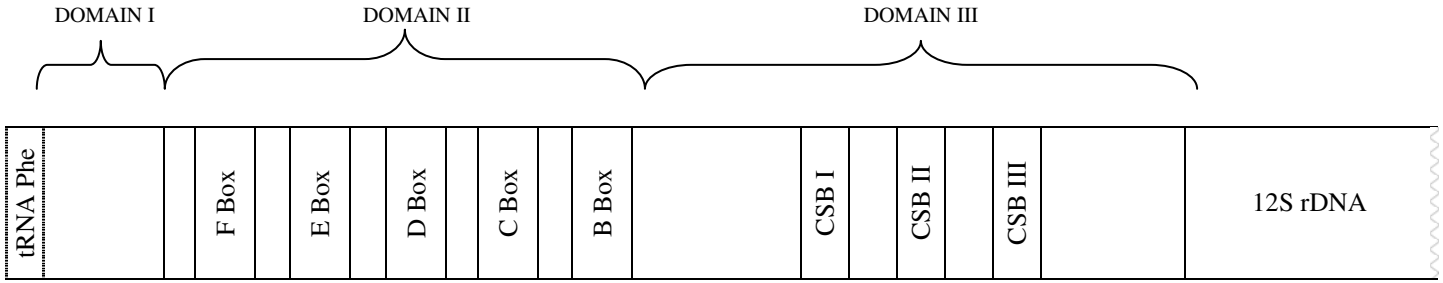


Figure 2.7: General structure of the crocodilian mtDNA control region. Gene arrangements from 5' to 3' adapted from Ray and Densmore (2002). The flanking tRNA-Phe at the 5' of the control region and the 12S rDNA at the 3' are also included.

gene (Ray and Densmore 2003). In the *Crocodylus*, only one form of motif was reported, the 'TAGG' motif, which is part of the VNTRs. The VNTRs showed shortening of sequences from 5' to 3' in the New World crocodiles and appeared constant in the Old World crocodiles (Ray and Densmore 2003). The presences of VNTR and repetitive regions for both gharials have not been previously reported and were not addressed in a related study of the crocodilian repetitive region (Ray and Densmore 2003).

2.2.4.1 mtDNA VNTRs Utiltiy

The variable number tandem repeats (VNTRs) are short sequences or motifs repeated in a tandem fashion, usually found in Domain I and Domain III of the control region (Lunt *et al*., 1998; Ray and Densmore, 2003) and are capable of folding into thermodynamically stable secondary structures (Lunt *et al*., 1998). This region has been used as a marker for intraspecific and interspecific variation studies (Arnason and Rand, 1992; Broughton and Dowling, 1994; Casane *et al*., 1994; Larizza *et al*., 2002; Ludwig *et al*., 2000; Mjelle *et al*., 2008; Nesbo *et al*., 1998; Ray and Densmore, 2002; Wilkinson *et al*., 1997) and in studies of replication models of the mtDNA genome (Buroker *et al*., 1990; Levinson and Gutman, 1987; Mundy *et al*., 1996; Taylor and Breden, 2000).

Study at population level has shown little substructure (Arnason and Rand, 1992; Broughton and Dowling, 1994) while variation at the family unit level showed mixed results. Heteroplasmy and sequence types in the European Vespertilonid

bat were stably transmitted from mother to offspring (Petri *et al*., 1996) but Wilkinson and Chapman (1991) found one case in the American evening bat, where a homoplasmic mother had a heteroplasmic offspring and both homoplasmic and heteroplasmic offspring did not have identical sequence repeats as the mother. In humans, heteroplasmy was stably transmitted in maternally related individuals due to either a wide bottleneck or joint transmission of two or more mtDNA (Lutz *et al*., 1999). However, in bovine, high mutations within one generation was observed owing to a narrow bottleneck (Hauswirth *et al*., 1984, Koehler *et al*., 1991). Due to these mixed results at intraspecific level, this marker has been suggested to be more appropriate as 'mtDNA fingerprint for individual' rather than for population or species level (Wenick *et al*., 1994).

Interspecific variation studies using mtDNA VNTRs reported the evolution of the VNTRs sequence between species suggesting the repeats are lineage specific either within order and family (Larizza *et al*., 2002) due to either concerted evolution of the sequence or functional constraints. In parrots, the mtDNA control region was duplicated and the two copies were identical in the *Amazona* genus suggesting concerted evolution (Eberhard *et al*., 2001) while in large cats, some repeat arrays were conserved among species which indicates either conserved functional roles or are influenced by concerted evolution (Jae-Heup et al. 2001).

2.2.4.2 Replication models of the D – loop

The length variations seen in the mtDNA repetitive region is due to the duplication of sequences and variations in the number of VNTR copies during the replication processes (Broughton and Dowling, 1994). Drift and tissue – specific selection can also cause length variations and heteroplasmy within an individual (Casane *et al*., 1994; Lunt *et al*., 1998).

Various models for the replication of the mtDNA genome depending of the location of the repeats, the sequence and its secondary structures stability have been suggested (Broughton and Dowling, 1994; Buroker *et al*., 1990; Chang and Clayton, 1985; Levinson and Gutman, 1987; Mundy *et al*., 1996).

The slipped strand mispairing (SSM) model by Levinson and Gutman (1987) was suggested in tandem repeats of non-coding regions characterized by either a tandem unit made up by a combination of different motifs, tandem arrangements of closely related motifs and imperfect copies of the original copy as it moves farther to 3' of the non-coding region. These patterns are explained by slipped strand mispairing and interhelical events like long tandem duplication or unequal-crossing over (UCO) caused by mutational changes. These create new motifs that are propagated further increasing in length and number of motifs. The Levinson and Gutman (1987) model was the likely model for the duplication of the tandem repeats for the *Crocodylus* as suggested by Ray and Densmore (2003) due to the shortening of the tandem repeat sequences from 5' to 3'.

The model by Taylor and Brenden (2000) is very similar to Levinson and Gutman (1987) but with the non-contiguous repeats as the site for misalignment. The tandem repeats are characterized by perfect tandem repeats and an imperfect repeat at the 3' region of the VNTRs in which the first portion of this imperfect repeat matches the first portion of the perfect repeats. An initial SSM by nucleotide substitution produces a non – contiguous repeat and subsequent SSM at this repeat is responsible for the length variations in this region of the control region. Taylor and Breden (2000) suggested that chance mutations alone, as seen in the model by Levinson and Gutman (1987) could not explain the formation of VNTRs for longer length sequences.

Mundy *et al*. (1996) suggested a model where the duplication of repeats occurs adjacent to the tandem repeats and involving inverted repeats. In the loggerhead shrikes, this inverted repeat was located at the tRNA-Phe which is upstream from the d-loop and the origin of H-strand replication. This model was proposed for *Caiman crocodiles* due to the presence of an intervening sequence between the 12S rDNA gene and the last tandem repeat (Ray and Densmore, 2003).

2.3 Intraspecific Variation Analyses

2.3.1 Definitions and Importance of Intraspecific Variation Analyses

There are various levels of genetic diversity namely the genetic variations among individuals within populations, genetic variation among populations within the

same species, and genetic variation between individuals from separate species. The genetic diversity in the first two instances above is also known as intraspecific variation while the latter is known as interspecific variation (Hillis *et al*., 1996). Changes in the genetic composition of populations and individuals are the primary mechanism of evolutionary change within species which help the population or species adapt to the changing environment and therefore increasing the possibility of its survival (Frankham *et al*., 2002; Mayr, 1963). The coalescent theory traces the evolutionary processes backward to the most recent common ancestor (MRCA) and is popularly used for within species or population level analyses (intraspecific variation) because it incorporates the demographic processes as well (Rosenberg and Nordborg, 2002).

Generally, low genetic variation especially in endangered species, in which numbers of surviving individuals is small, suggests susceptibility to extinction due to genetic drift (Snyder *et al*., 1996). But low genetic variation could also indicate that the current population or species has adapted to the current environment and is expanding. In the giant panda, the finding of a very low genetic variation was thought to be due to a population bottleneck in the past and together with current small numbers in the wild and ongoing habitat degradation indicates that this species may be on the edge of extinction (Zhang *et al*., 2002). However, neutrality tests, Tajima's D (1989) and Fu's F_S (1997) which are used for demographic analysis, indicated population expansion, suggesting that after the bottleneck in the past, the remaining populations have adapted to the

environment and are expanding (Zhang *et al*., 2002). This finding shows that apart from genetic variation alone, other analysis like demographic and coalescent analysis is necessary to make better inferences at the intraspecific level (Zhang *et al*., 2002).

A description of how haplotypes or the genetic variation of a species is geographically distributed is equally essential (Avise, 2000). In general, genetic variability and haplotypes or genotypes in many naturally occurring populations have been found to be geographically associated and is termed as phylogeography (Avise, 2000). This is useful in reintroduction programs and assigning geographical region of individuals of unknown origins (Venegas – Anaya *et al*., 2008).

In threatened or endangered species, intraspecific variation can indicate whether smaller management units exist below the species level. Management units (MUs) are populations with distinct haplotype and are demographically separated (Moritz, 1994). An example of this is the Hector's dolphins in New Zealand which had four populations, the east, west and south population of the South Island and North Island populations (Baker *et al*., 2002). Although there was substantial gene flow between the east and west population of the South Island population, all three (east, west and south) were considered separate management units due to the significant difference in haplotype frequencies between the populations (Baker *et al*., 2002). The evolutionary significant units (ESUs) are

populations that form a reciprocally monophyletic clade in the mtDNA phylogeny and are minimally connected or not connected by nuclear gene flow to other populations (Moritz, 1994). Fraser and Bernatchez (2001) defined ESUs as a lineage demonstrating highly restricted gene flow from other lineages of the species. The North Island population fit into the description of ESUs by Mortiz (1994) and is now recognized as a subspecies. Mixing this population with any of the other three could result in loss of local adaptation ability due to breeding with distant lineages and at the same time losing the genetic component of the evolutionary lineage (Baker *et al*., 2002).

However, the definitions for ESUs and MUs above have been criticised (Crandall *et al*., 2000; Paetkau, 1999) as many subspecies and distinct lineages have been found to be not reciprocally monophyletic. In the Orchard Oriole, two subspecies are identified based on morphology, breeding and wintering ranges (Baker *et al*., 2003). A polyphyletic mtDNA gene tree was recovered but the F_{ST} analysis showed a significantly high differentiation of these two subspecies. This finding suggests that these two Orchard Orioles just recently diverged and lineage sorting is incomplete, therefore the mtDNA tree was not reciprocally monophyletic (Baker *et al*., 2003). A recent speciation in which gene flow is absent, and the population size of the older and newer population can cause an evolutionary significant unit to not demonstrate a reciprocally monophyletic clade (Paetkau, 1999). Crandall *et al*. (2000) suggest a broader range of criteria to be included when defining an ESU; such as ecological information and genealogical histories

and exchangeabilities as this would indicate if the population is able to adapt and evolve over time enhancing the species survival.

The estimate of divergence time between lineages at the species level has also been used to help define management units or populations in *Elephas maximus* and *Pongo pygmaeus* (Fernando *et al*., 2003; Warren *et al*., 2001) in combination with other population analyses. The Bornean elephant population diverged from the other Asian elephants in the Pleistocene and had since been isolated, warranting them as an ESU (Fernando *et al*., 2003). Similarly, the four Bornean orangutan populations showed divergence of the same magnitude as the Bornean – Sumatran populations that are reproductively isolated with high F_{ST}, suggesting the four Bornean populations be managed separately (Warren *et al*., 2001). In the spectacled caiman, the phylogenetic analyses and divergence time among lineages suggest five subspecies (Venegas – Anaya *et al*., 2008). The basal clade which is a population from the Amazonia had a divergence of 6 million years indicating that this cryptic lineage be managed as a separate subspecies from the other Amazonia clade (Venegas – Anaya *et al*., 2008). These estimates were based on the *Alligator – Caiman* most recent common ancestor (MRCA) from estimates using morphological, mtDNA and nuclear gene data which showed similar results (Venegas – Anaya *et al*., 2008). The divergence estimates were based on mitogenomic analysis by Roos *et al*. (2007) which showed general consistencies with crocodilian fossil records.

In summary, conservation of genetic variation and the evolutionary processes involved in these variations are essential in preservation of genetic diversity at the intraspecific level.

2.3.2 Quantification of Genetic Diversity

There are a variety of techniques used to quantify genetic diversity such as using the mitochondrial DNA (mtDNA) nuclear DNA (nuDNA) sequences, restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), microsatellites and minisatellites. As seen in most phylogeography studies, the mtDNA sequencing is one of the most commonly used techniques (Avise, 2000). Similarly, in crocodilian population studies, mtDNA has been commonly used (Cedeno – Vazquez *et al*., 2008; Ray *et al*., 2000; Ray *et al*., 2004; Vasconcelos *et al*., 2006; 2008; Weaver *et al*., 2008). These findings were confirmed with microsatellite markers (de Thoisy *et al*., 2006, Dever *et al*., 2002, Eaton *et al*., 2009, Rodriguez *et al*., 2008). However, in the American alligators, microsatellite markers were able to separate populations according to large scale east-west phylogeographic separation with little population subdivision within the two regions (Davis *et al*., 2002). This population genetic structure was unable to be detected by mitochondrial marker which had very low variation (Glenn *et al*., 2002). One of the reasons for the incongruence could be the small sample size of only 25 individuals representing the entire occurrence range in the mtDNA study (Glenn *et al*., 2002) versus 14 to 59 samples collected in each localities from east to west of the occurrence range in the microsatellite study (Davis *et al*., 2002).

The common parameter to measure diversity within populations or species is the expected heterozygosity, or gene diversity defined by Nei (1987) as the probability that when two alleles are chosen at random from the population, they are different. Gene diversity refers to haplotype diversity, *h*, for the mtDNA genome. A moderate to high *h* is desirable for the population in the wild or in captivity to continue to adapt to the changes in environment. Nucleotide diversity, π, is the gene diversity at the nucleotide level and refers to the probability that when two homologous nucleotides are chosen randomly, they are different (Nei, 1987). A general pattern is seen in population studies using these two parameters (Grant and Bowen, 1998). A high *h* with low π indicates a population had a small effective population or population bottleneck followed by rapid expansion or population growth. In contrast, a species with low *h* and high π indicates divergence between geographically subdivided populations. If both these parameters are high, the species has had a large stable population with long evolutionary histories while a low *h* and π indicates a recent bottleneck or could be a founder effect of one or a few mtDNA lineages (Grant and Bowen, 1998). Therefore, these two parameters can be used to indicate population demography in combination with the neutrality tests (Fu and Li, 1993; Fu, 1997; Ramos and Rozas, 2002; Tajima, 1989) and the less sensitive mismatch distribution (Ramos and Rozas, 2002; Rogers and Harpending, 1992). As seen in the case of the Chinese panda, demographic analysis is an important part when deciding on conservation plans. Therefore, it is recommended to use a variety of test when analyzing populations.

The other common parameter for the population differentiation level is the Wright's (1951) fixation index, F_{ST}. This parameter reveals the connection between populations through gene flow and can be carried out at various hierarchical levels to identify units to be managed separately. A significantly high F_{ST} indicates low gene flow between populations while a low F_{ST} indicates that the various populations can be considered as a single panmixic unit (Slatkin, 1987). Gene flow may either constrain evolution by preventing adaptation to local conditions or promote evolution by spreading new genes (Slatkin, 1987). Therefore, an understanding of adaptive genetic differentiation among populations is necessary (Allendorf and Luikart, 2007).

In conclusion, the phylogenetic and genealogy analyses, and demographic analyses should be analysed together for better inferences of intraspecific variation. This would help to plan for better management, breeding and relocation programs.

2.3.3 Intraspecific Genetic Diversity of Crocodilians

2.3.3.1 Species Level Divergence within the Dwarf Crocodile (*Osteolaemus*)

The taxanomic classification of *Osteolaemus* at the genus and species level have been in conflict since the early 20th century (Ray *et al*., 2000). According to Schmidt (1919), *O. tetraspis osborni* should be placed in a separate genus, *Osteoblepharon*, while Mertens (1943) suggested that *O. t. osborni* and *O. t. tetraspis* should be considered as separate species within *Osteolaemus* (as cited in

Ray *et al*., 2000). A preliminary intraspecific variation study of *Osteolaemus* using mtDNA marker, ND 6 – $tRNA^{glu}$ – cyt *b*, was carried out with two individuals from Gabon and eight individuals of unknown origins (Ray *et al*., 2000). The high genetic divergence between the Gabon individuals and the unknown origin individuals was comparable with interspecific divergence albeit all 10 were morphologically indistinguishable (Ray *et al*., 2000).

Subsequent phylogeography study of samples originating from Central to West Africa, carried out with a combination of morphometric characters with mtDNA and nuclear gene markers, revealed strong support for two separate species (Eaton *et al*., 2009). In addition to the two species, *O. t. osborni* found in the Congo Basin and *O. t. tetraspis* from Ogooué Basin, another species, *O. sp. nov* was also detected. However, this lineage was not morphologically distinct from *O. t. tetraspis* (Eaton *et al*., 2009).

Since both these studies did not use a common marker, it is unclear if the eight individuals of unknown origins in the study by Ray *et al*. (2000) represent *O. sp. nov* or another distinct lineage within the genus (Eaton *et al*., 2009).

2.3.3.2 Low Genetic Diversity Trends in the American Alligator (*Alligator mississipiensis*) and the Nile Crocodile (*Crocodylus niloticus*)

In contrast to *Osteolaemus*, *A. missisipiensis* showed low genetic diversity and divergence using mtDNA, microsatellite and allozyme markers (Davis *et al*., 2002; Gartiside *et al*., 1977; Glenn *et al*., 2002). Population subdivision into East

and West of southeastern United States was only detected using microsatellite markers with a large sampling size (n = 359) over 13 sampling sites and using more polymorphic loci (Davis *et al*., 2002). Interestingly, even with a low genetic diversity, morphological differences were noticed in *Alligator* of Florida lakes that share a common drainage system (Davis *et al*., 2002). Epigenetic and environmental factors were suggested instead of direct inheritance for this observation (Davis *et al*., 2002).

Similarly, the farmed *C. niloticus* showed no genetic variation using 52 protein coding loci. Adaptation to a narrow environmental niche was one of the suggested reasons as mortality, hatchling abnormalities and growth rates were in normal ranges (Flint *et al*., 2000).

2.3.3.3 Population Structure in the Black Caiman (*Melanosuchus niger*)

The largest Neotropical predator, *M. niger*, previously threaten with poaching and habitat degradation is currently classified as vulnerable under IUCN (Farais *et al*., 2004). This crocodilian has a high genetic diversity and the population is at equilibrium (Farais *et al*., 2004).

Both the microsatellite and mtDNA cyt *b* markers showed high genetic diversity even though the population census indicated a marked reduction of wild populations. Extensive but recent exploitations are difficult to detect genetically and field surveys should be incorporated when interpreting genetic information

(de Thoisy *et al*., 2006). The dwindling population of the French Guiana and the stable population census of Angelique swamp showed similar genetic diversity (de Thoisy *et al*., 2006). Vasconcelos et al (2008) suggested that the reduction in population sizes was not detected by mtDNA due to either a historical population expansion which left a strong signature on the mtDNA genome, or the exploitation rate was too short with respect to the generation time of this caiman, or the difficulty in detecting genetic drift due to the historical expansion and long generation time.

The population differentiation detected was attributed with isolation – by – distance over larger geographical region and by ecological differences between the closely located white and black water region in French Guiana and Brazil (de Thoisy *et al*., 2006). On a global scale, though populations from Ecuador, Brazil and the French Guiana were significantly isolate, the gene flow levels were sufficient for recolonization processes (de Thoisy *et al*., 2006).

2.3.3.4 Population Structure and a Divergent Lineage of the Spectacled Caiman (*Caiman crocodilus*)

Similar to *M. niger*, *C. crocodilus* has a high genetic diversity in spite of reduced population size from census studies which showed distinct phylogeography trend (Farais *et al*., 2004; Vasconcelos *et al*., 2006; Venegas – Anaya *et al*., 2008). Using mtDNA COI and cyt *b*, populations of *C. c. fuscus* were differentiated into the Caribbean Costa Rica population and a wider population unit from the Caribbean coast of Colombia to the Pacific Costa Rica that corresponds to the

intervening mountains (Venega – Anaya *et al*., 2008). *C. c. fuscus* was also found to be expanding northward into *C. c. chiapasius* range due to habitat degradation which may result in hybridisation of the two caimans. A divergent and cryptic mtDNA lineage was detected but was indistinguishable from *C. c. crocodilus* based on morphology (Venega – Anaya *et al*., 2008).

2.3.3.5 Genetic Diversity Trends in Cases of Hybridization between Morelet's crocodile (*Crocodylus moreletii*), American crocodile (*C. acutus*) and Cuban crocodile (*C. rhombifer*)

These crocodiles faced severe exploitation previously and all three show the same trend of high genetic diversity and a population at equilibrium. The phylogenetic tree showed some of *C. moreletii* clustered together with *C. acutus* and had genetic divergence comparable to interspecific levels, suggesting hybridisation (Ray *et al*., 2004). The population at Banana Bank Lagoon was differentiated from other populations. But when the hybrid *C. moreletii* were not included in the analysis, population subdivision was not detected (Ray *et al*., 2004).

Cedeno – Vasquez *et al*. (2008) found that hybridisation of *C. moreletii* and *C. acutus* was higher at the Yucatan Peninsular. Using nuclear and mtDNA markers, hybridisation was found to be bidirectional with the initial generation consisting of female *C. acutus* cross with male *C. moreletii* (Rodriguez *et al*., 2008). Because *C. moreletii* haplotypes were detected with mtDNA markers, subsequent backcrossing of this hybrid generation with either crocodiles produced either *C. moreletii* or *C. acutus* haplotypes (Cedeno – Vasquez *et al*., 2008).

Hybrids of *C. rhombifer* and *C. acutus* were also detected using mtDNA markers and morphological characters (Weaver *et al*., 2008). Within *C. rhombifer*, two distinct lineages (α and ß) were detected with mtDNA markers. The microsatellite markers too detected two distinct lineges (I and II). The combination of this data suggest that the mtDNA α lineage consisted of hybrids of *C. acutus* with the I and II lineages (Weaver *et al*., 2008).

Therefore, crocodiles identified in the wild on field surveys may be pure *C. rhombifer*, *C. acutus*, *C. moreletii* or hybrid and the wild population size could be much lower than documented (Weaver *et al*., 2008)

In conclusion, the main trend in the intraspecific variation of crocodilian is a high genetic diversity (with the exception of *A. missisipiensis* and *C. niloticus*) even with small wild populations and most populations are at equilibrium, which does not reflect the census of crocodilians in the wild. Except when ecological factors are influencing population structure, sufficient gene flow exists in subdivided populations. Hybridisation and divergent lineages have also been detected in wild populations which directly affect the implementation of proper management strategies of these populations.

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample Collection

3.1.1 Sampling Sites

Blood samples of *Tomistoma* specimens were collected from various locations within Malaysia, Singapore, Hong Kong and Indonesia (Table 3.1). Samples from Sumatra (but in captivity in Jawa), Peninsular Malaysia, Sarawak and East Kalimantan were known to be from the respective geographical region. Though the precise location of the samples from Peninsular Malaysian is not conclusive, all samples are from the peninsular of Malaysia except for two hatchlings whose origin could not be ascertained.

Samples from Singapore, the Cikananga Wildlife Rescue Center in Jawa and Hong Kong Wetland Park were of unknown origins. Singapore's samples are thought to be from Malaysia (A. Matthews, pers. comm.) but were treated as unknown origin since documentations to support this claim were lacking. The samples from these holding areas were included in this study to document the genetic variability, identify which maternal lineage they belonged to and how these holding areas can contribute in conservation of this species.

Table 3.1: The blood collection localities and geographical origins for all *Tomistoma* samples. Figures in parentheses indicate the number of samples. The exact locations for samples of known origin from Peninsular Malaysia were not confirmed.

Region	Collection sites	N	Origins
Sarawak	Matang Wildlife Center	4	Kuching, Sarawak
	Miri Crocodile Farm	3	Miri, Sarawak
Peninsular Malaysia	Zoo Negara	6	Selangor (4), unknown origin (2)
	Melaka Zoo	4	Selangor
	Taiping Zoo	3	Selangor (1), Perak (1), Terengganu (1)
	Mini Zoo Temerloh	4	Selangor
Singapore	Singapore Zoological Gardens	10	Unknown
Hong Kong	Wetland Park, Hong Kong	4	Unknown (shipped from Samutprakarn Farm, Bangkok)
Jawa	PT Ekanindya Farm	2	Jambi, Sumatra
	Cikananga Wildlife Rescue Center	10	Unknown
East Kalimantan	CV Surya Raya Crocodile Farm	5	Wis Lake, West Kalimantan

3.1.2 Manual Restraint

Each *Tomistoma* was physically restraint before any blood was drawn. As a safety measure and ease of handling the crocodilian, the water in the moat was drained before pulling the *Tomistoma* with a lasso around both jaws to an area suitable for manual immobilisation. A gunny sac was then placed onto the eyes and head area of the crocodilian as visual barrier before three people can sit on the dorsal of the entire crocodile to hold it down and immobilize it. The snout was secured by covering the upper and lower jaw using parachute strings or 6 mm polystyrene rope together with the gunny sac. Smaller strings provide better security for the jaws as bigger ropes can loosen during immobilization. The fore and hind limbs are then tied to each other with 6 mm polystyrene rope or parachute strings to secure immobility before blood is drawn.

Release was done by removing the 6 mm polystyrene rope from the limbs and snout, followed by the 2 cm polystyrene rope around the body and mouth. The people sitting on these animals then moved away before the visual barrier is removed using a long rattan.

3.1.3 Blood Collection

Blood collection sites included the caudal vein (approached dorsally or ventrally) and the supravertebral vein at the post occipital site. For the tail bleed, the caudal vein is at the midline about 10 cm from the vent (Figure 3.1). For the ventral approach to the caudal vein, the crocodilian was restrained on its back (ventral

facing up) while for the dorsal approach to the caudal vein and supravertebral vein, the crocodile remained with its dorsal facing up. The site at the post occipital was at the dorsal midline from the cranium platform (about 1 – 3 cm in young adults to larger crocodiles).

Blood collection site was cleaned with alcohol and an 18 G spinal needle (SPINOCAN, B. Braun Medical Inc) was introduced vertically into the site until close to the vertebrae (Appendix 1). A syringe with 1 – 2 ml of 10 % EDTA was used to collect blood. For both the caudal and supravertebral veins, the venous sinus is located by aspirating blood with the syringe.

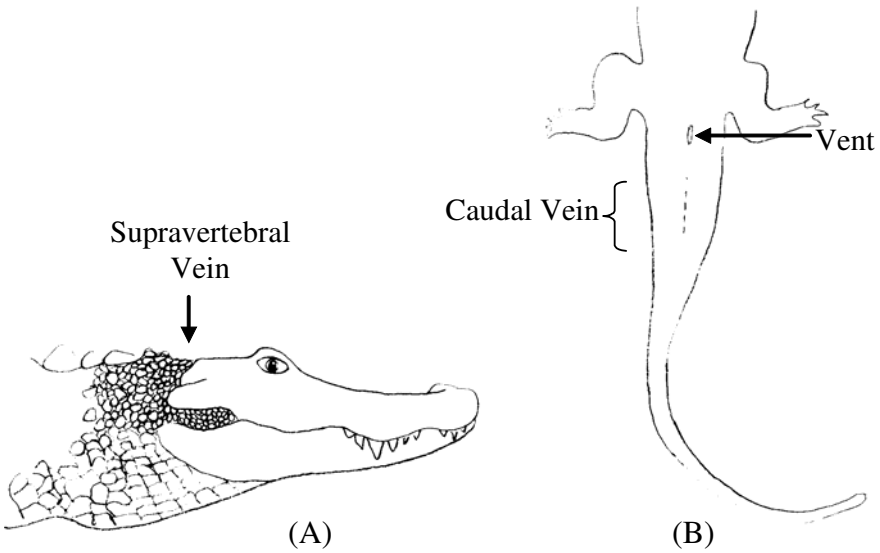


Figure 3.1: Location of the blood sampling sites. (A) The supravertebral vein at the post occipital site. (B) The caudal vein of the tail for the ventral approach.

A minimum of 10 ml blood was targeted to be collected. The blood collected from each *T. schlegelii* was placed into two 15 ml Falcon tubes and additional 1 – 2 ml of 10 % EDTA was added. Povidion Iodine and pressure are applied to the site of blood collection to minimize bleeding and infection after collection.

The Indonesian samples were collected and preserved on QIAcard FTA Spots (Qiagen). Only one drop of blood was placed onto each card for each individual. Because of the extensive travelling for samples collection in both Java and Kalimantan, the FTA cards, which provide convenience in transportation of samples and proper storage of samples and DNA at room temperature, were used.

3.1.4 Sample Storage

Blood was centrifuged at 4000 rpm for 20 minutes (EBA 21, Hettich Instruments) to separate the red blood cells (RBCs) and plasma. Both plasma and RBCs were stored in separate 15 ml Falcon tubes at -20ºC.

The Indonesian samples on the QIAcard FTA Spots (Qiagen) were stored at room temperature in the laboratory.

3.2 Primer Development

Primers for the cytochrome *b* – control region (cyt *b* – CR) and the protein coding ND 6 – $tRNA^{glu}$ – cyt *b* were specifically designed based on sequence of *Tomistoma schlegelii* from Genbank (AJ810455) using PrimerSelect in DNASTAR Lasergene version 7.0 (DNASTAR, Inc). Sequence positions and

limits were set manually while melting temperatures were set between 45 to 58ºC. Primer locations were restricted to 100 – 200 bp for each pairs while all other parameters were left as default. The primers were checked for the gene region, product length, primer dimers and hairpins formation. Melting temperature differences were limited to not more than 5ºC. As the sequencing for the repetitive regions in the mtDNA control region involved a large area (approximately 2.5 kb) three overlapping sets of primers were designed for the control region to cover the entire region.

3.3 Laboratory Analysis

3.3.1 DNA Extraction

Total DNA extraction was isolated following Chong *et al*. (2000) for whole blood. A total of 20 µl of RBCs were lysed with 1 % SDS and 400 µg/ml proteinase K incubated at 37°C overnight, followed by separation of DNA by precipitating proteins using phenol:chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). DNA was precipitated from the solution using pure ethanol. The precipitated DNA was eluted in 30 µl of 10 mM Tris – HCl buffer at 4°C overnight before storage at -20ºC.

The DNA from QIAcard FTA Spots was extracted following recommendation by the supplier for DNA extraction. Using a 1.2 mm coring device, a disc from the cards is removed and placed into PCR tubes. This disc was washed three to four times with the FTA Purification Reagent, incubating about 5 minutes each time at

room temperature. Subsequently, the disc was washed with 1X TE buffer for a total of three to four times using a pipette. Finally, the disc was added with 50 - 100 µl of 1X TE buffer in a fresh PCR tube and incubated at 37°C for half an hour. The disc was then discarded. The remaining TE buffer was quantified for the DNA concentration and used in the respective PCR protocols.

3.3.2 DNA Quantification

DNA concentration was determined with the Biorad SmartSpec^{TM} Plus spectrophotometer using absorbance at 260 nm. A total of 1 µl of extracts were diluted into 100 µl of 10 mM Tris – Cl and calculation was based considering this dilution factor. DNA purity was estimated by absorbance ratio of 260 / 280 nm. Readings were repeated three times and the average was calculated to improve accuracy.

Gel electrophoreses was carried out by running 1 µl of extracted DNA mixed with 1 µl of loading dye on a 1 % agarose gel at 80 V for 45 minutes to view the intactness of the extracts using the 1 kb ladder (Vivantis) as reference. After gel electrophoresis, the gel was stained in ethidium bromide solution for 5 minutes before viewing with GeneSnap version 7.0.4 (Synoptics Ltd).

3.3.3 PCR Optimization

One sample each from Peninsular Malaysia and Sarawak was used to optimise two different PCRs for the two fragments of the non – coding gene, and one each

for the protein coding fragment and the repetitive region at the 3' of the control region. The PCR parameters that were optimized were the Magnesium (Mg^{2+}), primer and DNA concentration and the annealing time (Table 3.2). For the mtDNA repetitive region, the PCR cycles were also optimized.

Table 3.2: List of optimum PCR parameters according to the primers sets.

Primer Set	Primer concentration (mM)	Annealing temperature (°C)	Elongation duration (sec)	PCR Cycles
Set I	0.3	54	45	35
Set II	0.3	55	25	35
Set III	0.2	55	35	35

An amplified DNA volume of 5 µl was mixed with 1 µl of loading dye before loading it onto a 1 % agarose gel (Vivantis). The gel was then stained with 1 % ethidium bromide and viewed with GeneSnap version 7.0.4 (Synoptics Ltd).

3.3.4 DNA Purification

SV spin columns kit by GENEALL (General Biosystems, Inc) was used in DNA purification before downstream processes. PB Buffer was added to the PCR mix at 5:1 ratio and centrifuged at > 12,000 rpm and the column was washed with NW Buffer and centrifuged at > 12,000rpm. An additional centrifuge step to ensure proper removal of NW buffer was carried out before elution buffer was placed at the centre of the column. After a standing period of 10 minutes, the column was centrifuged and eluted DNA was stored at -20°C. If smearing was seen on the

PCR products gel image, the PCR products were run on 1 % agarose gel and the band of interest, estimated using the 100 bp DNA ladder, was excised by cutting the gel after electrophoresis at 80 V for an hour. The gel slice was weighed and the GB Buffer was added at 3:1 ratio of buffer to gel. The gel slice was incubated at 50°C until gel was completely melted (5 – 10 minutes). The mixture was then placed into the SV column and centrifuged at > 12,000 rpm. The SV column was centrifuged again with GB Buffer to ensure removal of all traces of agarose gel before the washing of column procedure, similar to the direct gene clean method.

3.3.5 Cloning

Cloning was carried out for the PCR products of the VNTRs region using the Invitrogen Cloning Kit following the regular chemical transformation protocol, under cloning of large PCR products, provided by the suppliers. Luria – Bertani (LB) agar with 50 µg / ml Ampicillin was prepared on petri dishes and once solidified the Petri dishes were inverted and left overnight at room temperature. The purified PCR product was then mixed with plasmid vector (pCR2.1–TOPO) and salt solution and incubated at room temperature before adding 2 µl of this mixture into the thawed competent cells, *E. coli* strain TOP10 (Invitrogen). This mixture was then treated with heat shock for 30 seconds at 42°C after incubation on ice. The commercially prepared Super Optimal broth with Catabolite repression (S.O.C) media (Invitrogen) was added to these cells and incubated at 37°C before spreading on pre-warmed selective agar plates which has been spread with X-gal. Plates were incubated at 37°C overnight. Each of the white (positive)

colonies were then individually spread onto separate petri dishes containing LB agar and incubated at 37°C overnight. About 10 colonies were selected from each petri dish and incubated overnight at 200 rpm and 37°C in LB Broth containing 50 µg / ml Ampicillin.

3.3.6 Alkaline Lysis Plasmid Extraction and Restriction Enzyme Digestion

The plasmids were extracted using Alkaline Lysis method following the protocol by Sambrook and Russell (2001) before confirmation of insert using restriction enzyme (RE) digestion. The bacterial cells were lysed when the plasmid is incubated on ice with 0.2 N NaOH and 1 % SDS and the DNA is separated from the cell debris by adding potassium acetate. The mixture was centrifuged using Microfuge^{R} 22R Refrigerated Microcentrifuge (Beckman Coulter Inc) and the supernatant was separated from the pelleted debris. The DNA was precipitated using isoporpanol containing 5 M potassium acetate and centrifuged again. The pelleted DNA was separated from the supernatant and washed with 70 % ethanol before resuspension of DNA in 10 mM Tris – HCl.

RE digestion was carried out using 1 µl of extracted plasmid with 1µl of *Eco*R1 for 1 – 3 hours at 37°C followed by inactivation at 65°C for 20 minutes. The *Eco*R1 sites flank the insert and therefore produced two bands, a 3.9 kb vector and the 2.5 – 3.0 kb insert.

3.3.7 Sequencing

Sequencing was done using ABI 3730XL DNA Analyser in both directions with PCR primers for the two fragments of the control region and the protein coding region. For the repetitive region, in addition of the PCR primers, internal primers were used to sequence homoplasmic bands while the M13 primers were used for the heteroplasmic bands that were cloned.

3.4 Data Analysis of mtDNA for Intraspecific Variation

3.4.1. Sequence Identity and Alignment

Sequences identity was checked using FinchTV version 1.4 by manual comparison of the chromatograph with the sequences generated. A BLAST search for gene homology was conducted before doing the multiple sequence alignment and nucleotide composition using ClustalW in MEGA version 4 (Tamura *et al*., 2007).

3.4.2 Assembling Sequences of the Two Sets of the Control Region Primers

A single contig comprising of 888 bp was obtained with SeqMan program in DNASTAR Lasergene version 7.0 (DNASTAR, Inc) when the sequences from the 701 bp and the 417 bp PCR product for the control region (primer set I and II) were overlapped.

3.4.3. Partition Homogeneity Test

A partition homogeneity test to decide if concatenation of the two different genetic markers for the intraspecific variation gene markers (cyt *b* – CR and the ND 6 – $tRNA^{glu}$ – cyt *b*) was possible was done using PAUP 4.0 beta version (Swofford, 2003) with parsimony criteria and branch and bound search.

3.4.4 Genetic Diversity

3.4.4.1 Polymorphic Sites and Number of Haplotype

Polymorphic sites and haplotypes were identified using DnaSP version 5 (Librado and Rozas, 2009) for each marker separately.

3.4.4.2 Haplotype and Nucleotide Diversity

Standard indices of genetic variation, haplotypic diversity, *h*, and nucleotide diversity, π, defined by Nei (1987) was calculated using DnaSP version 5 (Librado and Rozas, 2009). These two parameters were estimated first, for a total number of samples and then separately for the geographically associated samples of known origins only followed by for each collection sites, considering all samples held in collection each site as a population.

3.4.5 jModeltest

jModeltest software (Posada, 2008) was used to search for the best fit model for the data using only ingroup as to maximise the accuracy of the model for the Maximum Likelihood tree. The likelihood settings were set at default while the

Akaike Information criterion (AIC) was set to calculate parameters importance and model averaging.

3.4.6 Genetic Divergence

Pairwise genetic distance between all haplotypes was calculated using the Tamura Nei (1993) model which incorporates transition (Ti) and transversion (Tv) substitution rates, and the differences in nucleotide frequencies. *Gavialis gangeticus* (Genbank Accession AJ810454) was used as an outgroup to compare the distances of between genus and within species of *Tomistoma*.

For comparison with intraspecific data of other crocodilian, we used the Tajima – Nei distance (Tajima and Nei, 1984) which assumes an equality of substitution rates among sites and between substitution implemented in MEGA version 4 beta (Tamura *et al*., 2007) for the ND 6 – $tRNA^{glu}$ – cyt *b* fragment. The K2 model (Kimura, 1980), the uncorrected pairwise distance method (Nei and Kumar, 2000) and HKY85 model were estimated for the cyt *b* – CR data. The K2 model considers the Ti and Tv substitution rates with equal nucleotide frequencies and equal substitution among sites while the uncorrected pairwise distance method considers the proportion of nucleotide sites (*p*) at which two sequences being compared are different. The HKY85 (Hasegawa *et al*., 1985) distance was calculated using PAUP over 543 bp of control region sequences while the K2 distance was based on 842 bp and uncorrected pairwise distance was based on 459 bp of the control region.

3.4.7 Phylogenetic Tree

Phylogenetic trees were generated using PAUP version 4b (Swofford, 2003) for distance and character based approaches. TREEVIEW (Page, 1996) was used to view and edit the Neighbour Joining and Maximum Parsimony trees while MEGA version 4 (Tamura *et al*., 2007) was used for the Maximum Likelihood trees. *Gavialis gangeticus* (Genbank Accession AJ810454) was used as an outgroup to root the trees.

3.4.7.1 Neighbour Joining Tree

The distance based Neighbour Joining (NJ) tree was generated using the Tamura – Nei model (Tamura and Nei, 1993) with 1000 bootstrap replicates.

3.4.7.2 Maximum Parsimony Tree

The maximum parsimony trees were carried out by heuristic search using the tree bisection reconnection (TBR) with all characters treated as unordered with equal weight. A 50 % majority rule command was used to search for the best tree from all the trees produced. The confidence value for the branches was generated by 1000 bootstraps replicates (Felsenstein, 1985).

3.4.7.3 Maximum Likelihood Tree

Using the results form jModeltest, the Maximum Likelihood (ML) tree was generated by heuristic search using TBR function with 100 bootstrap replicates.

3.4.8 Haplotype trees

3.4.8.1 Parsimony Based Haplotype Networks

Haplotype connectivity based on statistical parsimony was done with TCS (Clement *et al*., 2000) at 95 % limits. When networks were not connected, connection limits were manually adjusted until all haplotypes were connected.

3.4.8.2 Distance Based Haplotype Networks

Distance based network connection were generated using minimum spanning network (Kruskal, 1956, Prim, 1957) in Arlequin 3.1 (Excoffier *et al*., 2005) and viewed with TreeView (Page, 1996).

3.4.9 Gene Flow and Population Division

3.4.9.1 Nearest Neighbour Statistic (S_{NN})

The extent of population subdivision and gene flow was examined using nearest – neighbour statistic, S_{NN}, which is a measure of how often the nearest neighbours sequences are from the same locality in geographic region. It is useful when population size is small and haplotype diversity is high as in our case (Hudson, 2000). This was analysed by considering only known origins according to geographical regions (with and without considering the distant lineage, H4) using DnaSP version 5 (Librado and Rozas, 2009).

3.4.9.2. Nucleotide Based Pairwise F_{ST}

The pairwise F_{ST} and gene flow, N_M were estimated using Hudson *et al*. (1992) equation based on sequence data in DnaSP version 5 (Librado and Rozas, 2009). This analysis is more sensitive than the chi square analysis. The pairwise F_{ST} analysis was carried out by 10,000 permutations and pseudorandom number seed, for the population groupings as in S_{NN}.

3.4.10 Neutrality Test

All the neutrality tests were carried out using DnaSP (Librado and Rozas, 2009).

3.4.10.1 Fu and Li's D

The D test statistic is based on the differences between the number of mutations appearing only once among the sequences and the total number of mutations (Fu and Li, 1993) while the significance values were obtained by computer simulations assuming that the true value of theta falls into the two tailed test by DnaSP.

3.4.10.2 Fu's F_S

This test is based on haplotype frequency (Fu, 1997) instead of nucleotide frequency as the Fu and Li's D and was carried out using segregating sites. The significance of the D values was estimated with 10,000 replicates using the coalescent simulations which assumes a neutral infinite-sites model and a large constant population size (Hudson, 1990).

3.4.10.3 Ramos and Rozas's R2

The R2, which is suitable for small population sizes (Ramos and Rozas, 2002) was carried out for both the population growth-decline and constant sizes, using the pairwise number of differences and significant values were also estimated using the coalescent simulations with 10,000 replicates.

3.5 Data Analysis for mtDNA Repetitive Regions

3.5.1 Band Size Estimation

Precise band sizes on the gel image were identified using AlphaEaseFC Imaging System version 6.0.2 (Alpha Innotech Corp) by comparing the band sizes of the DNA ladder in the gel image.

3.5.2 Sequence Alignment

Similar to the intraspecific sequence alignment, sequences identity was checked using FinchTV version 1.4 by manual comparison of the chromatograph with the sequences generated and BLAST search for gene homology. The nucleotide composition and multiple sequence alignment were carried out using ClustalW in MEGA version 4 (Tamura *et al*., 2007). Motifs and repetitive sequences were located using the Find Motif function in MEGA version 4 beta.

3.5.3 Secondary Structure and Free Energy Estimation

RNAstructure version 4.6 (Mathews *et al*., 2004) was used to generate secondary structure and to estimate the stable energy levels. The structures are generated based on minimising the free energy level.

CHAPTER 4

RESULTS

4.1 Primer Development

The primer sets I and II were successful in generating sequences flanking the 3' of cyt *b* to the region just before the mtDNA repetitive region for PCR and sequencing. Set I generated 701 base pairs (bp) from the 3' of cyt *b* and Set II generated 417 bp to the CSB III in control region. For the ND 6 – $tRNA^{glu}$ – cyt *b* gene, only one primer set was required, Set III was also successful in generating 570 bp of the target region (Table 4.1).

For the mtDNA repetitive region, located between the CSB III of control region and the 12S rDNA gene, the forward primer of Set II and a reverse primer located at the 12S rDNA (H491) were able to amplify the required region for PCR and sequencing. For the heteroplasmic bands, instead of the PCR primers, M13 primers were used for sequencing. Due to the large band size generated for all the individuals with PCR (2.5 to 3.0 kb), internal primers designed in an attempt to sequence the entire length of the products (Table 4.2 and Figure 4.1), also successfully produced the targeted sequences.

Table 4.1: The PCR primers location, sequence and the PCR band size generated for the cyt *b* – CR, the ND 6 – $tRNA^{glu}$ – cyt *b* and the mtDNA repetitive region.

Primer set	Primer location (and PCR product size)	Primer name	Primer sequence (5' to 3')	Fragment size
Set I	58bp of 3' cyt *b* to Domain II (before B Box)	L14930	AGCGGGCAAAATAGAAAACTGA	701 bp
		H15630	ATAGAGATGCCGGGATTACGAA	
Set II	Within D Box of Domain II to just after CSB III	L15516	TATCACTCTCATGTACTCTTCTTG	417 bp
		H15932	AGCTATTTTCATTTTTATTTCTAT	
Set III	The last 285bp of ND 6 to 203bp of 5' cyt *b*	L13457ND6	CCCCACAAACACCAACCAA	570 bp
		H14027CB	CGACGGATGCGAAGGCTATG	
Set IV	Within D Box of Domain II to 491 bp of 12S rRNA	L15516	as above	~ 2.5 – 3.0 kb
		H 491	TCTCTGGCGGGTGTAGTATGTAAT	

Table 4.2: Internal primers used for sequencing of both homoplasmic and heteroplasmic bands. M13 primers were used for initial sequencing of heteroplasmic bands instead of PCR primers (Primer Set IV)

Primer name	Primer sequence (5' to 3')
L15790	TCTATATTTCAGCTATGCCC
H-int	GGTGTAGATGTGCCTGATGCC
M13 Forward (-20)	GTAAAACGACGGCCAGTG
M13 reverse	CAGGAAACAGCTATGACC

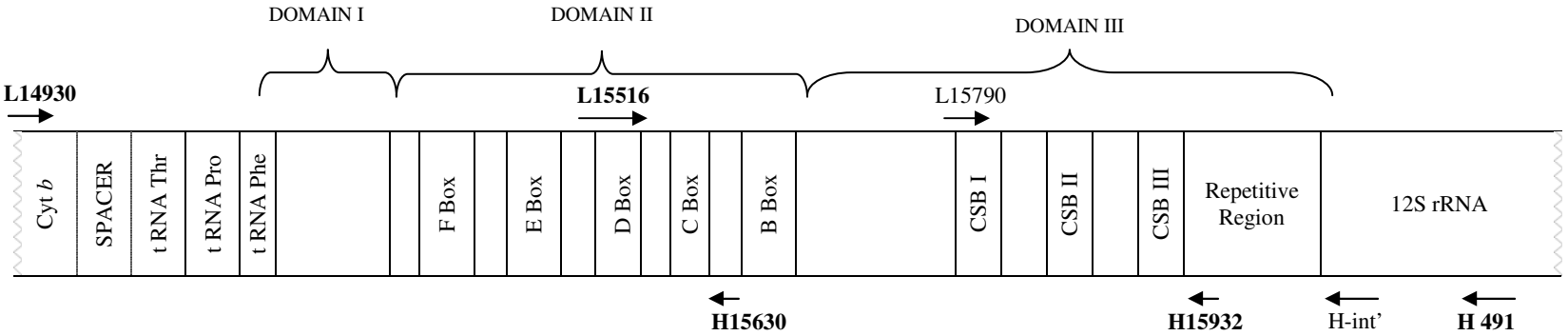


Figure 4.1: Locations of the PCR and internal primers for the mtDNA non – coding repetitive region. Bold font indicates the PCR primers while normal font indicates internal primers.

4.2 Laboratory Analysis

4.2.1 DNA Extraction and DNA Quantification

Intact DNA with a purity of 1.7 to 1.9 (260 / 280) were obtained with the manual DNA extraction protocol (Figure 4.2). The DNA concentration ranged between 35 000 ng/ul to 540 000 ng/ul which was diluted to 50 ng/ul for PCR.

4.2.2 PCR Optimisation and DNA Purification

For the Set I, II and III primers, the concentration for MaxTaq at 0.5 U, $MgCl_2$ at 1 mM, 0.2 mM dNTPs and 50ng of DNA was suitable to generate the required PCR products. The concentration of primer varied for the different primer sets. The optimized PCR conditions for each primer set generated clear bands with minimum smearing (Figure 4.3).

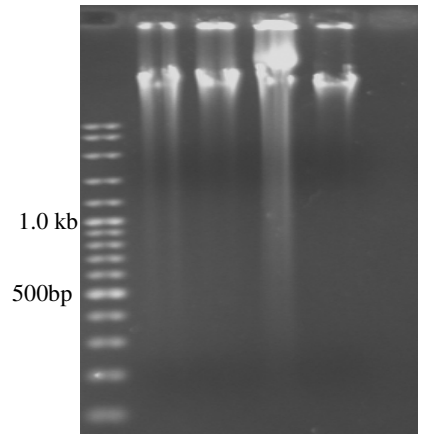


Figure 4.2: Gel image of extracted DNA. A 100 bp DNA ladder is on the left of the gel.

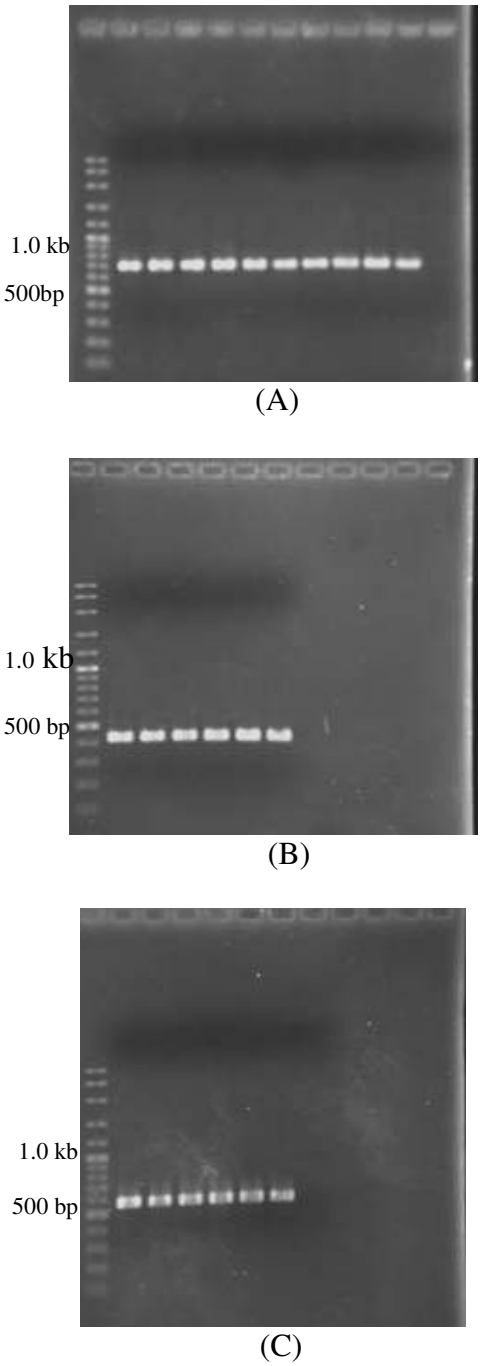


Figure 4.3: Gel image of PCR products of the mtDNA control region. (A) Primer Set I from the 3' cyt *b* to B Box of Domain II of the mtDNA control region, generating approximately 700 bp fragments. (B): Primer Set II from the B Box of Domain II to the CSB region in Domain III of the mtDNA control region, generating approximately 420 bp fragments. (C) PCR products of the ND 6 – $tRNA^{glu}$ – cyt *b* marker using primer Set III from the 3' of ND 6 to 5' of cyt *b*. A 100 bp DNA ladder is on the left of agarose gel.

4.2.3 DNA Purification and DNA Sequencing

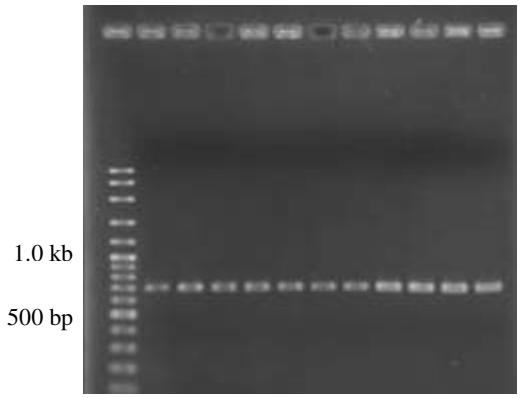
The PCR products were purified before sequencing (Figure 4.4). The strong bands obtained after purification produced good sequencing results with strong, clear peaks with no or minimal noise. Sequencing was done in both directions (forward and reverse) to confirm nucleotide identity.

4.3 Data Analysis for Intraspecific Variation

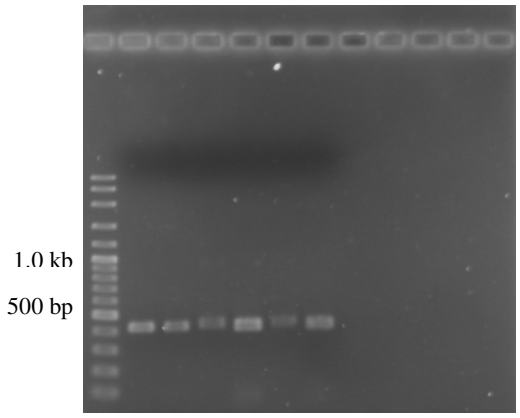
4.3.1 Sequence Identity and Alignment

The comparison of sequences obtained for the forward and reverse sequences with the chromatograph showed identical sequences. BLAST search results showed a maximum identity of 98 – 100 % over a query coverage of 100 % with the available *Tomistoma* sequence in Genbank. The Set I and II markers were overlapped to generate a single contig. The final alignment for Set I and Set II as a contig was 888 bp from tRNA – Thr to CSB II while the final alignment for Set III primers was 431 bp.

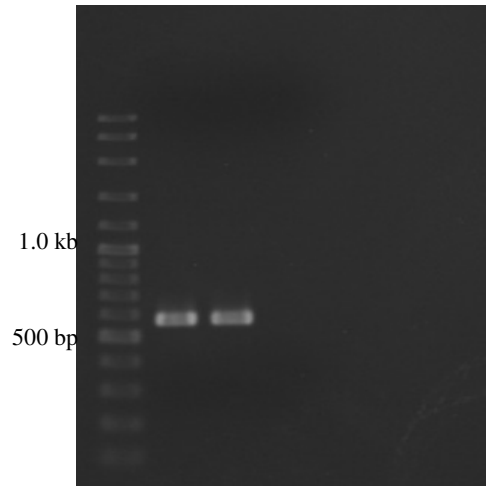
Sequence obtained, 888 bp from the cyt *b* – CR and 431 bp from the ND 6 – $tRNA^{glu}$ – cyt *b* gene, had been deposited into the Genbank (Appendix 2). Nucleotide composition for the cyt *b* – CR marker was T (31.3 %), C (26.8 %), A (28.9 %) and G (13.0 %) while ND 6 – $tRNA^{glu}$ – cyt *b* was T (23.7 %), C (29.9 %), A (34.3 %) and G (12.1 %).



(A)



(B)



(C)

Figure 4.4: Gel image of purified PCR products of the mtDNA control region. (A) Primer Set I and (B) Primer Set II. (C) Primer set III (ND 6 – $tRNA^{glu}$ – cyt *b*). A 100 bp DNA ladder is on the left of agarose gel.

4.3.2 Partition Homogeneity Test

Partition homogeneity test for the total length of 1319 bp from the concatenate data indicated the two markers were significantly congruent with a *p* value of 1.0. Since using multiple genes provide better estimates than individual genes, three sets of data consisting of the cyt *b* – CR marker, ND 6 – $tRNA^{glu}$ – cyt *b* marker and a concatenation of these two were used in the subsequent analysis. These three data sets were congruent in all analyses except for the population structure analysis.

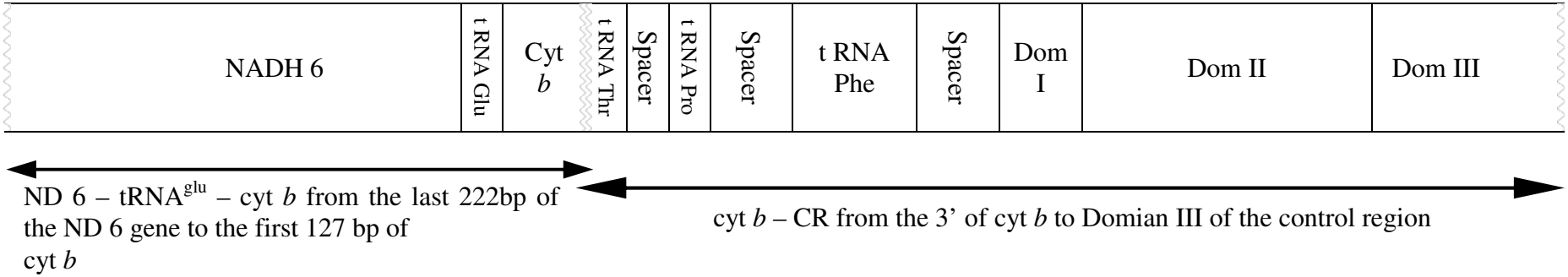
4.3.3. Genetic Diversity

4.3.3.1 Polymorphic Sites and Number of Haplotypes

There were 22 polymorphic sites with 20 transitions, one transversion and an indel, which resulted in five haplotypes for the cyt *b* – CR marker. For the ND 6 – $tRNA^{glu}$ – cyt *b* marker, 12 polymorphic sites with 10 transitions and two transversions also resulted in five haplotypes (Table 4.3). The sequence alignment of 1319 bp from the concatenate data revealed six haplotypes. The H1 and H2 haplotypes differed by one transition in the cyt *b* – CR but had identical sequence over the 431bp of the ND 6 – $tRNA^{glu}$ – cyt *b* marker. However, H1 and H3 differed by one transition in the ND 6 – $tRNA^{glu}$ – cyt *b* region. The ND 6 – $tRNA^{glu}$ – cyt *b* marker was equally sensitive as the control region as it was able to detect a different haplotype from some individuals with H1 haplotype of the mtDNA control region. The haplotype H4 had seven and eight unique base substitutions in the ND 6 – $tRNA^{glu}$ – cyt *b* and the cyt *b* – CR marker

Table 4.3: Location of polymorphic sites for the ND 6 – tRNA^{glu} – cyt *b* and cyt *b* – CR sequences. Six haplotypes, H1 to H6 were detected from the total of 56 samples.

	Position of polymorphic sites																																		N
	13522	13554	13557	13567	13620	13687	13693	13715	13735	13797	13828	13935	15029	15107	15123	15180	15193	15223	15241	15254	15293	15295	15418	15442	15590	15608	15620	15655	15700	15704	15729	15768	15805	15857	
H1	C	A	G	A	G	T	T	C	C	G	G	T	T	C	C	T	T	C	C	G	C	A	C	T	A	T	C	C	A	T	T	A	T	A	21
H2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	13
H3	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	9
H4	G	G	T	.	.	C	C	T	T	.	A	C	.	T	.	C	C	T	.	A	T	G	T	C	G	C	T	.	.	.	C	G	.	.	3
H5	.	.	.	.	A	C	.	.	T	A	.	.	G	T	.	C	.	.	T	.	.	G	T	C	.	.	.	T	G	C	C	.	-	G	2
H6	.	.	.	.	.	C	.	.	T	A	.	.	G	T	.	C	.	.	T	.	.	G	T	C	.	.	.	T	G	C	.	.	.	G	8



respectively (Table 4.3). These nucleotide sites clearly distinguished H4 from the other haplotypes. The H5 (of unknown origins) and H6 haplotypes found in East Kalimantan differed by only one transition in each marker. When populations of known origin (Peninsular Malaysia, Sarawak, East Kalimantan and Sumatra) were compared, each population had one haplotype which was distinct between geographical regions with the exception of Peninsular Malaysia (Table 4.4). Peninsular Malaysia had three haplotypes, which consisted of the most divergent haplotype, H4, the most commonly found haplotype, H1 and the Sarawak haplotype, H2 which differs with H1 by one base substitution. The two specimens of unknown origins had the H5 haplotype. Results from locations of unconfirmed origins (Jawa, Singapore Zoo and Hong Kong) did not reveal any new haplotypes for the *Tomistoma* in this study.

4.3.3.2 Haplotype and Nucleotide Diversity

The haplotype diversity, *h* was similar for both markers at 0.6 and the nucleotide diversity, π were 0.6 % and 0.5 % for the cyt *b* – CR and ND 6 – $tRNA^{glu}$ – cyt *b* marker respectively when all samples were considered. When only samples of known origins were considered, *h* for the cyt *b* – CR marker was slightly higher at 0.70 compared to 0.6 for ND 6 – $tRNA^{glu}$ – cyt *b* marker, while π was 0.66 and 0.60% for the cyt *b* – CR marker and ND 6 – $tRNA^{glu}$ – cyt *b* marker respectively (Table 4.5).

Table 4.4: Haplotype distribution from the respective sampling regions.

Origin	Peninsular Malaysia	Sarawak	Sumatra	Kalimantan	unknown	unknown	unknown	unknown	Total
Collection Sites / Haplotypes	Peninsular Malaysia	Sarawak	Jawa, Indonesia	Kalimantan, Indonesia	Singapore	Hong Kong	Peninsular Malaysia	Jawa, Indonesia	
HI	10				6	1		4	21
H2	2	7			4				13
H3			3			3		3	9
H4	3								3
H5							2		2
H6				5				3	8
Total	15	7	3	5	10	4	2	10	56

Table 4.5: List of haplotype (*h*) and nucleotide (π) diversities of the two mtDNA markers and concatenate data. Diversities estimates are for a total number of 56 samples. The diversities estimate for 30 samples of known origin is also presented.

Groups of samples	All samples		Samples with known geographical origins	
Diversity parameters / Markers	*h*	π (%)	*h*	π (%)
cyt *b* – CR	0.646	0.556	0.708	0.66
ND 6 – $tRNA^{glu}$ – cyt *b*	0.592	0.485	0.570	0.60
Concatenate	0.769	0.535	0.777	0.64

Overall haplotype diversity, using the concatenate data, was 0.77 with Peninsular Malaysia showing highest haplotype and nucleotide diversity (h = 0.63, $\pi = 0.00770$). In terms of holding areas, the highest haplotype diversity was seen in Zoo Negara, Malaysia (h = 0.867, $\pi = 0.01093$) followed by Cikananga Wildlife Rescue Center, Jawa (h = 0.733, $\pi = 0.00531$). Singapore Zoo and Hong Kong Wetland Park had two haplotypes each while Sumatra, West Kalimantan, Sarawak (2 collection sites) and Mini Zoo Temerloh had one haplotype each resulting in no genetic diversity. When geographical regions were considered, only Peninsular Malaysia showed genetic variation (Table 4.6).

4.3.4 jModeltest

The model revealed by jModeltest for the concatenate data and the cyt *b* – CR data was the TPM2uf+G model. However, for the concatenate data the proportion

of invariable sites was 0.8627. The ND 6 – $tRNA^{glu}$ – cyt *b* data had equal rates with the TPM2uf model (Table 4.7).

Table 4.6: Haplotype and nucleotide diversities of the sampling localities using concatenated data.

Holding areas	*n*	*h*	π
Hong_Kong	4	0.500	0.00038
Singapore	10	0.533	0.00040
Jawa	10	0.733	0.00531
Sumatra (PT Ekanindya Crocodile Farm)	3	0	0
Kalimantan (Surya Crocodile Farm)	5	0	0
Sarawak	7	0	0
Zoo Negara	6	0.867	0.01093
Zoo Melaka	4	0.667	0.01162
Taiping Zoo	3	0.667	0.00051
Mini Zoo Temerloh	4	0	0
Total	**56**	**0.769**	**0.00535**
Geographical region (using only known origins)			
Sumatra	3	0	0
Peninsular Malaysia	15	0.632	0.00770
Sarawak (West Borneo)	7	0	0
Kalimantan (East Borneo)	5	0	0

Table 4.7. The jModeltest results for the three data sets.

Marker	Concatenate Data	cyt *b* – CR Data	ND 6 – $tRNA^{glu}$ – cyt *b* Data
Model	TPM2uf+G	TPM2uf+G	TPM2uf
partition	010212	010212	010212
-lnL	1977.1867	1325.7426	643.0076
freqA	0.3061	0.2870	0.3458
freqC	0.2774	0.2676	0.2977
freqG	0.1285	0.1317	0.1214
freqT	0.2880	0.3136	0.2352
R(a) [AC]	0.0000	0.0000	0.0000
R(b) [AG]	9.4167	9.6042	4.0795
R(c) [AT]	0.0000	0.0000	0.0000
R(d) [CG]	1.0000	1.0000	1.0000
R(e) [CT]	9.4167	9.6042	4.0795
R(f) [GT]	1.0000	1.0000	1.0000
gamma shape	0.0150	0.0150	-

4.3.5 Genetic Divergence

The genetic distance calculated with concatenate data was between 1.2 to 1.8 % for the H4 haplotype while the other haplotypes had a genetic divergence that ranged between 0.1 to 1.3 % using the Tamura Nei model (1993) (Table 4.8). Similarly, with the cyt *b* – CR and the ND 6 – $tRNA^{glu}$ – cyt *b* data, the H4 haplotype showed the highest genetic divergence (Appendix 3).

Table 4.8: Genetic distance of the concatenate data using Tamura-Nei distance. Tamura and Nei, (1993) genetic distance is based on unequal base frequencies and unequal ratios of transitions to transversions (Ti:Tv) implemented in MEGA v.4 (Kumar *et al*., 2004)

	H1	H2	H3	H4	H5	H6
H1	-					
H2	0.00076	-				
H3	0.00076	0.00152	-			
H4	0.01777	0.01856	0.01855	-		
H5	0.01230	0.01308	0.01308	0.01778	-	
H6	0.01074	0.01153	0.01152	0.01777	0.00152	-
Gavialis	0.15510	0.15617	0.15612	0.15494	0.15501	0.15709

Using Tajima – Nei (1984) model, the genetic distance of the ND 6 – $tRNA^{glu}$ – cyt *b* marker ranged from two to ten fold higher when other haplotypes were compared to the H4 haplotype. The lower range of the genetic divergence from the cyt *b* – CR of *Tomistoma* (0.1%) is comparable with the *Osteolaemus*

population (0 – 0.7 %) (Eaton *et al*., 2009) but was six times lower from the divergence between *Osteolaemus* species (Eaton *et al*., 2009) (Table 4.9).

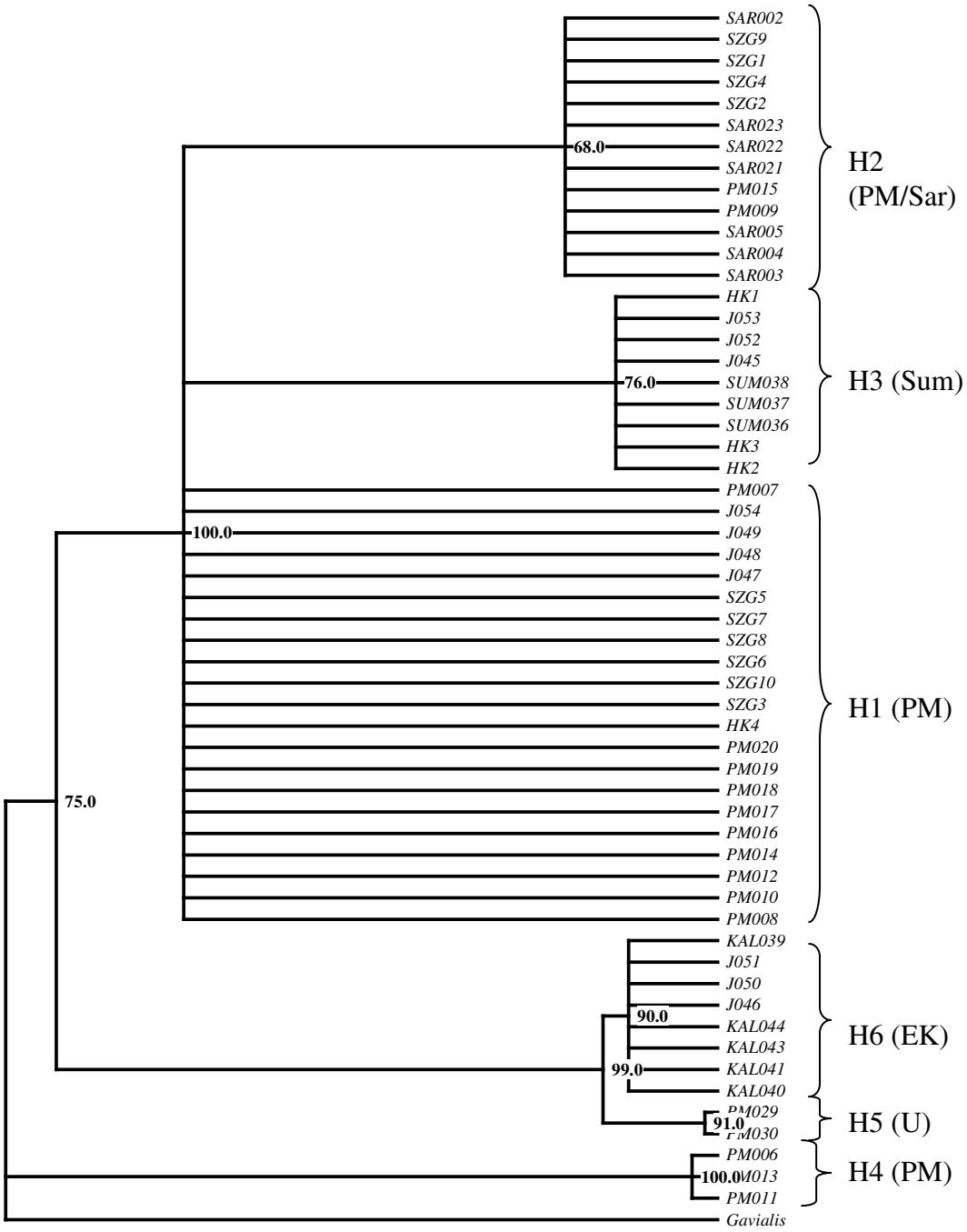
Table 4.9: Genetic distance in percentage using Tajima Nei, Kimura 2 Parameter, the uncorrected pairwise distance and the HKY model. The Tamura Nei method was carried out the total base pairs generated in this study for the respective genes. The cyt *b* – CR was truncated to 842 bp, 459 bp and 543 bp for the Kimura 2 Parameter, the uncorrected pairwise distance method and the HKY model respectively for comparisons with *Osteolaemus* (Ray *et al*., 2000; Eaton *et al*., 2009), *Crocodylus rhombifer* (Weaver *et al*., 2008) and *C. moreletti* (Ray *et al*., 2004)

Distance models	Species	cyt *b* – CR	ND 6 – $tRNA^{glu}$ – cyt *b*
Tajima – Nei	*Tomistoma*	-	0 – 2.4
	Osteolaemus	-	0.3 – 9.8
K2P	*Tomistoma*	0.1 – 1.8	-
	Osteolaemus (within species)	0 – 0.4	-
	Osteolaemus (between species)	11.5 – 12. 4	-
Uncorrected pairwise distance	*Tomistoma*	0 – 2.4	-
	C. rhombifer	1.6	-
	C. rhombifer vs *C. acutus*	3.4 – 4.1	-
HKY85	*Tomistoma*	0 – 0.0188	-
	C. acutus	0.0048	-
	C. moreletti	0.0062	-
	C. acutus vs *C. moreletti*	0.0553	-

4.3.6 Phylogenetic Trees

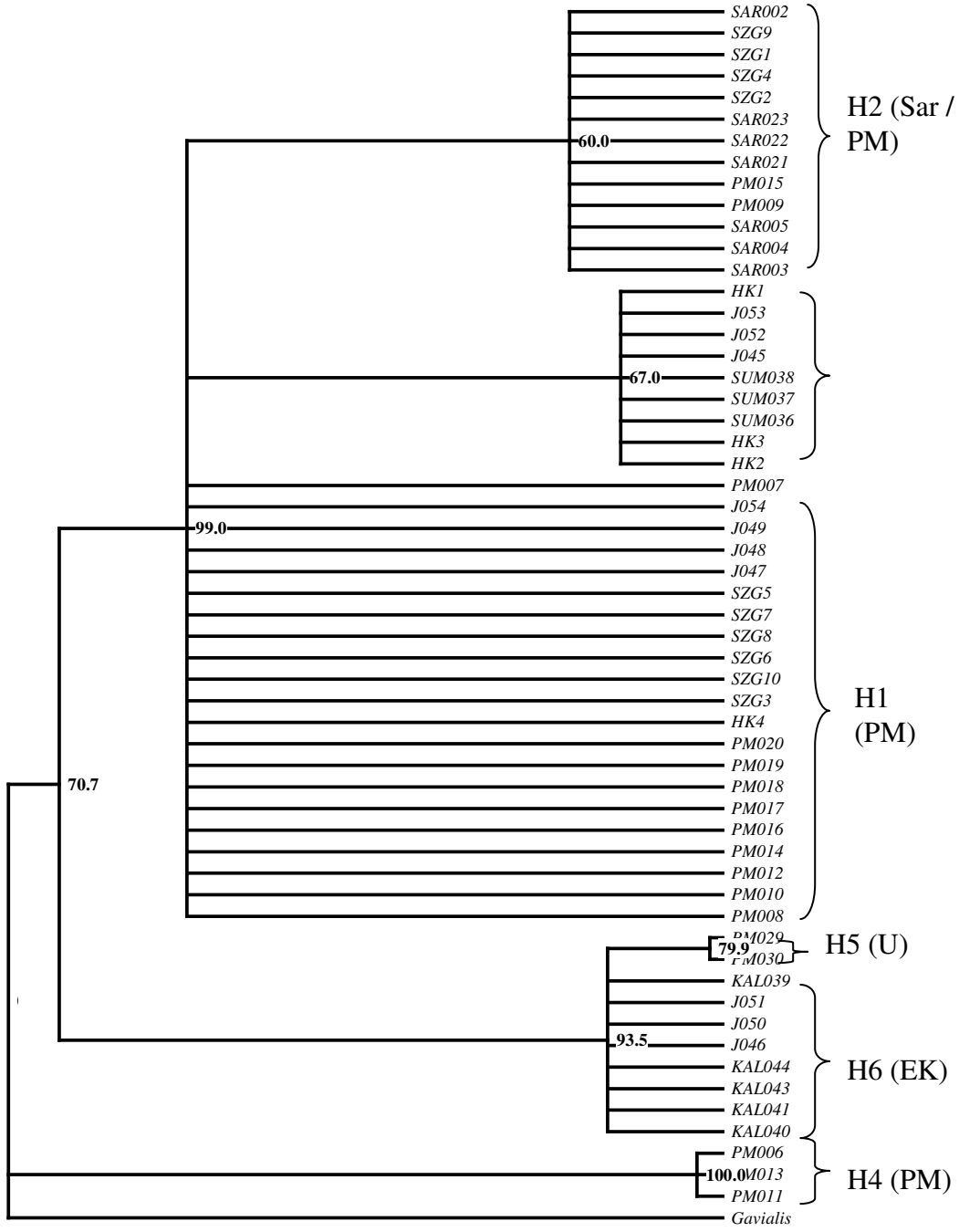
The Neighbour Joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) phylogenetic trees had congruent topology for the concatenate data (Figure 4.5) and individual markers data (Appendix 4). Three main clades were seen in all the trees with the H4 lineage as the basal clade with very high bootstrap values of either 100 % or 99 % (Figure 4.5). The other two major clades consisted of subclades comprising haplotypes that differed by only one base substitution in both the genetic markers. The H1 haplotype from Peninsular Malaysia, H2 haplotype from Peninsular Malaysia and Sarawak and the H3 haplotype from Sumatra grouped into one major clade whereas the H5 haplotype (unknown origins) and H6 haplotype from East Kalimantan always clustered into another major clade. The two individuals whose origins were unknown (H5 haplotype) were always grouped into the East Kalimantan clade in all trees.

Samples with unknown origins from Singapore Zoo clustered with the subclades representing Peninsular Malaysia and Sarawak while samples from Jawa clustered with the subclades representing Sumatra, Peninsular Malaysia and Kalimantan. Samples from Hong Kong clustered with those of Sumatra and Peninsular Malaysia.



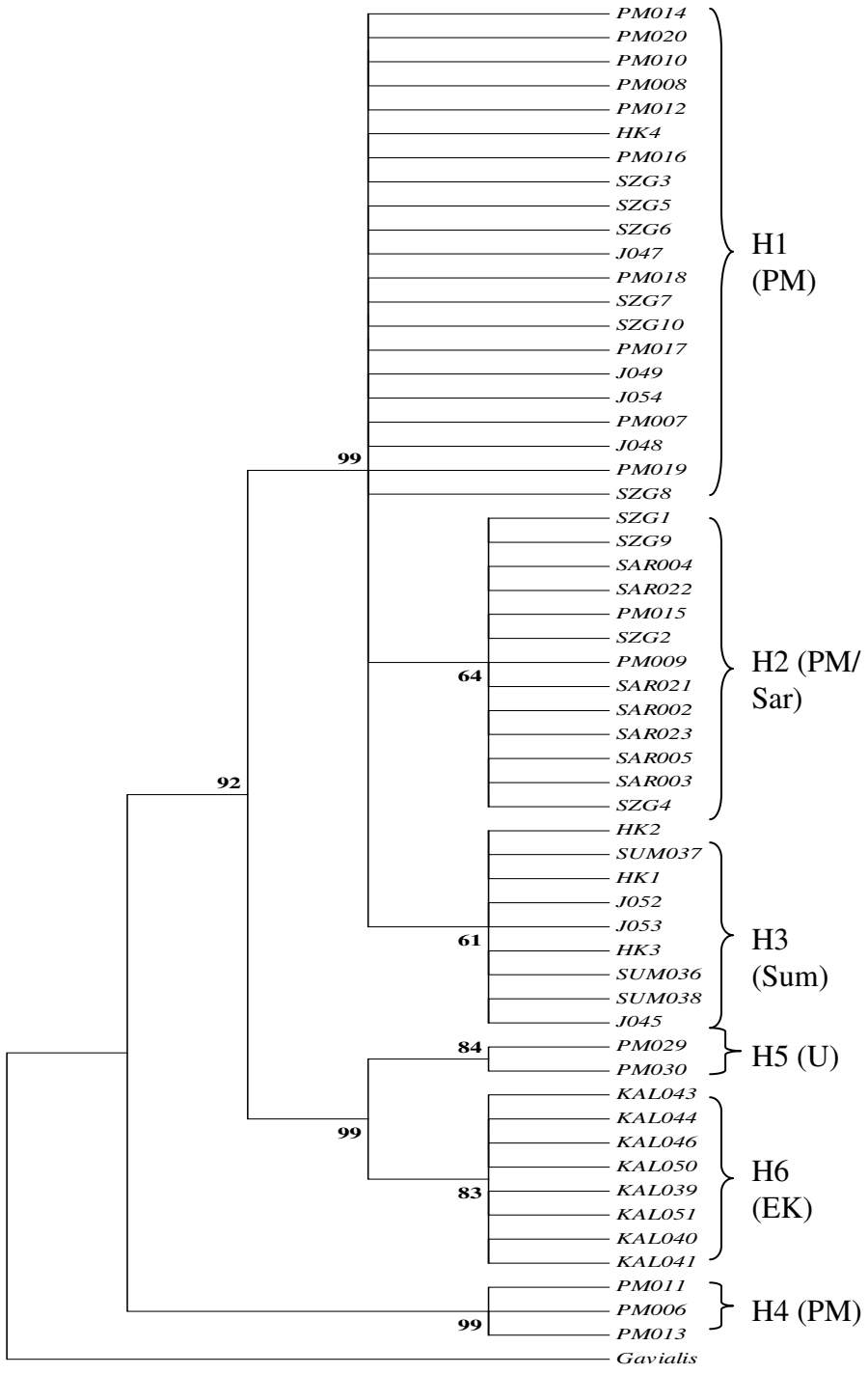
(A)

Figure 4.5: Phylogenetic trees generated with concatenate data. Trees generated with PAUP v4.0b using *Gavialis* as an outgroup. Clades are indicated following lineages (H1 to H6) and geographical regions are bracketed. (PM = Peninsular Malaysia, Sar = Sarawak, Sum = Sumatra, EK = east Kalimantan, U = Unknown). (A) The Neighbour Joining tree following Tamura Nei (Tamura and Nei, 1993) model. (B) The Maximum Parsimony tree. (C) The Maximum Likelihood tree.



(B)

Figure 4.5 - Continued



(C)

Figure 4.5 – Continued

4.3.7 Haplotype Trees

4.3.7.1 Parsimony and Distance Based Haplotype Network

The H4 haplotype could not be reliably connected to the other haplotypes at 95 % probability level for the concatenate (Figure 4.6) and cyt *b* – CR data (Appendix 5). The 95 % connection limit for *Tomistoma* was 8 steps for the ND 6 – $tRNA^{glu}$ – cyt *b* marker which connected all haplotypes (Appendix 5). The concatenate data haplotype network could only be connected manually at 23 steps (Figure 4.7) and for the cyt *b* – CR marker at 14 steps (Appendix 5). Haplotype connections within the distinct (95 %) networks remained unchanged in the manually joined networks. This lineage appeared on a different evolutionary branch in the minimum spanning network while the other haplotypes were placed together on a separate evolutionary branch for the concatenate data (Figure 4.8) and individual marker data (Appendix 5).

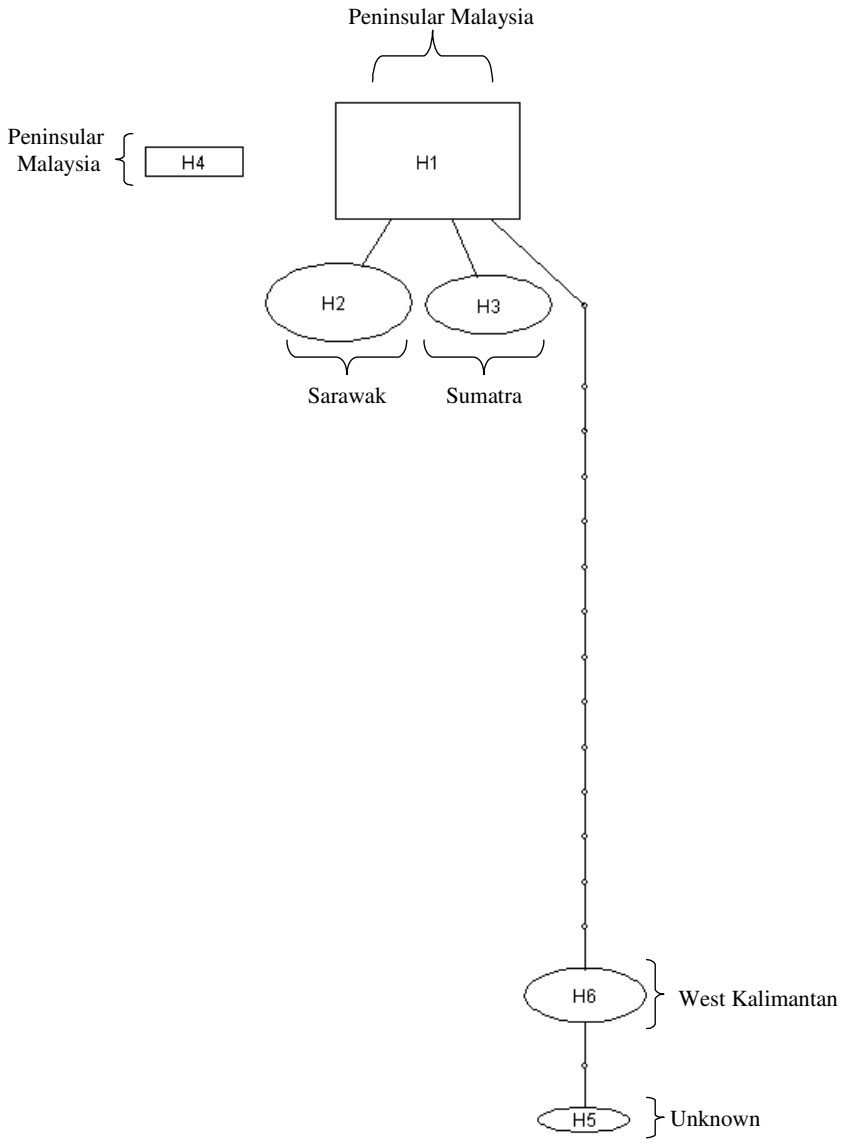


Figure 4.6: Haplotype network using concatenate data. The H4 haplotype was not parsimoniously connected at the 95 % limit with TCS.

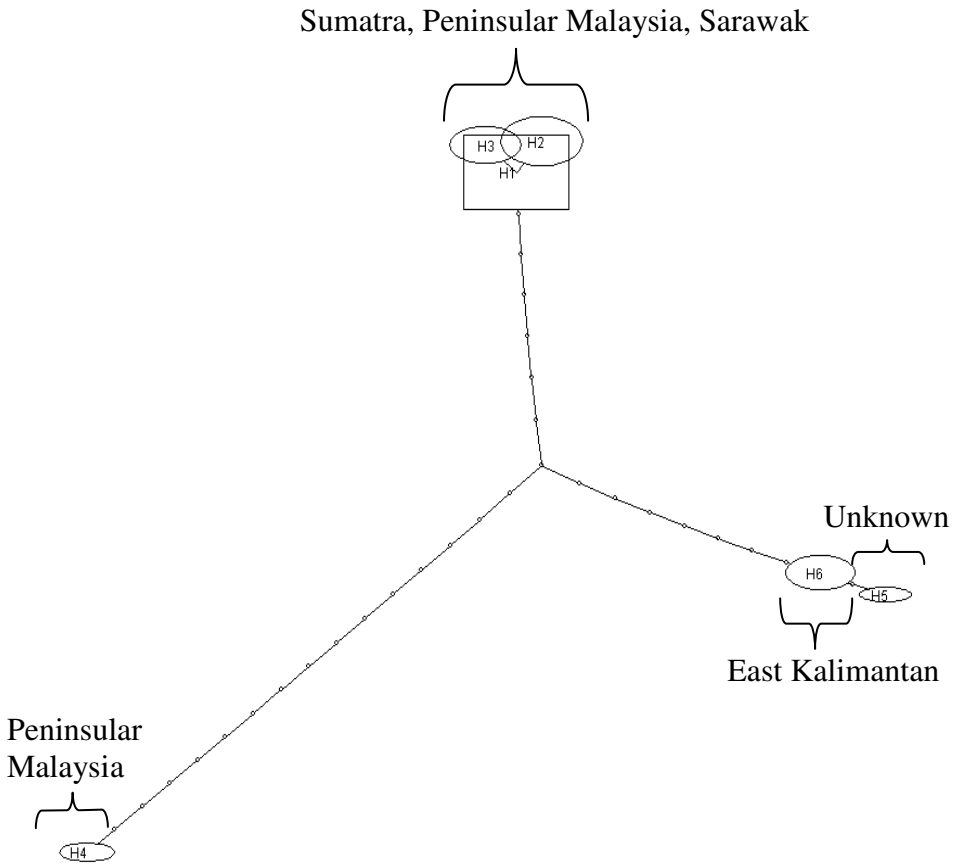


Figure 4.7: Haplotype network using concatenate data connected manually at 23 step connection limit.

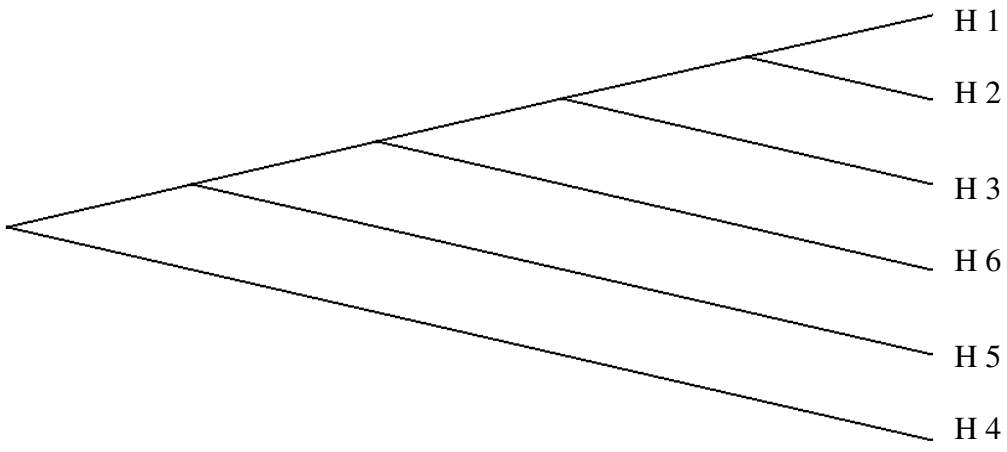


Figure 4.8: Distance based haplotype tree using concatenate data. H4 lineage is on a different evolutionary branch and is reciprocally monophyletic from other lineage.

4.3.8 Gene Flow and Population Division (S_{NN} and Pairwise F_{ST})

The S_{NN} analysis and pairwise F_{ST} for ND 6 – $tRNA^{glu}$ – cyt *b*, cyt *b* – CR and the concatenate data were congruent for a well differentiated East Kalimantan population from other regions.

S_{NN} results showed that the populations from the four geographical regions (Sumatra, Peninsular Malaysia, Sarawak and East Kalimantan) were significantly differentiated with almost no gene flow using the concatenate data (Table 4.10 and Table 4.11). The cyt *b* – CR also showed four main populations for *Tomistoma* that are geographically associated (Appendix 6). However, the S_{NN} analysis using the ND 6 – $tRNA^{glu}$ – cyt *b* data found only three well differentiated populations, the Sumatran population, Peninsular Malaysia – Sarawak population and the East Kalimantan population (Appendix 6). The incongruence between the Sarawak and Peninsular Malaysian populations with the ND 6 – $tRNA^{glu}$ – cyt *b* could be due to the failure of this marker to differentiate the two different haplotype, H1 and H2 which were detected in the cyt *b* – CR and concatenate data. Therefore, in this study, the concatenated data would be used to discuss the population analysis of *Tomistoma*.

The pairwise F_{ST} findings were congruent with the S_{NN} analysis for the concatenate data set which separated populations of *Tomistoma* into the four geographical regions. However, the pairwise F_{ST} was incongruent between the two individual markers for the populations from Sumatra, Peninsular Malaysia

and Sarawak. The pairwise F_{ST} estimates using the cyt *b* – CR data set found that the Sumatran and Peninsular Malaysian population had substantial gene flow between them whereas the ND 6 – $tRNA^{glu}$ – cyt *b* data set showed that the Sarawak and Peninsular Malaysian population were undifferentiated.

The reason for the incongruence between the S_{NN} and the pairwise F_{ST} estimates could be due to the small sampling size in which the S_{NN} analysis is more powerful (Hudson, 2000) and therefore the discussion for the population structure would be based on the S_{NN} estimates.

Table 4.10: Population structure estimates using concatenate data. Analysis carried out following geographical regions using samples of known origins, excluding the H4 haplotype. Above diagonal is the S_{NN} values while below diagonal are the F_{ST} values. The significant value of the S_{NN} and F_{ST} estimates are $p < 0.0001$ and $p < 0.001$. The sample size for each population is 12 samples for Peninsular Malaysia, seven for Sarawak, three for Sumatra and five for East Kalimantan.

Locations	Peninsular Malaysia	Sarawak	Sumatra	East Kalimantan
Peninsular Malaysia	-	0.82	1.00	1.00
Sarawak	0.82	-	1.00	1.00
Sumatra	0.87	1.00	-	1.00
East Kalimantan	0.99	1.00		-

Table 4.11: Gene flow estimates using concatenate data. Gene flow estimated between populations following $N_m = 1- F_{ST} / 2 F_{ST}$ (Hudson *et al*., 1992)

Locations	N_m from F_{ST}
Peninsular Malaysia X Sarawak	0.11
Peninsular Malaysia X Sumatra	0.07
Peninsular Malaysia X East Kalimantan	0.01
Sarawak X Sumatra	0
Sarawak X East Kalimantan	0
Sumatra X East Kalimantan	0

4.3.9 Neutrality Test

Neutrality tests were carried out when considering all samples as a population and subsequently with populations of known origins. The Peninsular Malaysia samples were treated as a population. The neutrality tests could not be carried out for the other regions because each region only had one haplotype.

The significantly positive Fu and Li's D indicated that the total populations and Peninsular Malaysian were at balancing selection, population structure or a recent bottleneck (Table 4.12). The neutrality tests for the cyt *b* – CR and the ND 6 – $tRNA^{glu}$ – cyt *b* data are in Appendix 7. Though Rozas R2 and Fu's Fs were positive, they were not significant when tested with coalescent simulations ($p > 0.05$) indicating population at equilibrium. However, when the H4 lineage was not considered in Peninsular Malaysian population, the Fu and Li's D value was

positive but not significant. A non significant neutrality test is unable to reject the null hypothesis, which assumes a population at equilibrium. For small samples, the R2 is a more powerful test and therefore for subsequent discussion, the R2 would be used for discussion.

Table 4.12: Neutrality test using concatenate data and samples of known origins only. Bold font indicates significance at $p < 0.05$. Value in brackets are excluding H4 lineage in Peninsular Malaysia.

	Fu and Li's D	Fu's Fs	Rozas R2
Total	**1.88496**	9.682	0.1069
Total Peninsular Malaysia	**1.58616**	9.492	0.1694
	(0.752)	(0.297)	(0.1515)

4.4 mtDNA Control Region Repetitive Region

4.4.1 PCR Optimisation, DNA Purification and Sequencing

The Set IV primers required higher concentration of MaxTaq (1 U), $MgCl_2$ (3mM) and dNTPs concentration was 0.2 mM while other chemicals concentration was the same with the other primer sets. The optimum annealing temperature was at 55°C with a 90 seconds elongation time in a total of 25 cycles.

The target PCR bands were strong with only one individual showing heteroplasmy (Figure 4.9a) and produced suitable purified DNA for sequencing (Figure 4.9b). Sequencing for highly repetitive regions in each direction generated 800 – 1000 bp using both PCR or M13 primers and internal primers. Only 14 individuals from the 30 individuals that were amplified for PCR products were

selected for sequencing. Five of these were hatchlings from a maternal parent while the other represented the different PCR band sizes.

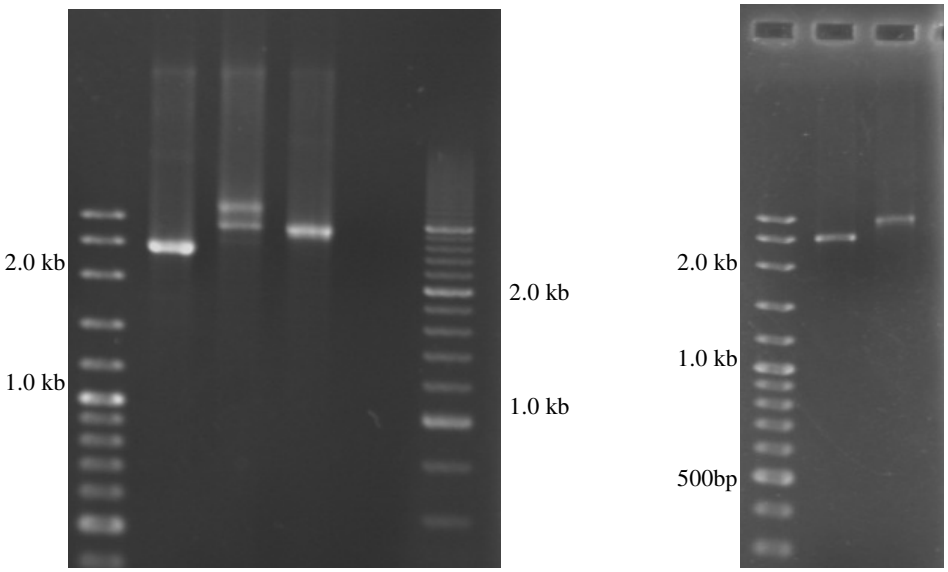


Figure 4.9: Gel image of PCR and DNA purification products for repetitive regions. (A) PCR products using the Set IV primers which amplifies the region of D Box of Domian II to the 491 bp of 12S rDNA at its 5' region. (B) Gene clean products for the homoplasmic bands of the mtDNA control region repetitive region. A 100 bp DNA ladder is on the left of both images while a 200 bp DNA ladder is on the right of image (A).

4.4.2 Cloning and Restriction Enzyme Digestion

The heteroplasmic bands were cloned prior to sequencing because after indirect DNA purification, the band intensity was not ideal for sequencing. The RE digestion on the extracted plasmid produced the two bands of which the 3.9 kb band is the vector while the 2.5 – 3.0 kb is the insert size (Figure 4.10).

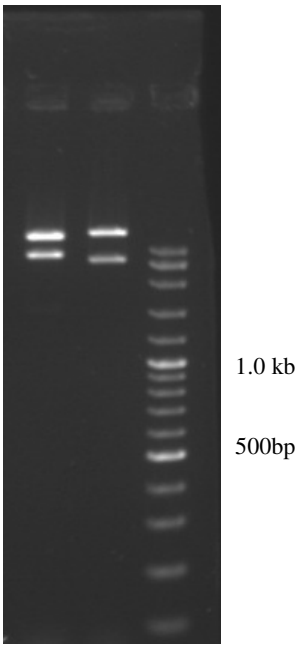


Figure 4.10: Gel image of the restriction enzyme products. A 3.9 kb vector and an approximately 2.5 – 3.0 kb insert of the heteroplasmic PCR product is produced with *Eco*R I. A 100bp DNA ladder is on the right.

4.4.3 Sequence Identity and Alignment

The comparison of sequences obtained with the chromatograph and with BLAST confirmed that the sequences are indeed of the mtDNA VNTR of *Tomistoma*. BLAST results were between 89 – 93 % for maximum identity while the maximum coverage was only 45 – 53 % with the *Tomistoma* sequence available in Genbank. The maximum coverage was higher (46 – 65 %) when comparing with *Gavialis* but maximum identity was lower (52 – 60 %). The forward PCR primer and forward internal primers were able to overlap to generate a single contig. Similarly the reverse PCR primers and reverse internal primers were also overlapped to generate a single contig. The multiple alignment for the region after CSB III is in Appendix 8.

Although internal primers were used, the sequences obtained did not overlap at the VNTR region from both directions due to technical difficulty of sequencing large sequence sizes of repetitive bases ranging from 2.5 to 3.0 kb as seen in this study.

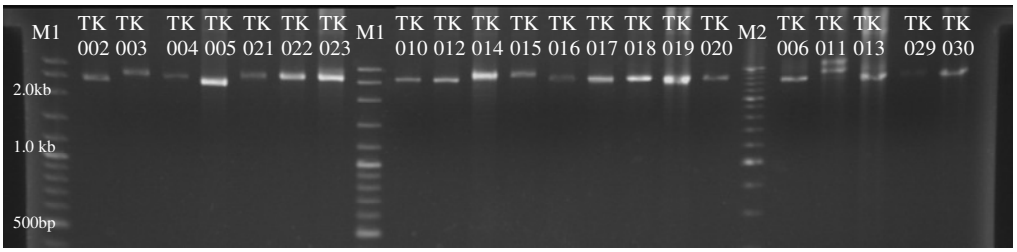
4.4.4 General Structure

The amplified fragments flanking the repetitive region of the mtDNA CR from 30 individuals of *Tomistoma* showed length variation (Figure 4.11A). The shortest homoplasmic band was 2.5 kb while the longest was 2.7 kb (Table 4.13). The 'family unit' showed a uniform band size of around 2.6 kb (Figure 4.11B). The 2.6kb band was also the most common size amongst all individual followed by the 2.7 kb and 2.8 kb band. Only one individual was heteroplasmic with bands longer than the homoplasmic bands, at 2.8 kb and 3.1 kb in length.

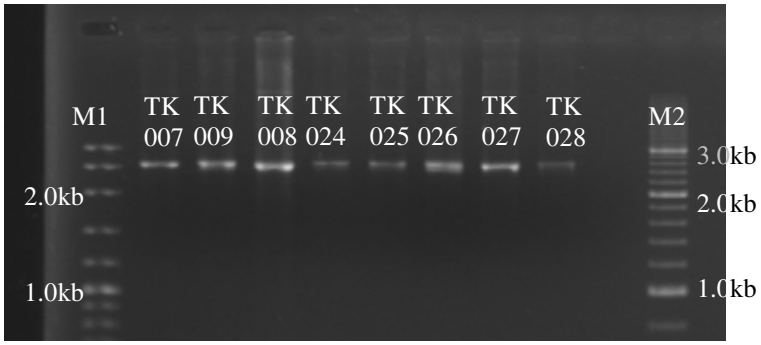
Sequencing results revealed nucleotide composition of the repetitive region (after the CSB III region) as AT rich with adenine (41.1 %), tyrosine (31.9 %), cytosine (16.4 %) and guanine (8.2 %).

Generally, the repetitive region of *Tomistoma* has two main parts. The region closer to the CSB III (5' of the repetitive region) was represented by more than one form of motifs which is repeated between two to four times while the region

closer to the 12S rDNA gene (3' of the repetitive region) consisted of the tandem repeats duplicated at least eight times (Figure 4.12 and Figure 4.13).



(A)



(B)

Figure 4.11: Agarose gel image showing the bands generated using PCR primers Set IV. (A) Band sizes of unrelated individuals showing the size variation and heteroplasmy in *Tomistoma schlegelii*. (B) Family unit showing equal band size. TK007 and TK009 are adult males sharing the pond with the sole female, TK008. TK024 – TK027 are the first nest while TK028 is from the subsequent nest. M1: 100 bp DNA ladder. M2: 200 bp DNA ladder.

Table 4.13: Band sizes of PCR products from Figure 4.11 (A) and (B). The respective size classes, the total number of individuals in each class, the voucher number of selected individuals from the different classes used for sequencing and their respective Genbank accession numbers is presented. GU217576, GU217574 and GU217575 are sequences generated using the reverse PCR primers while the other forward and reverse sequences were generated using internal primers.

Class	Band Size (kb)	Indivi-duals	Voucher	Genbank accession (forward and reverse sequencing)
1	2.47	1	TK005	GQ421353 and GU217577
2	2.52	1	-	
3	2.56	15	TK006	GU220807 and GU217576
			TK007	GQ421351 and GU217578
			TK008	GQ421344 and GU217579
			TK009	GQ421350 and GU217580
			TK010	GQ421352
			TK024	GQ421345 and GU217581
			TK025	GQ421346 and GU217582
			TK026	GQ421347 and GU217583, GU217574
			TK027	GQ421348
			TK028	GQ421349 and GU217584
4	2.61	3	-	
5	2.66	4	TK014	GQ421354
6	2.71	1	-	
7	2.76	3	TK030	GQ421355
8	2.81 and 3.08	1	TK011_short, TK011_long	GQ421356 and GU217585 GQ421357 and GU217586, GU217575

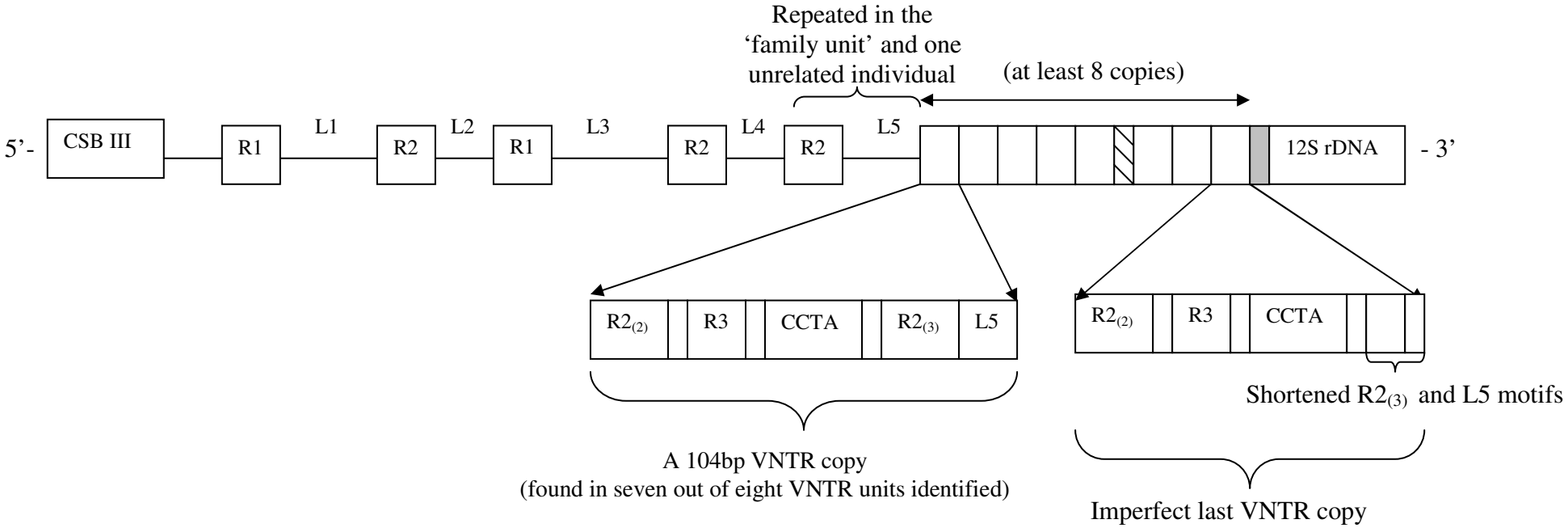


Figure 4.12: Schematic diagram of the repetitive region after the CSB III. The repetitive region consists of two parts; the motif region and the tandem repeats. The motif region consists of motif R1 and R2 followed by their short AT chains, L1, L2, L3, L4 and L5. The region that was not overlapped by both forward and reverse sequencing, marked with jagged line. The size of the non – overlapping region is between 500 bp for the shortest band size to approximately 1 kb for the larger heteroplasmic band. The schematic illustration of unrelated individuals, the family unit and an unrelated individual, TK010 which have duplication of R2 motif and the AT chain, L5 before the VNTRs. The motifs within each 104 bp VNTR and the imperfect VNTR copy at the 3' end are illustrated. The non-repetitive region before the 12S rRNA is shaded in grey.

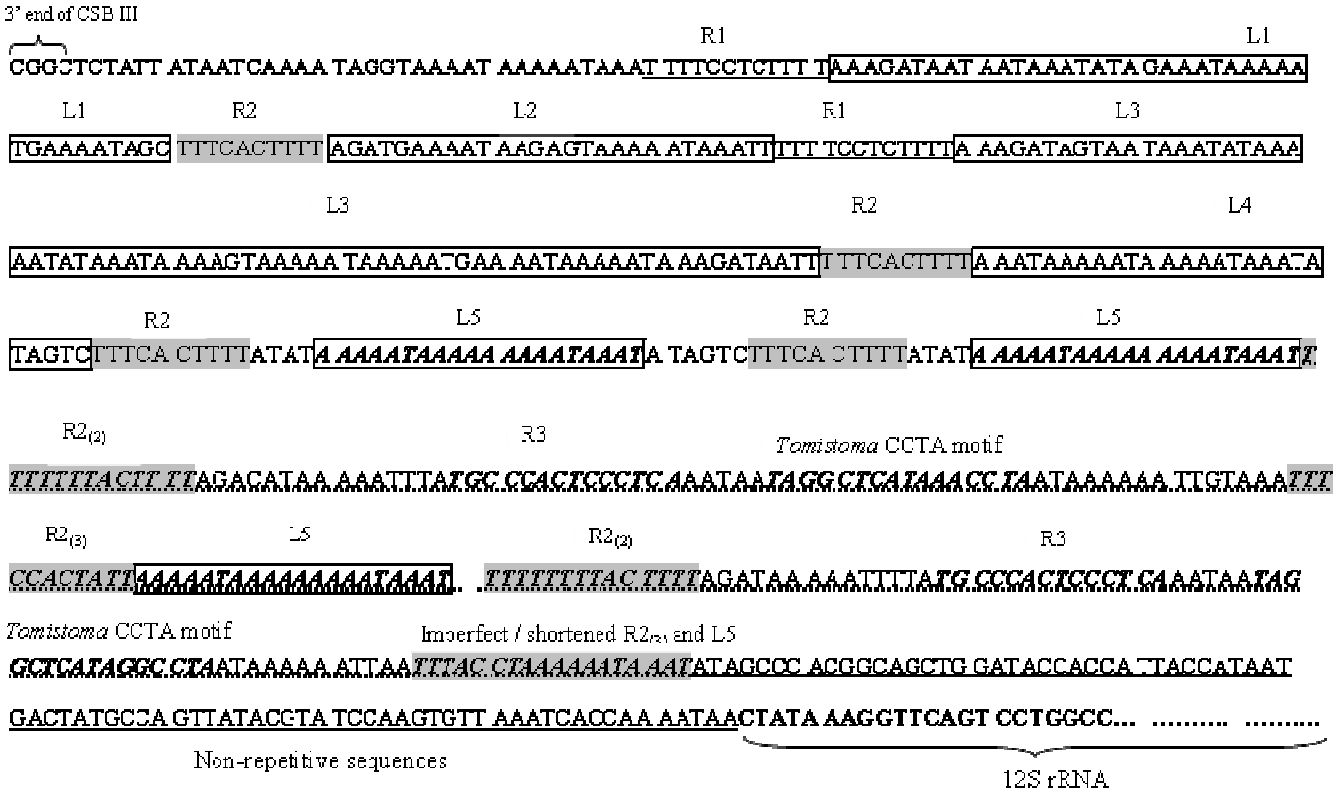


Figure 4.13: Nucleotide sequence of the repetitive region and the flanking CSB III and 12S rRNA gene of *Tomistoma schlegelii* as illustrated in Figure 4.12. The motif region begins approximately 35 bp after the CSB III with motif R1 (underlined). The R2 motif in the motif region and the $R2_{(2)}$ and $R2_{(3)}$ motifs in the VNTRs are shaded for sequence comparison. All the AT chains adjacent to the motifs are boxed. The VNTRs are underlined with dotted line while motifs in the VNTRs are in bold and italic. Only the first and the last VNTR are shown. The last VNTR is imperfectly duplicated where the $R2_{(3)}$ and the L5 sequences are shortened. The non-repetitive region with a length of 82 bp (indicated) lies between the last VNTR and the 12S rRNA gene.

Each tandem repeat was 104 bp in length. The forward sequencing results revealed that the band size of 2.5 to 3.0 kb generated with PCR primers consisted of approximately 300 bp of the 3' end of Domain III and a 270 – 310 bp of motif region which flanks the VNTRs region at its 5'end. Five VNTR copies were seen with the forward sequencing. The reverse sequencing revealed approximately 500 bp of the 5' end of 12S rDNA gene and a short non repetitive region of 82 bp which flanks the VNTRs at the 3' end (Figure 4.12 and Figure 4.13). Three copies of VNTR were seen with the reverse sequencing in which the last copy is imperfectly duplicated (Figure 4.12 and Figure 4.13). Highly conserve patterns were observed from sequences flanking the VNTRs and the numbers of VNTR copies (eight copies) seen in all the individuals sequenced (homoplasmic and heteroplasmic), indicates that the length variation is due to the region in which the forward and reverse sequence did not overlap.

4.4.4.1 Motif Region

The motif region starts with TTTTCCTCTTTT (R1), approximately 35 bp after the CSB III. R1 is repeated twice but is alternated with another motif TTTCACTTTT (R2) (Figure 4.12 and Figure 4.13). Motifs R1 and R2 are followed by short adenine and thymine chains interrupted by guanines. Though there is elongation of these chains from 5' to 3', R1 motifs had longer AT chains compared to R2. The third repeat of R2 and the AT chains (L5), AAAAATAAAAAAAAATAAATTT, found after the third repeat of R2 is perfectly duplicated in the maternal parent (Figure 4.12). This duplication of R2

and L5 is inherited in all her hatchlings from two consecutive nests with base substitution occurring towards the 3' of the sequence (Figure 4.12 and Figure 4.13). One unrelated individual (TK010) also showed the identical repeat pattern as in the family unit.

4.4.4.2 VNTRs Region

The variable number tandem repeats in *Tomistoma* is a 104 bp unit which consists of four different motifs and an AT chain (Figure 4.12). The VNTRs starts after the motif region mentioned above with a motif $R2_{(2)}$ which differs from the original R2 with an insertion of two tyrosine and a transition of the first cytosine from C to T of R2. This is followed by motif R3, TGCCCACTCCCTCA, and by another adjacent motif, '*Tomistoma* CCTA' motif. The *Tomistoma* CCTA motif showed base substitutions within individuals and therefore a consensus for this motif is TAGGCTYATARRCCTA. The last motif in the VNTRs is $R2_{(3)}$, another version of R2 with an insertion of a tyrosine and a cytosine after the third tyrosine and a transversion at the eighth nucleotide of R2. Motif $R2_{(3)}$ is followed by the AT chains (L5) that are found after the R2 motif at the region before the VNTRs.

The last VNTR repeat has an imperfect or shortened sequence of both the $R2_{(3)}$ and L5. Adjacent to the imperfect VNTR was a non repetitive region consisting of 82 bp. The 5' region of the 12S rDNA gene was observed after the non repetitive region (Figure 4.13). The placement of the imperfect tandem repeat at the 3'

region of the repetitive region has also been reported in other species (Brown *et al*., 1996; Nesbo *et al*., 1998)

In the heteroplasmic (TK011) and one homoplasmic individual (TK006), the R3 is TGCCCACTTCCTCA, with a transition at the ninth nucleotide when compared with the R3 of the other homoplasmic individuals. The L5 (AAAAATAAAAAAAAATAAAT) is significantly shortened in the heteroplasmic and TK006 individuals where the last ten nucleotides are deleted (Appendix 8). Both TK006 and TK011 are members of the H4 lineage.

4.4.5 Secondary Structures

Stable secondary structures were produced by both the short motifs and the tandem repeats. Motifs R1 with L3 and the third / forth repeat of R2 with its L5 were able to form secondary structures though with lower energy level (Figure 4.14A and Figure 4.14B).

The free energy levels were very much dependent on the AT chains adjacent to the motifs (either R1 or R2) to form the stem – loop structure. The tandem repeat also formed a stable stem-loop structure consisting of two stem-loop structures. The first stem – loop is made up of the first motif in the VNTRs, $R2_{(2)}$ and L5 while the second is between the $R2_{(3)}$ and *Tomistoma* CCTA motif (Figure 4.14C). Depending on where the *Tomistoma* CCTA motif was placed, the free energy level ranged from -9.9 – 12.5 kcal/mol. When *Tomistoma* CCTA motif was

placed at the 3', a similar secondary structure with *Crocodylus* was seen (Figure 4.15). The higher level of free energy in the tandem repeat suggests that it could be the source of duplication events.

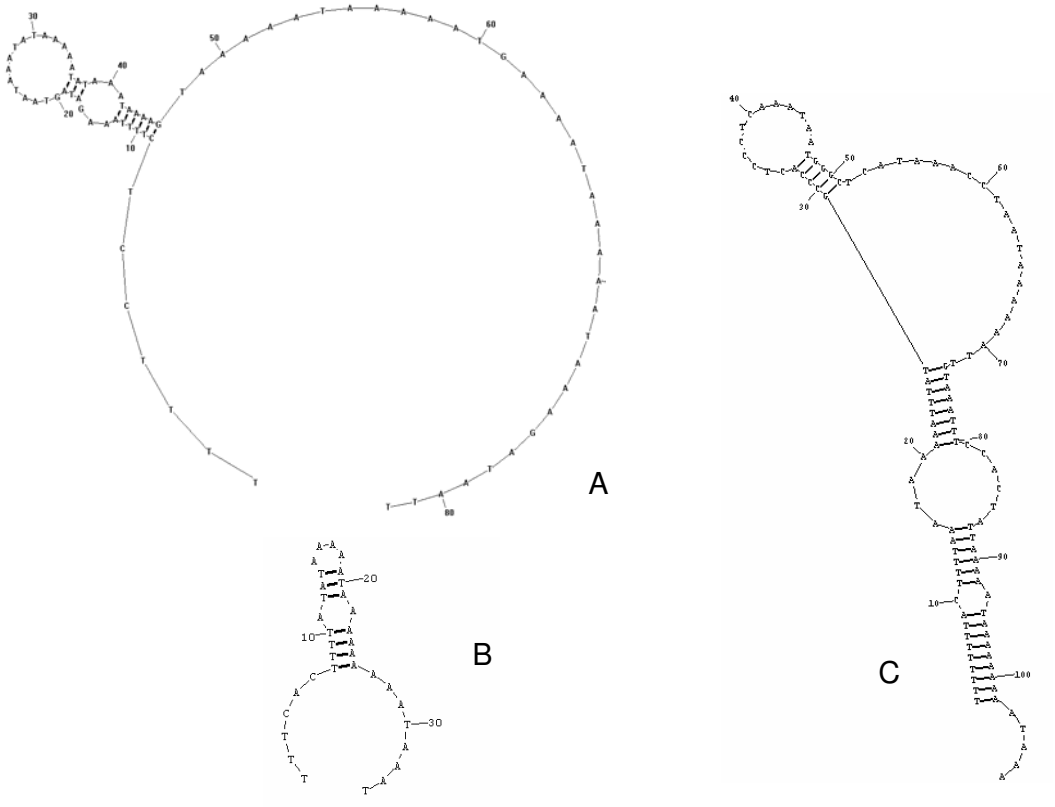


Figure 4.14: Stable secondary structures and its free energy level calculated using RNAstructure version 4.6. (A) Secondary structure of the second repeat of motif R1 (with the longer AT chains, L3) had free energy of -0.8 kcal/mol. (B) Stable secondary structures of the third / forth repeat of R2 and its L5 with free energy of -2.0 kcal/ml. (C) Stable secondary structures of the VNTRs with the motif arrangement as in Figure 4.13 with a free energy level of -12.5 kcal/mol.

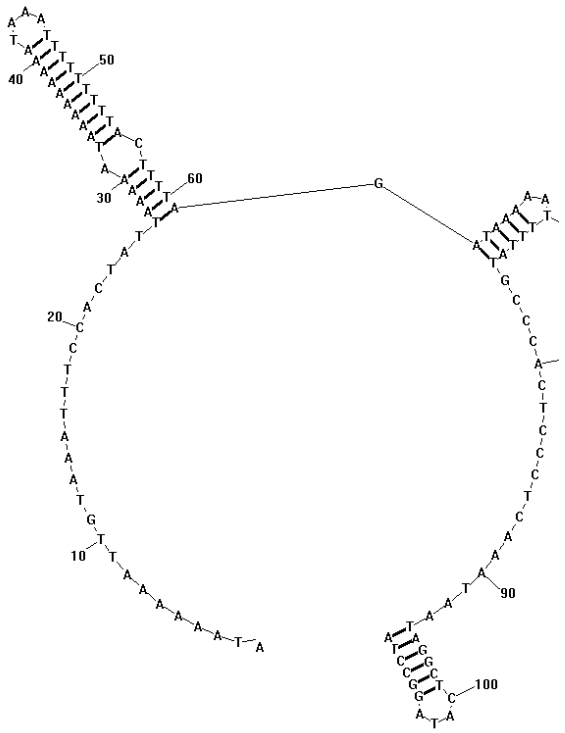


Figure 4.15: Stable secondary structures when the VNTRs with the *Tomistoma* CCTA motif at the 3'. Free energy level calculated using RNAstructure version 4.6 for this VNTR sequence arrangement was -11.7 kcal / mol.

CHAPTER 5

DISCUSSION

5.1. Methodology Development

The newly designed primers were successful in amplifying the target bands for all the regions in this study without difficulty in optimizing the PCR conditions. Sequencing was also straightforward except for the heteroplasmic band for the mtDNA repetitive region. Indirect DNA purification consistently produced poor intensity bands that were not ideal for downstream analysis. This was overcome by cloning the purified DNA fragment.

5.2 Intraspecific Variation

5.2.1 Genetic Diversity at Species, Population and Captive Holding Locality

Overall haplotype diversity was high (h = 0.77) while overall nucleotide diversity (π) was low (0.54%). Although the genetic diversities inferred from individual markers were slightly lower than from concatenate data, h and π were still high. This pattern of genetic diversities is consistent with that reported in previous crocodilian population studies (Farais *et al*., 2004; Ray *et al*., 2004; Vasconcelos *et al*., 2006; Vasconcelos *et al*., 2008). In most of these studies, either in small (Farais *et al*., 2004) or large sampling size (Vasconcelos *et al*., 2006; Vasconcelos *et al*., 2008), the high h and low π were associated with expansion of the crocodile population. In contrast, Ray *et al*. (2004) found this pattern in populations of *C.*

moreletti that was at equilibrium. The marker used by Ray *et al*. (2004) was the mtDNA d loop with a sampling size similar to Vasconcelos *et al*. (2006, 2008). Similarly, the high haplotype diversity seen in our data coupled with positive but not significant neutrality tests, suggests that *Tomistoma schlegelii* population is at equilibrium, contrary to the assumption that genetic diversity would be low due to the small remaining wild population of this freshwater crocodilian. When considering the total *Tomistoma* samples as a population or with samples of known origins in Peninsular Malaysia, the demographic analysis inferred from genetic data does not appear to reflect the census studies which indicated gradual decline in numbers (Bezuijen *et al.*, 2001, Stuebing *et al*., 2006, Sebastian, 1993; Simpson *et al*., 1998).

Explanations for similar census and genetic diversity findings in other crocodilian were that recent exploitations are difficult to detect genetically due to historical population expansion which left a strong signature on the mtDNA genome and therefore is unable to detect the recent exploitations, and longer generation time compared with the rate of exploitations which is not able to detect genetic drift even though populations are small (Vasconcelos *et al*., 2008).

de Thoisy *et al*. (2006) suggested that census studies should be incorporated when interpreting genetic information for better inferences. For *Tomistoma*, census study has been limited to previously documented localities of occurrence, which are now faced with habitat destruction (Bezuijen *et al*., 2001; Bezuijen *et al*.,

2004; Sebastian, 1993; Simpson *et al*., 1998). For a species that is particularly sensitive to human activities (Bezuijen *et al*., 2002), a decline in sightings may not reflect actual population size. When faced with threats from humans, the possibility that this species could have translocated to another area which may be in accessible to humans cannot be discounted altoghether. In addition to the limited areas surveyed for *Tomistoma* sightings, the duration of the surveys have also been limited from one night to a maximum of 10 nights (Auliya *et al*., 2006; Bezuijen *et al*., 2001; Bezuijen *et al*., 2004; Sebastian, 1993; Simpson *et al*., 1998), a relatively short duration for reliable inferences from the census studies.

Genetic diversity of most holding localities was high except for the farms (Sumatra, Sarawak and Kalimantan) and Mini Zoo Temerloh. In the case of farmed crocodilian, a low genetic diversity does not necessarily reflects inbreeding or an effect of genetic drift due to small remaining population as seen in *C. niloticus* (Flint *et al*., 2000) but could indicate adaptation to the farm conditions. In this study, all individuals from the farms and Temerloh Zoo animals are from the wild and not bred in the farm. The low genetic diversity from these four localities could still be due to adaptation to the restricted habitat that they are left with in the wild.

Only *Tomistoma schlegelii* population from Peninsular Malaysian showed genetic variation while the other geographical regions were not genetically diverse. This could be due to the fact that Peninsular Malaysia has been reported as an area of

overlap for most vertebrates of this region. The snakes and frogs of Peninsular Malaysian have been shown to share species and haplotypes with both Sumatra and Borneo, in which the latter two regions show lesser similarities with each other (Inger and Voris, 2001).

5.2.2 Geographical Association of Haplotypes

The haplotypes for *Tomistoma* were geographically distinct except for the H2 haplotype which was found in both Peninsular Malaysia and Sarawak. This trend of populations or lineages overlapping across Sumatra, Peninsular Malaysia, Sarawak and West Kalimantan is also seen in several fish species due to historical association. A haplotype of *H. hoevenii* was found to be distributed from Kapuas River (west Kalimantan) to Palembang (southeastern Sumatra) and Johor (south Peninsular Malaysia) suggesting a recent Pleistocene exchange (Dodson *et al*., 1995). Similarly, a haplotype of the freshwater fish *Tor tambroides* was found in Sarawak and Peninsular Malaysia suggesting the ancient North Sunda River system as a means for gene flow (Esa *et al*., 2008). Another freshwater fish *Hampala macrolepidota* showed low population differentiation between Sarawak and Peninsular Malaysia and haplotypes from both regions clustered in one clade on the phylogenetic tree (Ryan and Esa, 2006). The distribution of H2 haplotype in the two geographically separated regions (Peninsular Malaysia and Sarawak) seems to indicate an association to the ancient river system, the North Sunda River, which drained these two regions when the Sunda Shelf was exposed (Voris, 2000).

The likely origins of individuals whose geographical origins were not known from Singapore Zoo, Hong Kong Wetland Park and Cikananga Wildlife Rescue Centre, Jawa were able to be resolved based on the phylogenetic trees inferred from individuals of known origins. Data from this study suggests samples from the H3 lineage in Hong Kong Wetland Park originated from Sumatra, while the other three individuals of the H1 lineage originated from Peninsular Malaysia. From a total of ten *Tomistoma* held in Singapore Zoo, six were from Peninsular Malaysian while four others from Sarawak. The geographical origins for individuals from Cikananga Wildlife Rescue Center ranged from Sumatra, Peninsular Malaysia and Sarawak. The two specimens from Zoo Negara of unknown origins (public surrender specimens) were always found in the same clade as West Kalimantan in the phylogenetic trees with very little genetic divergence between these two populations. This study was limited to only one sampling site in the whole of East Kalimantan and therefore could not identify if these two specimens are from East Kalimantan or other parts of Kalimantan. However, when data of this study was compared with data of wild caught *Tomistoma* from West, Central and East Kalimantan over 451bp of control region (H. Kurniati, unpubl. data), the H5 haplotype clustered together with the East Kalimantan individuals. Apart from this, the H1 and H2 haplotypes was also seen in West Kalimantan, reinforcing the suggestion that the shared haplotype in Peninsular Malaysia, Sarawak and West Kalimantan is due to historical river system connection. Therefore, the H1 haplotype found from confiscated specimens in Cikananga Wildlife Rescue Center could have originated from West

Kalimantan, Sarawak or Peninsular Malaysia. Similarly, the HI samples from Hong Kong Wetland Park and samples from Singapore Zoo (H1 and H2 haplotype) could be from any of these three regions as well.

Based on the three data sets, the concatenate data was the most useful in identifying likely origins of the samples from unknown locations. This is because the individual markers, cyt *b* – CR and the ND 6 – $tRNA^{glu}$ – cyt *b* region were not able to distinguish the H2 and H3 lineage respectively. Therefore when using either one of these markers individually, instead of having distinct haplotypes based on geographical regions, the Sumatra and Sarawak regions would have shown similar haplotypes with Peninsular Malaysian lineages.

5.2.3 Population Structure

The S_{NN} analysis however indicated the populations from the four geographical regions (Sumatran, Peninsular Malaysia, Sarawak and East Kalimantan) are different even though the H2 haplotype is found in both Peninsular Malaysia and Sarawak. In rats, haplotype sharing in well differentiated population has been reported and was due to historical association rather than ongoing gene flow (Patton *et al*., 1994). The division of *Tomistoma* population according to the geographical regions is also seen in large endangered land mammals. The tiger for instance, there are different subspecies in the respective geographical region (Luo *et al*., 2004) while the Bornean Orangutans are also differentiated into the Sarawak and northwest Kalimantan, south west Kalimantan, Central Kalimantan

and Sabah populations (Warren *et al*., 2001). Sharing of alleles was also seen and in Orangutans found across its geographical range probably due to retention of polymorphism or recent gene flow (Kanthaswamy and Smith, 2002). Such a division has been attributed by the type of landscape and habitat ideal for these mammals. Natural barriers like mountains (Schwaner and Crocker Ranges in Borneo, Titiwangsa Mountains in Peninsular Malaysia and the Barisan mountain range in Sumatra) are reported to restrict the movements of lowland mammals such as tigers and elephants and therefore restrict the gene flow between populations (Luo *et al*., 2004; Fleischer *et al*., 2001). The Schwaner and Crocker mountain ranges are likely to act as land barrier for *Tomistoma* populations in Borneo (East Kalimantan and Sarawak) as well. However, the natural barriers for populations in Sumatra, Peninsular Malaysia and Sarawak which separated during the late Pleistocene (Voris, 2000), are the likely the Melaka Strait and South China Sea. Further investigation with larger sample size of known origins should be carried out to identify if finer scale population structure exists and the cause of such structure.

5.2.4 Divergent Lineage

The data from this study shows that the H4 lineage is highly divergent. The genetic divergence compared to other haplotypes was the highest for all three data sets. Moreover, the genetic divergence seen in the cyt *b* – CR data is comparable to that of the Cuban crocodile (Weaver *et al*., 2008) and a magnitude higher when compared with the American and Morelet's crocodile (Ray *et al*., 2004). In the

haplotype network, except for the ND 6 – $tRNA^{glu}$ – cyt *b* data which connected all lineages, the H4 haplotype was not parsimoniously connected to the other haplotypes using cyt *b* – CR and concatenate data. Unconnected haplotype networks are seen in cases of hybridization or cryptic species (Hart and Sunday, 2007). With the distance based haplotype network placing the H4 on a different evolutionary branch from the other lineages and forming a basal and reciprocally monophyletic clade in the mtDNA trees partly fit the description of an ESU according to Moritz's definition (1994) in which the mtDNA alleles have to be reciprocally monophyletic. Furthermore, when the mtDNA VNTRs sequence was investigated, individuals from this haplotype were distinct from the rest with indels of the AT chain within the VNTRs region.

The genus *Tomistoma* previously had more members that ranged from northern Africa to Japan as indicated from the fossil records (Brochu, 2003; Kobayashi *et al*., 2005; Piras *et al*., 2007). Although the divergence of this lineage was lower than that of *Osteolaemus* species (Eaton *et al*., 2009), the genetic distance of 2.4% for the cyt *b* – CR data of *Tomistoma* is higher than the distance between hybrid and pure *C. rhombifer* and just slightly lower than when these two forms of *C. rhombifer* were compared to *C. acutus* (Weaver *et al*., 2008). Therefore, there is a possibility of hybrids in wild *Tomistoma* populations. The phylogenetic tree however does not seem to indicate a possible hybridisation with other crocodilian of this region (*C. siamensis*, *C. porosus* and *C. mindorensis*) as the divergent H4 lineage is placed between the other lineages and *Gavialis*, a sister taxa. Therefore,

if this lineage is indeed a hybrid, it is likely that it is a hybrid with another Tomistominae. This possibility is based on the bidirectional hybridisation of *C. moreletti* and *C. acutus* and in which the hybrids were fertile (Cedeno – Vasquez *et al*., 2008). Assuming this can also happen in *Tomistoma*, the hybrids of Tomistominae would have survived even if other members of this family are extinct.

Small population that diverged from other populations over a considerable period of time can become highly divergent and result in an ESU (Moritz, 1994; Snyder *et al*., 1996). Since the exact location of the three individuals comprising this H4 lineage is unknown, it is difficult to confirm if this lineage diverged from another lineage of *Tomistoma* due to drift caused by small population size.

The H4 lineage was morphologically similar to the other lineages suggesting a possible cryptic species. Cryptic species were characterized as lineages that have high genetic divergence and are not connected in the parsimony based haplotype trees (Hart and Sunday, 2007), which is also demonstrated by the H4 lineage. However, when genetic divergence was compared with the newly discovered *Osteolaemus* lineage, *O. sp. nov* with *O. t. tetraspis*, lineages with no apparent morphological differences (Eaton *et al*., 2009), the divergence of the H4 lineage is one fold lower than between *O. sp. nov* and *O. t. tetraspis*. Further investigation into this lineage is required to confirm the status of this lineage.

As most of captive *Tomistoma* are intended for breeding and conservation purposes, it is particularly important to identify the status of this lineage and to detect if other holding localities have this lineage. In an endangered species, it is important to minimize inbreeding and outbreeding for better breeding and survival success (Frankham *et al*., 2002). Therefore, this lineage should be treated separately until the status of this lineage is verified.

5.3 mtDNA Repetitive Region

The higher A and T composition observed in this study is common for the mtDNA control region in general (Ray and Densmore, 2003; Zhang *et al*., 2009). Similarly, the mtDNA repetitive region has nucleotide composition which is also AT bias and has length variations and presence of a heteroplasmic band has also been reported in other crocodilians (Ray and Densmore, 2003) and in other animals (Arnason and Rand, 1992; Delport *et al*., 2002; Rand and Harrison, 1989; Roques *et al*., 2004).

Since each VNTR is 104 bp long and the length difference seen between bands is from 50 or 100 bp, it is likely that the length variation in *Tomistoma* is due to partial or complete VNTR indels. Similarly in the komodo dragon and *Crocodylus*, the likely patterns in mtDNA repetitive regions that were difficult to sequence were indicated using information from gel image and conservation of sequences flanking the VNTRs (Kumazawa and Endo, 2004; Ray and Densmore, 2003).

The two heteroplasmic bands were identical in sequence pattern with no base substitution between the two bands. The homoplasmic individuals showed high conservation of VNTRs sequence pattern (either with the heteroplasmic pattern or homoplasmic pattern) but single base substitution was seen within the VNTR units. The base substitution occurring within the family unit was also reported in Vespertilonid bats where heteroplasmy was stably inherited (Petri *et al*., 1996). The inheritance of the duplicated R2 and L5 in all members of the family unit suggests a stable transfer of repetitive regions from mother to offspring although such pattern may not necessarily be unique to the family unit as it was also seen in one unrelated individual (TK010). A similar observation was reported in humans as individuals from unrelated maternal lineage have been shown to have the same haplotype for the repetitive regions (Soong *et al*., 1997).

The VNTRs pattern in the heteroplasmic individual differed from all homoplasmic individuals, except for one (TK006), with a substitution in the R3 and indels within the L5. The heteroplasmic individual and TK006 are members of the H4 lineage. In *Crocodylus moreletii*, the base indels reported in heteroplasmic haplotype was in the Poly A region and not repeated as VNTRs while the homoplasmic haplotypes only differed in the number of VNTRs (Ray and Densmore, 2003). In fact, the VNTR sequences between different species of *Crocodylus* (*C. porosus* and *C. palustris*) were found to be generally conserved (Ray and Densmore, 2003) unlike *Tomistoma* in which individuals differed in bases (R3) and the indel of the AT chains within L5 resulting in two different

types of tandem repeats within *Tomistoma.* In the VNTRs of *Crocodylus*, the trend of AT chain indels was only seen when the New World species were compared to the Old World species. Therefore, the repetitive region of this crocodilian was able to detect this highly divergent lineage.

The stem – loop structures made up by *Tomistoma* VNTRs is similar to the stem – loop structures by poly A and T in *Crocodylus* adjacent to each TAGG motif repeat. The most probable model for the replication for *Crocodylus* suggested was the Levinson and Gutman (1987) model due to the secondary structure and difference in the number of VNTRs repeats (Ray and Densmore, 2003). Because of the noncontiguous motifs in the tandem repeats of *Tomistoma* ($R2_{(2)}$ and $R2_{(3)}$) with an imperfect tandem repeat at the 3', the formation of stable secondary structures could be due to the replication model suggested by Taylor and Breden (2000). This model adopts the slip strand mispairing model by Levinson and Gutman (1987) but at noncontiguous repeats involving minisatellites.

5.3.1 Homology and Variations with Other Species Patterns and Repeats

Crocodylus

The poly A-T after the CSB III found in *Crocodylus* (Ray and Densmore, 2003) is absent in *Tomistoma*. An inverted form of the *Crocodylus* TAGG motif (Ray and Densmore, 2003) is present in *Tomistoma* (referred as *Tomistoma* CCTA motif). Similar to the *Crocodylus* TAGG, the *Tomistoma* CCTA motif has the first four nucleotides at 5' and 3' inverted. Secondary structure produced when the CCTA

motif was placed at the 3' of the tandem repeat was similar to that of *Crocodylus* secondary structure when the *Crocodylus* TAGG motif was arranged at the 3' (Ray and Densmore, 2003).

Alligator

Based on the sequence alignment, the repetitive region of *Tomistoma* after CSB III is similar to *Alligator* in which the distinct Poly-A followed by poly-T in the *Crocodylus* is absent. Instead, it is replaced by the short motif repeats in *Tomistoma* and *Alligator* but in *Alligator*, the motifs are tandem repeats with short AT chains adjacent to the motif. The first motif is repeated three times followed by the second motif by 11 times (Ray and Densmore, 2003). These two genera also share a similar form of the CCTA motif, *TAGG*SY*YATARR*YY*TA* but the inverted repeats are only 2bp long at the 5' and 3' nucleotides in the *Alligator* (shaded font indicates nucleotides conserved between the two genera). Similar to *Tomistoma*, this CCTA motif is found in the alligator's VNTRs.

Caiman crocodilus

Caiman's VNTRs consist of three copies of a 287 bp sequence and a truncated copy which is located closer to the 12S rDNA (Ray and Densmore, 2003). Within these copies, a short sequence of ATTATA is found in the motifs of *Alligator*. This sequence is also found immediately after the CSB III in *Tomistoma* in all the individuals sequenced. However, there are no other repeats of this sequence found

subsequently in both the motif and VNTRs regions of the *Tomistoma*. This sequence is not found in *Gavialis* and *Crocodylus*.

Gavialis

The CSB III region is highly conserved between *Tomistoma* and *Gavialis*. The region after CSB III in *Gavialis gangeticus* contains two parts, a short motif region followed by the VNTRs. The motif present in the *Gavialis gangeticus* is the '*Tomistoma* R1' with the fifth nucleotide having a transition from T to C. In *Gavialis*, this short motif region consists of only one imperfect tandem repeat followed by perfect tandem repeats (VNTRs) whereas in *Tomistoma* the imperfect tandem repeat is at the last VNTR units (3' end). The *Gavialis* VNTRs consisted of two sets of R1 and ends with a TAGG motif which is similar to the *Crocodylus* (CCTAGGSYWAAATASG) in which the ninth and the fifteenth nucleotide have base substitution. However, the *Tomistoma* CCTA motif is not seen in the *Gavialis*. The perfect duplicate of VNTRs for *Gavialis* starts approximately at the same site as the VNTRs of *Crocodylus*. The secondary structure formed by the VNTRs was also similar to *Crocodylus* secondary structure when the TAGG motif is placed at the extreme 3' end. Both *Tomistoma* and *Gavialis* have 85% similarities at the non-repetitive sequences between the last VNTR and the 12S rRNA.

5.3.2 Conservation of Crocodilian mtDNA Repetitive Region.

In marsupials, the conservation of the repeats was used to indicate evolutionary histories (Nilsson, 2009). The highly conserved sequences in marsupials were easily aligned for lineages that diverged 20 million years ago (Ma) and could indicate a recent event as compared to placental mammals which showed lesser conservation (Nilsson, 2009). In crocodilian, the presence of the R1 motif sequence and the high conservation of the non – repetitive region at the 5' of 12 S rDNA gene in *Tomistoma* and *Gavialis* are consistent with previous molecular work on crocodilians in which these two are closely associated, diverging some 28 – 22 Ma (Roos *et al*., 2007). Similarly, the short ATTATA sequence seen in every repeat copy of *Caiman* and *Alligator* suggest closer association between these two genera although it is estimated that these two diverged some 68 – 73 Ma (Roos *et al*., 2007). The presence of TAGG motif in *Crocodylus* and *Gavialis* also supports their molecular data classification as Crocodylidae (Janke *et al*., 2005; Roos *et al*., 2007). With the exception of *Caiman*, the TAGG motif and the inversion of this motif, the CCTA motif which are found in all crocodilian that diverged before K/T boundary, approximately 90 to 106 Ma (Brochu, 2009; Roos *et al*., 2007), could be due to concerted evolution. Concerted evolution was suggested for the conservation of sequences of the mtDNA repetitive region of large cats (Jae-Heup *et al*., 2001), and nuclear genes of primates that diverged over 35 Ma (Pavelitz *et al*., 1995). The repetitive patterns of *Tomistoma* are interesting as it consisted of motifs and inversion of motifs found in all other crocodilian irrespective of their phylogenetic classification.

CHAPTER 6

CONCLUSION

In conclusion, the genetic diversity study on *Tomistoma* found that the overall haplotype diversity is high with population structure especially associated with geographical region. We were able to identify the geographical regions for samples of unknown origins from Singapore Zoo, Hong Kong, Jawa, and Peninsular Malaysia. The findings also suggest that *Tomistoma* populations are geographically differentiated into Sumatra, Peninsular Malaysia, Sarawak and East Kalimantan. The divergent H4 haplotype should be managed separately and should be investigated further using microsatellite to confirm the status of this lineage.

The type of mtDNA repetitive patterns present in *Tomistoma* which are unique due to the presence of two different types of patterns, the motif region and VNTRs within a species that is not seen in the other crocodilian that diverged during the Pre K/T boundary. Indels of complete motifs (R2 and L5) were seen in the motif region (before the VNTRs). The VNTR repeat sequence varies in base pattern and probably in the number of VNTR copies which caused the length variation of the band sizes. Though not as common, heteroplasmy does occur in this taxon. The H4 lineage (heteroplasmic and one homoplasmic individual)

differed from other lineages at R3 with base substitution and L5 with base indels. The repetitive region consisted of a combination of patterns which are specific to different crocodilian and shares similar secondary structure with *Crocodylus* and *Gavialis*. Finally, this marker has a potential to detect maternally related siblings due to the stable inheritance of VNTRs sequence from maternal to offspring and the highly divergent H4 lineage.

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Appendix 1 Ventral approach to the caudal vein

Figure 1: Ventral approach to the caudal vein using an 18 G Spinocan needle. The needle is inserted approximately 10 cm from the vent.

Appendix 2 Genbank accession number for the respective specimens for cyt *b* – CR and the ND 6 – tRNA^{glu} – cyt *b* marker.

Table 1: Laboratory voucher number and Genbank Accession Number following geographical origins for the respective gene markers.

Gene marker	Origin	Lab voucher	Genbank Accession Number
cyt *b* – CR	Sarawak	SAR002	HM593977
	Sarawak	SAR002	HM593978
	Sarawak	SAR004	HM593979
	Sarawak	SAR005	HM593980
	Peninsular Malaysia	PM006	HM593981
	Peninsular Malaysia	PM007	HM593982
	Peninsular Malaysia	PM008	HM593983
	Peninsular Malaysia	PM009	HM593984
	Peninsular Malaysia	PM010	HM593985
	Peninsular Malaysia	PM011	HM593986
	Peninsular Malaysia	PM012	HM593987
	Peninsular Malaysia	PM013	HM593988
	Peninsular Malaysia	PM014	HM593989
	Peninsular Malaysia	PM015	HM593990
	Peninsular Malaysia	PM016	HM593991
	Peninsular Malaysia	PM017	HM593992
	Peninsular Malaysia	PM018	HM593993
	Peninsular Malaysia	PM019	HM593994
	Peninsular Malaysia	PM020	HM593995
	Sarawak	SAR021	HM593996
	Sarawak	SAR022	HM593997
	Sarawak	SAR023	HM593998
	Unknown	PM029	HM593999
	Unknown	PM030	HM594000
	Hong Kong	HK1	HM594001
	Hong Kong	HK2	HM594002
	Hong Kong	HK3	HM594003
	Hong Kong	HK4	HM594004
	Unknown	SZG3	HM594005
	Unknown	SZG10	HM594006
	Unknown	SZG6	HM594007
	Unknown	SZG2	HM594008
	Unknown	SZG8	HM594009
	Unknown	SZG7	HM594010
	Unknown	SZG5	HM594011
	Unknown	SZG4	HM594012

	Unknown	SZG1	HM594013
	Unknown	SZG9	HM594014
	Sumatra	SUM036	HM594015
	Sumatra	SUM037	HM594016
	Sumatra	SUM038	HM594017
	West Kalimantan	KAL039	HM594018
	West Kalimantan	KAL040	HM594019
	West Kalimantan	KAL041	HM594020
	West Kalimantan	KAL043	HM594021
	West Kalimantan	KAL044	HM594022
	West Kalimantan	KAL045	HM594023
	Unknown	J046	HM594024
	Unknown	J047	HM594025
	Unknown	J048	HM594026
	Unknown	J049	HM594027
	Unknown	J050	HM594028
	Unknown	J051	HM594029
	Unknown	J052	HM594030
	Unknown	J053	HM594031
	Unknown	J054	HM594032
ND 6 – $tRNA^{glu}$ – cyt *b*	Sarawak	SAR002	HM594033
	Sarawak	SAR002	HM594034
	Sarawak	SAR004	HM594035
	Sarawak	SAR005	HM594036
	Peninsular Malaysia	PM006	HM594037
	Peninsular Malaysia	PM007	HM594037
	Peninsular Malaysia	PM008	HM594039
	Peninsular Malaysia	PM009	HM594040
	Peninsular Malaysia	PM010	HM594041
	Peninsular Malaysia	PM011	HM594042
	Peninsular Malaysia	PM012	HM594043
	Peninsular Malaysia	PM013	HM594044
	Peninsular Malaysia	PM014	HM594045
	Peninsular Malaysia	PM015	HM594046
	Peninsular Malaysia	PM016	HM594047
	Peninsular Malaysia	PM017	HM594048
	Peninsular Malaysia	PM018	HM594049
	Peninsular Malaysia	PM019	HM594050
	Peninsular Malaysia	PM020	HM594051
	Sarawak	SAR021	HM594052
	Sarawak	SAR022	HM594053
	Sarawak	SAR023	HM594054
	Unknown	PM029	HM594055
	Unknown	PM030	HM594056
	Hong Kong	HK1	HM594057

Hong Kong	HK2	HM594058
Hong Kong	HK3	HM594059
Hong Kong	HK4	HM594060
Unknown	SZG3	HM594061
Unknown	SZG10	HM594062
Unknown	SZG6	HM594063
Unknown	SZG2	HM594064
Unknown	SZG8	HM594065
Unknown	SZG7	HM594066
Unknown	SZG5	HM594067
Unknown	SZG4	HM594068
Unknown	SZG1	HM594069
Unknown	SZG9	HM594070
Sumatra	SUM036	HM594071
Sumatra	SUM037	HM594072
Sumatra	SUM038	HM594073
West Kalimantan	KAL039	HM594074
West Kalimantan	KAL040	HM594075
West Kalimantan	KAL041	HM594076
West Kalimantan	KAL043	HM594077
West Kalimantan	KAL044	HM594078
West Kalimantan	KAL045	HM594079
Unknown	J046	HM594080
Unknown	J047	HM594081
Unknown	J048	HM594082
Unknown	J049	HM594083
Unknown	J050	HM594084
Unknown	J051	HM594085
Unknown	J052	HM594086
Unknown	J053	HM594087
Unknown	J054	HM594088

Appendix 3 Genetic Divergence for the cyt *b* – CR and ND 6 – tRNA^{glu} – cyt *b* data

Table 1: Genetic distance of the cyt *b* – CR data using Tamura-Nei distance (Tamura and Nei, 1993), based on unequal base frequencies and unequal ratios of transitions to transversions (Ti:Tv) implemented in MEGA (v.4, Kumar *et al*., 2004)

	H1 / H3	H2	H4	H5	H6
H1 / H3	-				
H2	0.001	-			
H4	0.017	0.017	-		
H5	0.015	0.015	0.016	-	
H6	0.014	0.014	0.017	0.001	-
Gavialis	0.127	0.127	0.125	0.127	0.127

Table 2: Genetic distance of the ND 6 – tRNA^{glu} – cyt *b* data using Tamura-Nei distance (Tamura and Nei, 1993), based on unequal base frequencies and unequal ratios of transitions to transversions (Ti:Tv) implemented in MEGA (v.4, Kumar *et al*., 2004)

	H1 / H2	H3	H4	H5	H6
H1 / H2	-				
H3	0.002	-			
H4	0.021	0.024	-		
H5	0.009	0.012	0.021	-	
H6	0.007	0.009	0.019	0.009	-
Gavialis	0.222	0.225	0.229	0.215	0.218

Appendix 4 Phylogenetic Trees for the cyt *b* – CR and ND 6 – tRNA^{glu} – cyt *b* data

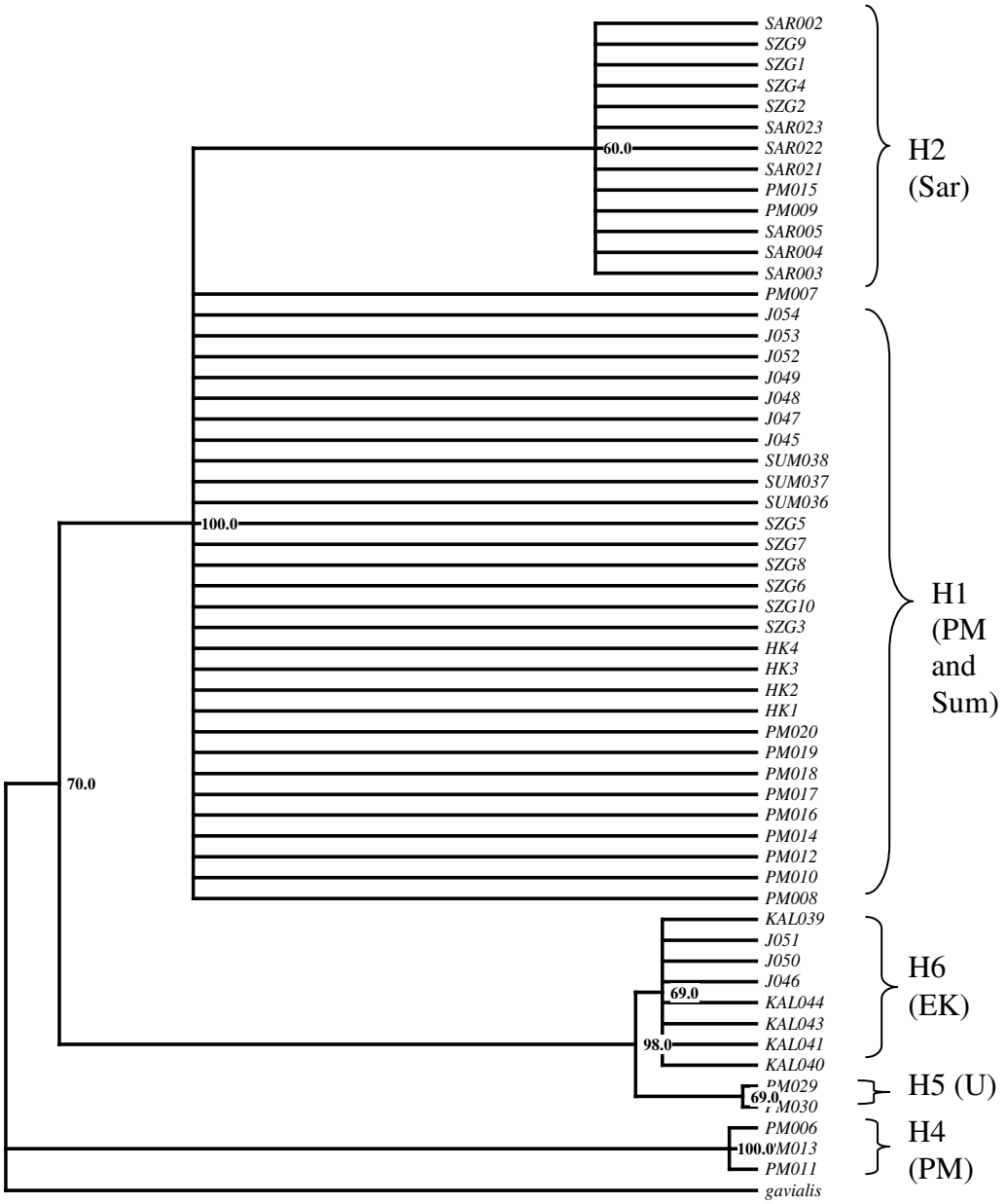


Figure 1. Neighbour Joining tree for the cyt *b* – CR data following Tamura Nei (Tamura and Nei, 1993) model generated with PAUP v4.0b using *Gavialis* as an outgroup. Clades are indicated following lineages (H1 to H6) and geographical regions are bracketed. (PM = Peninsular Malaysia, Sar = Sarawak, Sum = Sumatra, EK = east Kalimantan, U = Unknown)

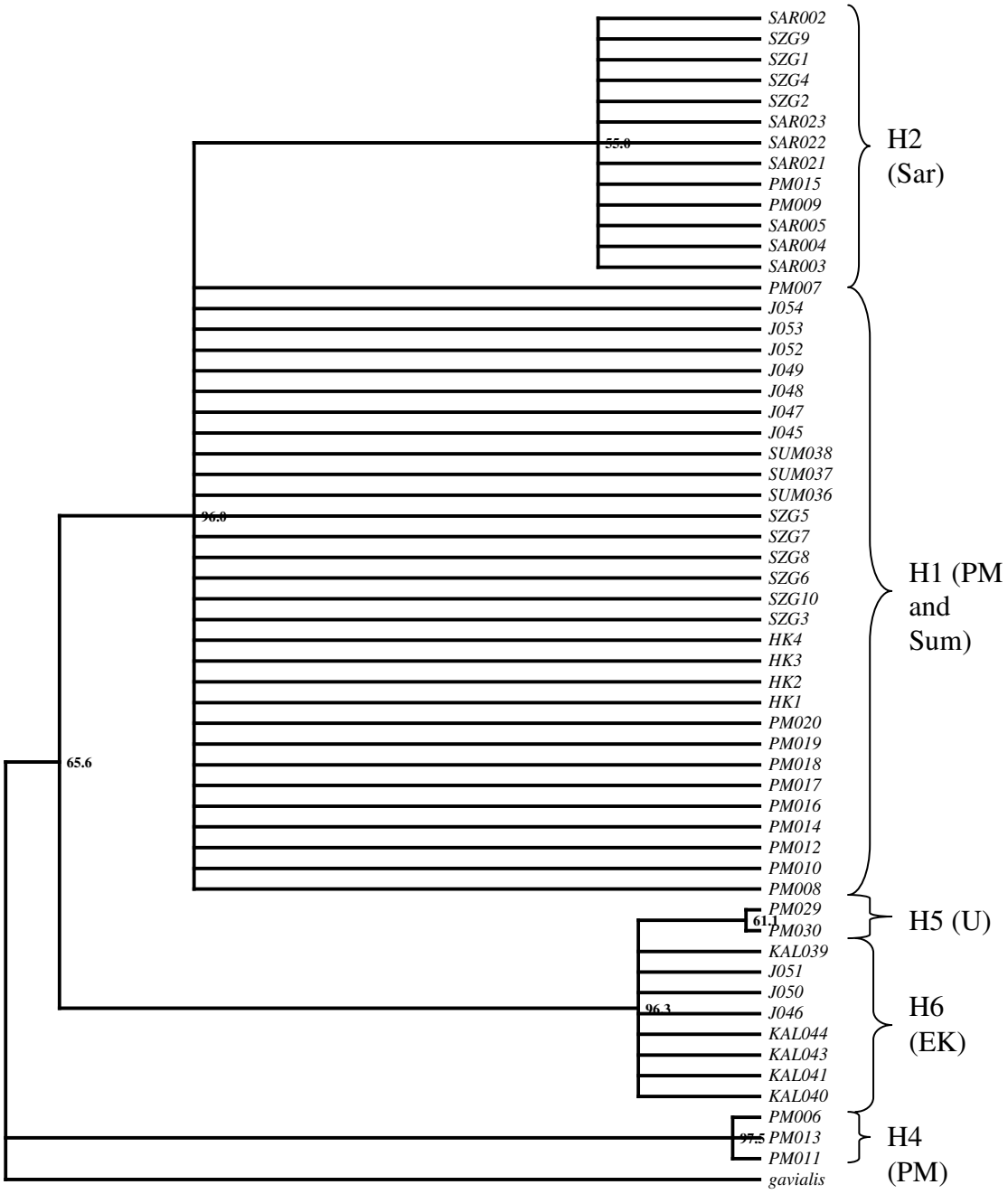


Figure 2: Maximum Parsimony tree generated for the cyt *b* – CR data with PAUP v.4.0b using *Gavialis* as an outgroup. Clades are indicated following lineages (H1 to H6) and geographical regions are bracketed. (PM = Peninsular Malaysia, Sar = Sarawak, Sum = Sumatra, EK = east Kalimantan, U = Unknown).

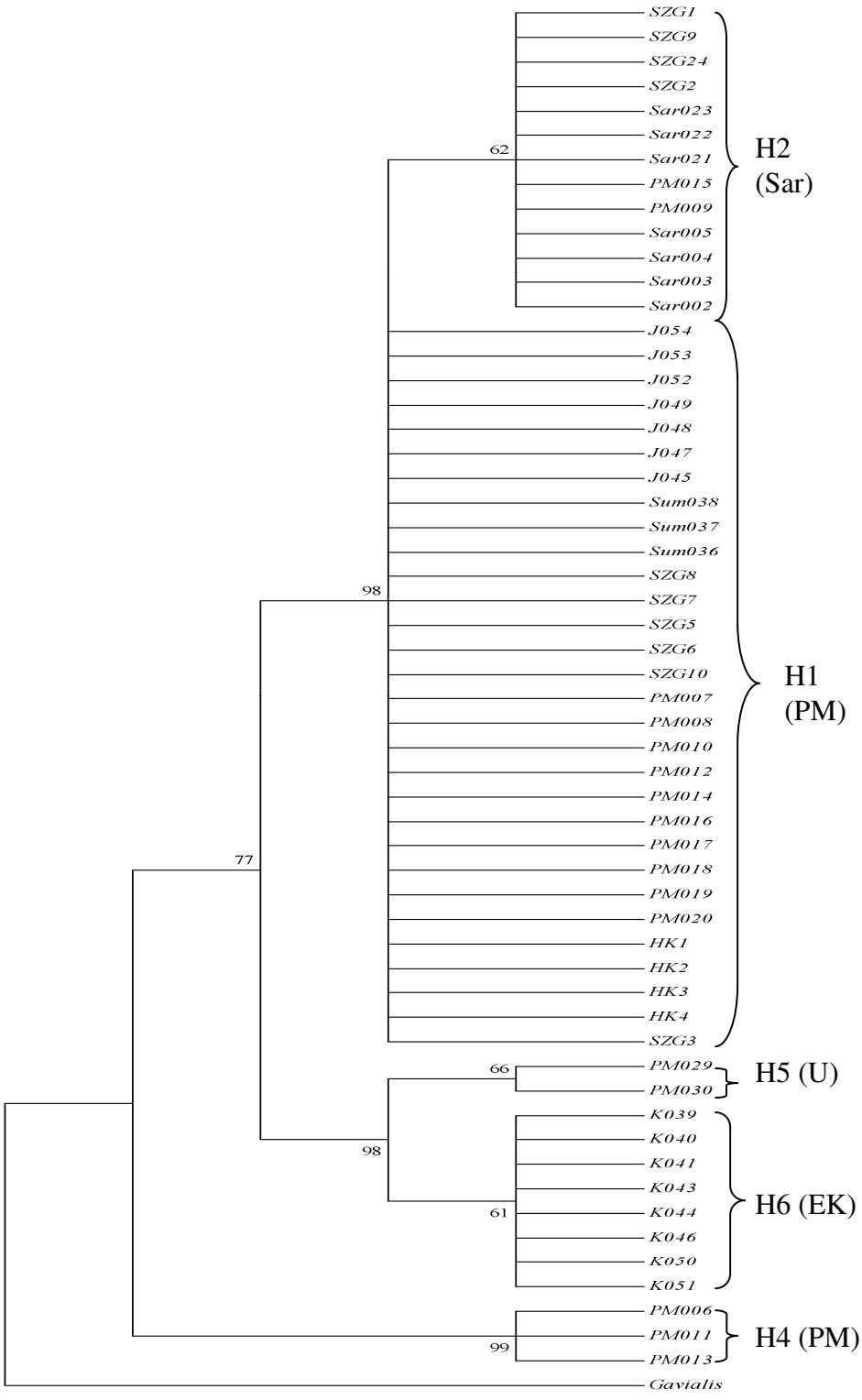


Figure 3: Maximum Likelihood tree for the cyt *b* – CR data generated with PAUP v4.0b using *Gavialis* as an outgroup. Clades are indicated following lineages (H1 to H6) and geographical regions are bracketed. (PM = Peninsular Malaysia, Sar = Sarawak, Sum = Sumatra, EK = east Kalimantan, U = Unknown)

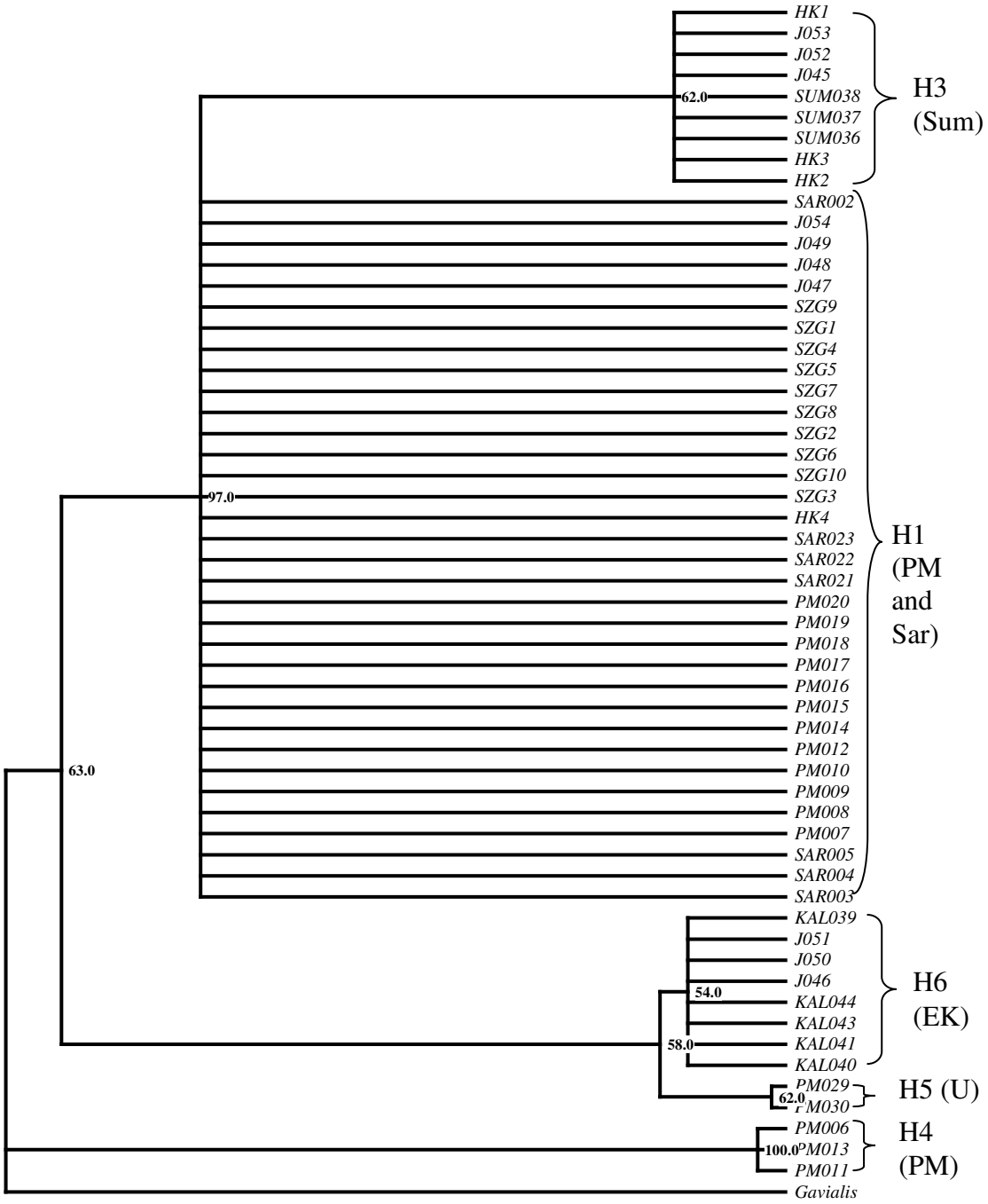


Figure 4: Neighbour Joining tree ND 6 – $tRNA^{glu}$ – cyt *b* data following Tamura Nei (Tamura and Nei, 1993) model generated with PAUP v4.0b using *Gavialis* as an outgroup. Clades are indicated following lineages (H1 to H6) and geographical regions are bracketed. (PM = Peninsular Malaysia, Sar = Sarawak, Sum = Sumatra, EK = east Kalimantan, U = Unknown)

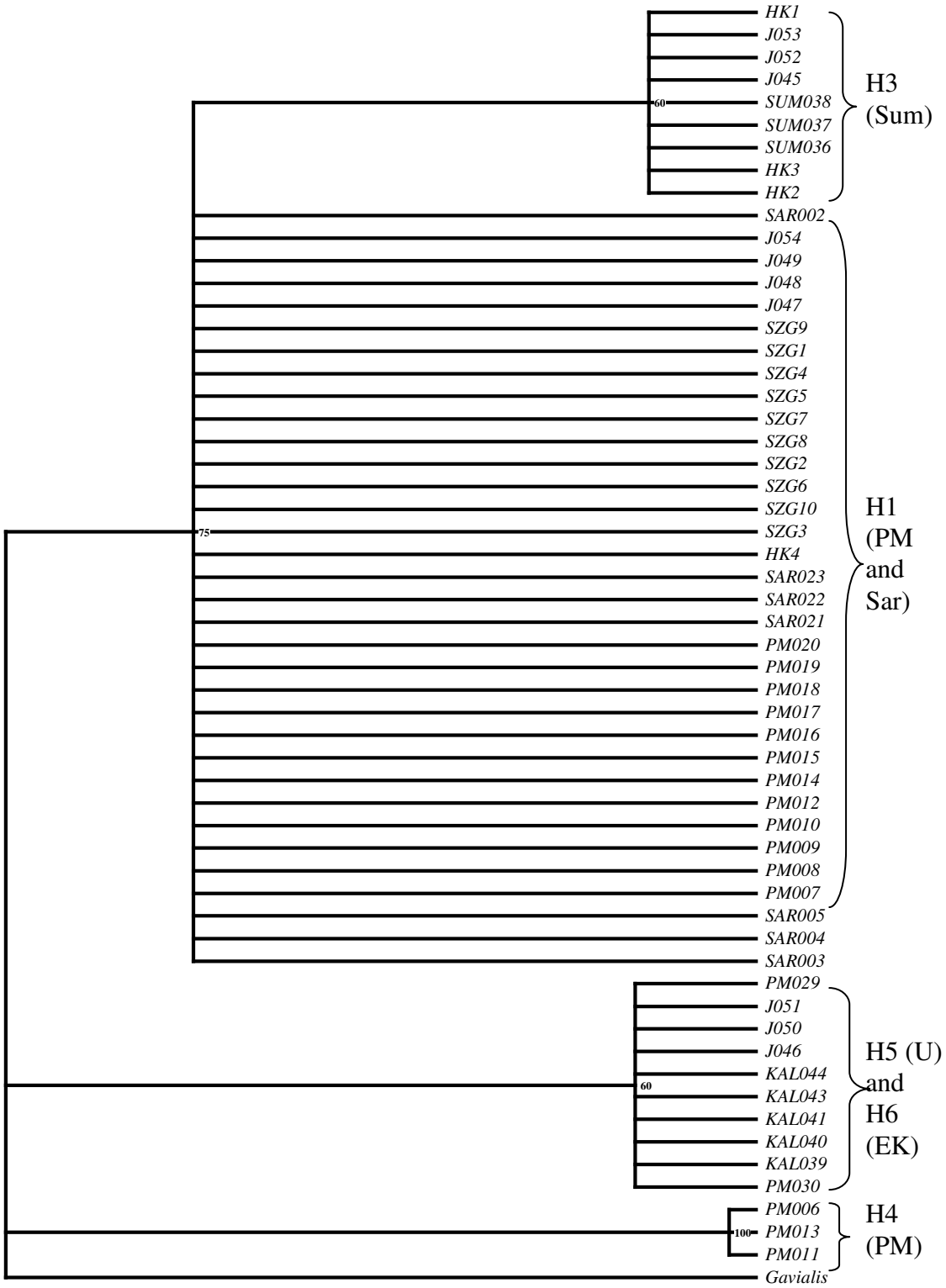


Figure 5: Maximum Parsimony tree generated for ND 6 – $tRNA^{glu}$ – cyt *b* data with PAUP v.4.0b using *Gavialis* as an outgroup. Clades are indicated following lineages (H1 to H6) and geographical regions are bracketed. (PM = Peninsular Malaysia, Sar = Sarawak, Sum = Sumatra, EK = east Kalimantan, U = Unknown).

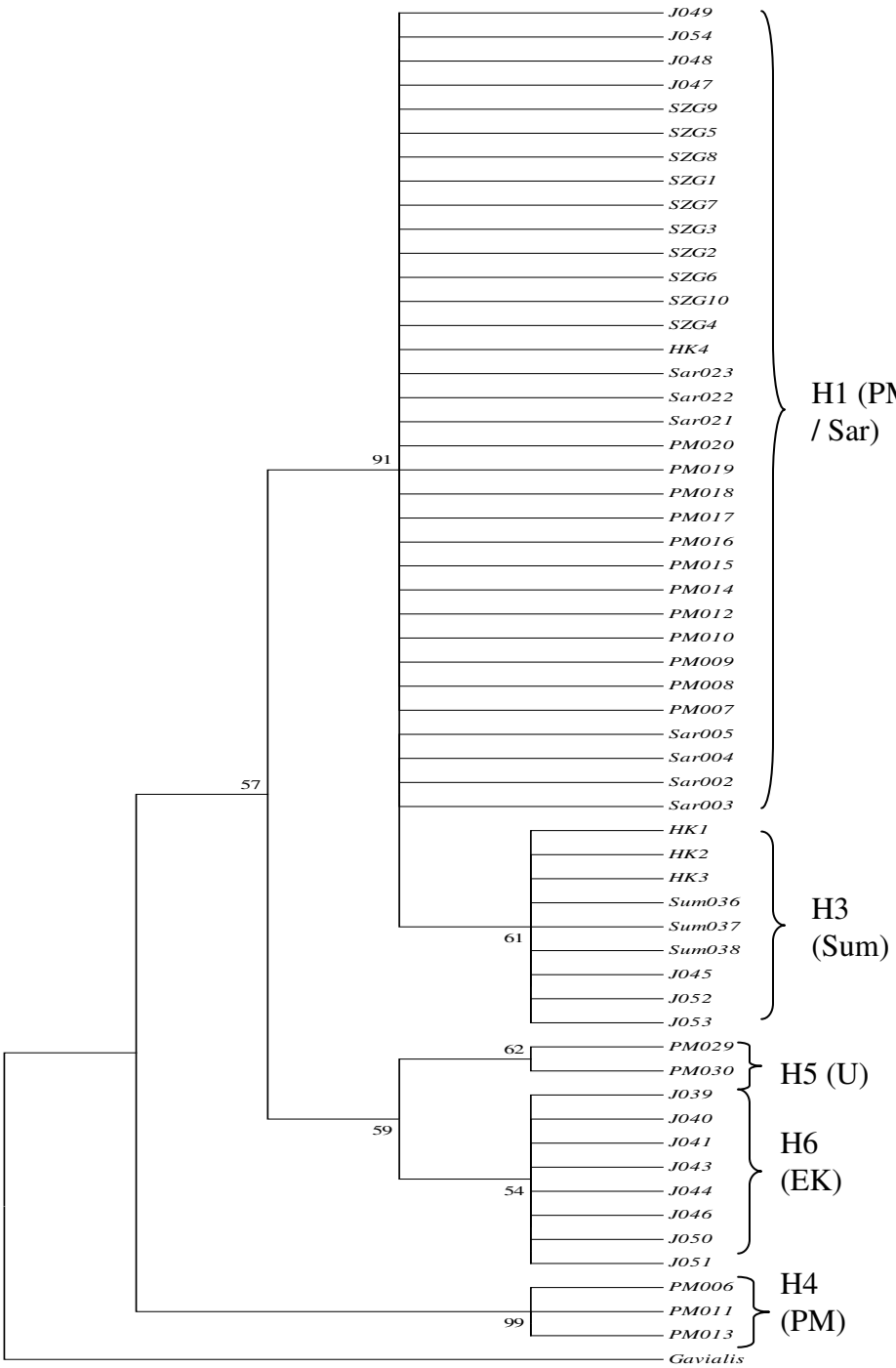


Figure 6: Maximum Likelihood tree generated for ND 6 – $tRNA^{glu}$ – cyt *b* data with PAUP v4.0b using *Gavialis* as an outgroup. Clades are indicated following lineages (H1 to H6) and geographical regions are bracketed. (PM = Peninsular Malaysia, Sar = Sarawak, Sum = Sumatra, EK = east Kalimantan, U = Unknown).

Appendix 5 Haplotype Networks based on parsimony and distance cyt *b* – CR and ND 6 – tRNA^{glu} – cyt *b* data

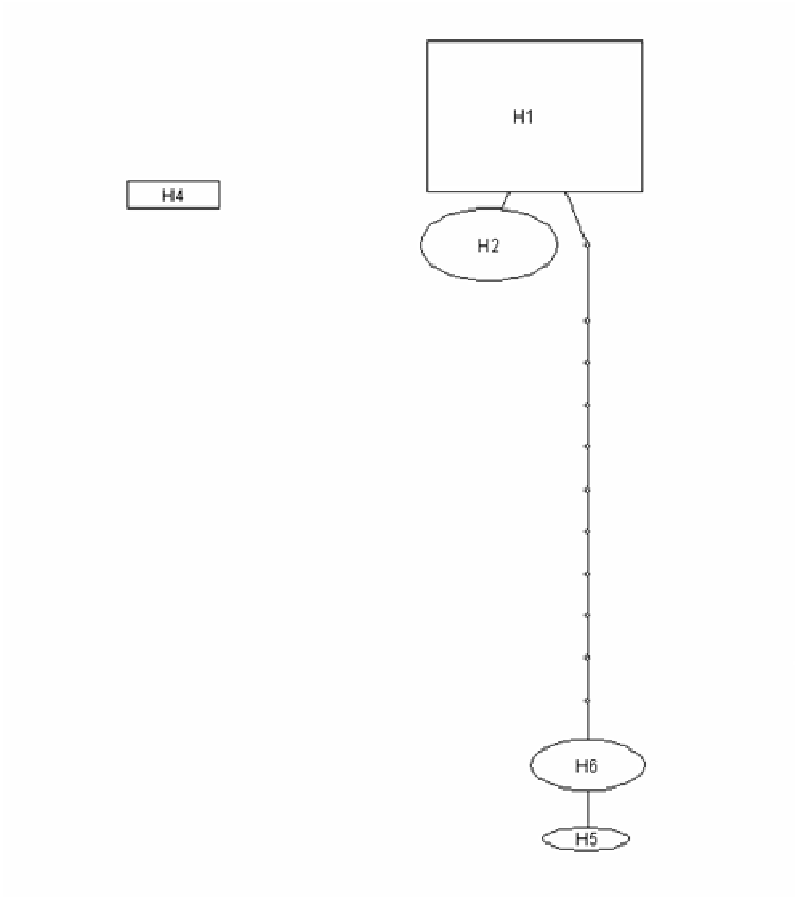


Figure 1: Haplotype network using TCS for the cyt *b* – CR data. The H4 haplotype was not parsimoniously connected at the 95 % limit.

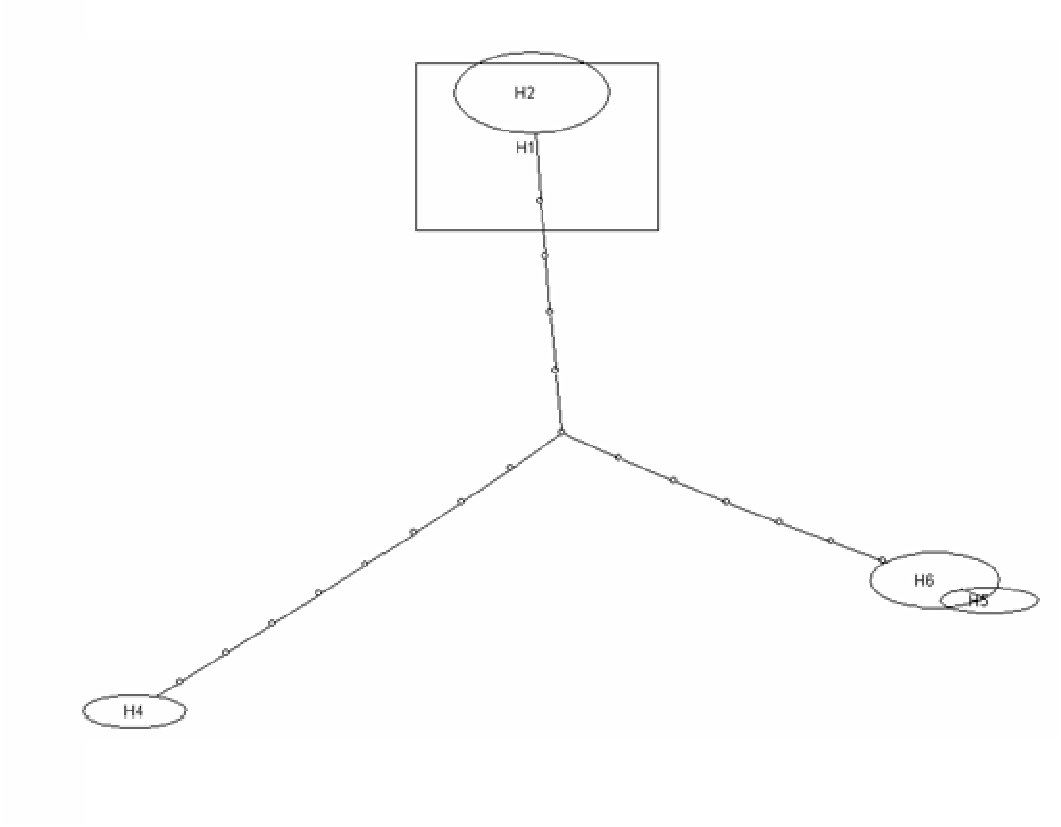


Figure 2. Haplotype network using TCS for cyt *b* – CR data showing the H4 lineage connection with other lineages at 14 step manual connection limit

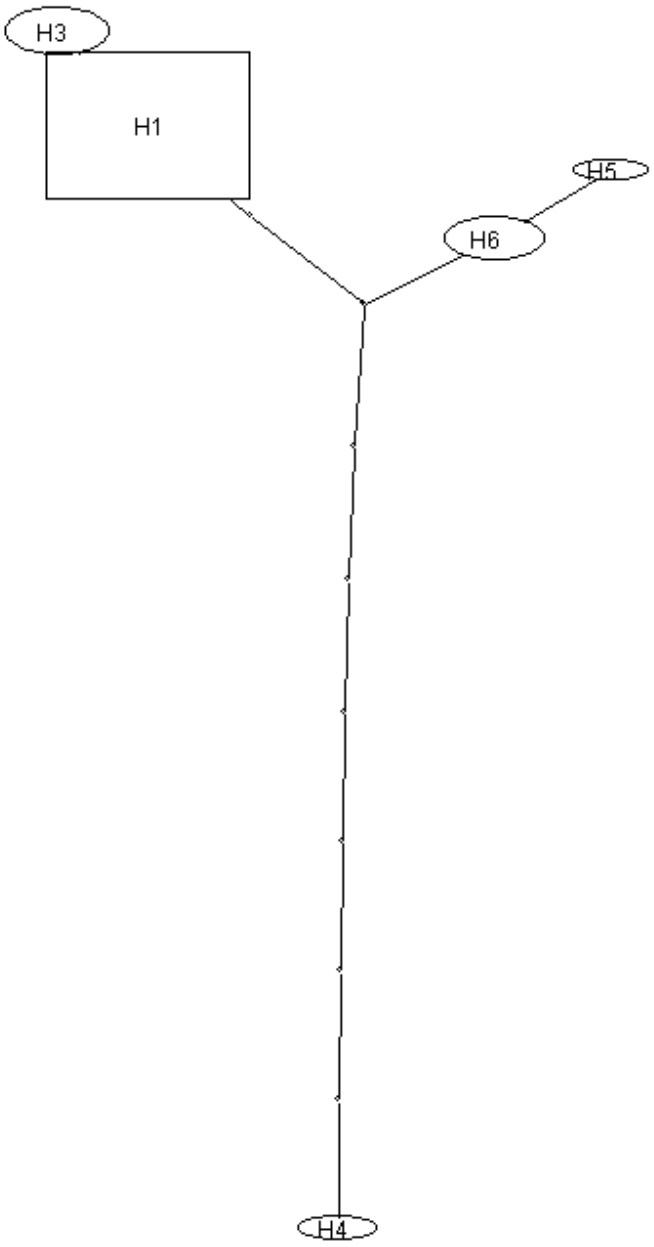


Figure 3: Haplotype network using TCS for the ND 6 – $tRNA^{glu}$ – cyt *b* data showing all haplotypes parsimoniously connected at 95% limit

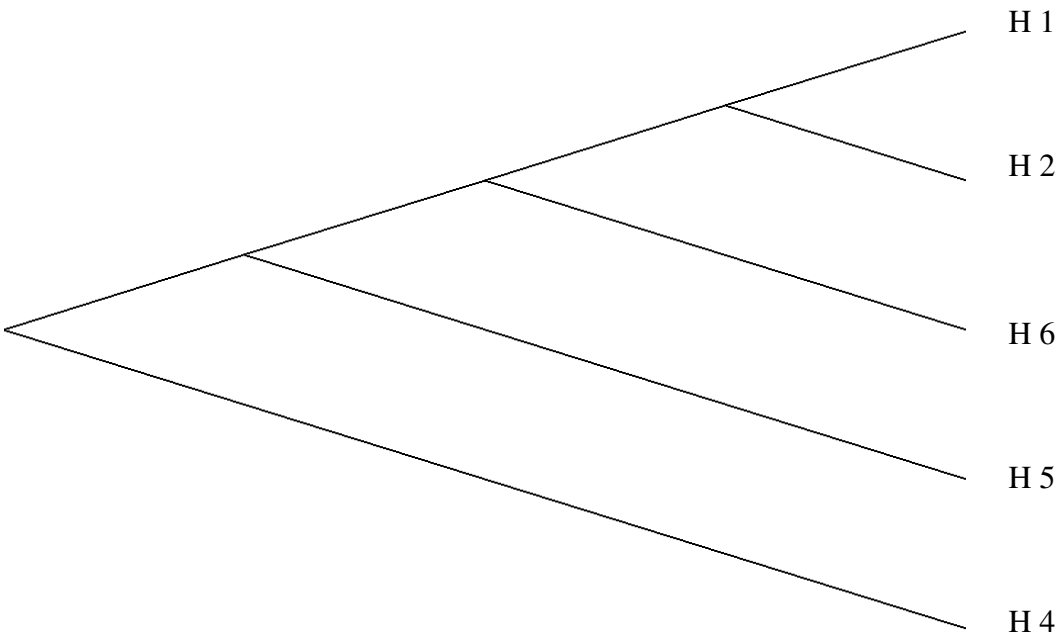
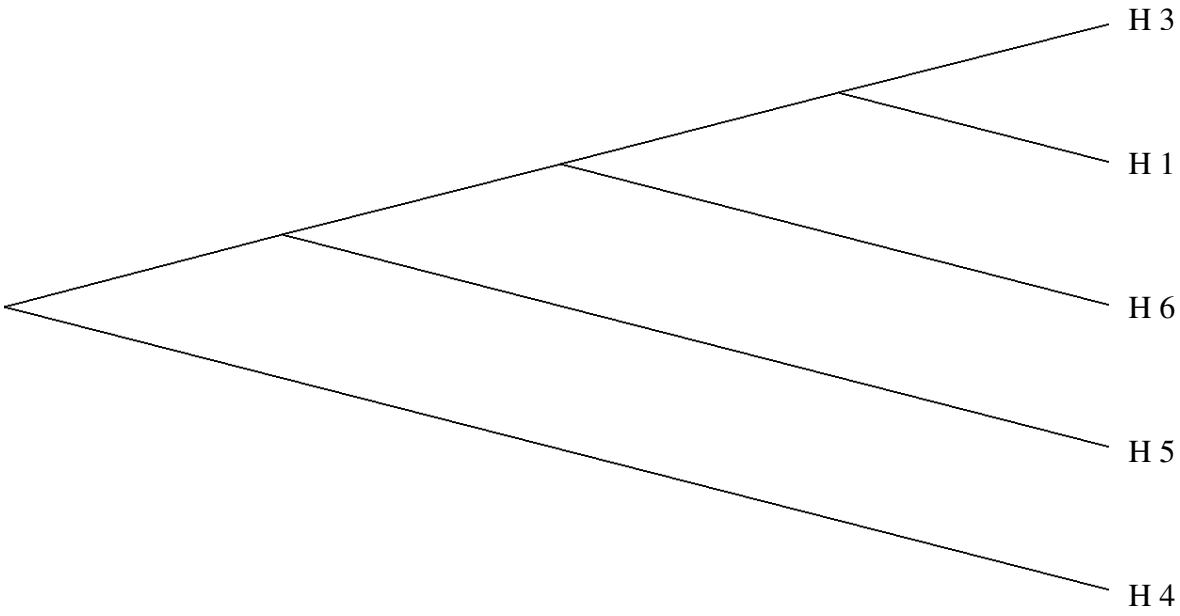


Figure 4 . Minimum spanning network using cyt *b* – CR data showing the H4 lineage on a different evolutionary branch.



Appendix 4.10 . Minimum spanning network using ND 6 – $tRNA^{glu}$ – cyt *b* data showing the H4 lineage on a different evolutionary branch.

Appendix 6 Population subdivision and gene flow analysis for the cyt *b* – CR and ND 6 – $tRNA^{glu}$ – cyt *b* data

Table 1a: Population structure estimates using the cyt *b* – CR data following geographical regions using samples of known origins, excluding the H4 haplotype in the analysis. Above diagonal is the S_{NN} values while below diagonal are the F_{ST} values. The significant value of the S_{NN} and F_{ST} estimates are $p < 0.0001$ and $p < 0.001$. The sample size for each population is 12 samples for Peninsular Malaysia, 7 for Sarawak, 3 for Sumatra and 5 for East Kalimantan.

Locations	Peninsular Malaysia	Sarawak	Sumatra	east Kalimantan
Peninsular Malaysia	-	0.84	0.67	1.00
Sarawak	0.82	-	1.00	1.00
Sumatra	0.09	1.00	-	1.00
East Kalimantan	0.99	1.00		-

Table 1b: Gene flow estimates following $N_m = 1- F_{ST} / 2 F_{ST}$ (Hudson et al. 1992) using the cyt *b* – CR data.

Locations	N_m from F_{ST}
Peninsular Malaysia X Sarawak	0.11
Peninsular Malaysia X Sumatra	5.00
Peninsular Malaysia X East Kalimantan	0.01
Sarawak X Sumatra	0
Sarawak X East Kalimantan	0
Sumatra X East Kalimantan	0

Table 2a: Population structure estimates using Protein coding 3' ND6 – 5' cyt *b* region data following geographical regions using samples of known origins, excluding the H4 haplotype in the analysis. Above diagonal is the S_{NN} values while below diagonal are the F_{ST} values. The significant value of the S_{NN} and F_{ST} estimates are $p < 0.0001$ and $p < 0.001$. The sample size for each population is 12 samples for Peninsular Malaysia, 7 for Sarawak, 3 for Sumatra and 5 for East Kalimantan.

NGC

Locations	Peninsular Malaysia	Sarawak	Sumatra	east Kalimantan
Peninsular Malaysia	-	1.00	1.00	1.00
Sarawak	NS	-	1.00	1.00
Sumatra	1.00	1.00	-	1.00
East Kalimantan	1.00	1.00		-

Table 2b: Gene flow estimates following $N_m = 1- F_{ST} / 2 F_{ST}$ (Hudson *et al*., 1992) using 3' ND6 – 5' cyt *b* region data.

Locations	N_m from F_{ST}
Peninsular Malaysia X Sarawak	-
Peninsular Malaysia X Sumatra	0
Peninsular Malaysia X East Kalimantan	0
Sarawak X Sumatra	0
Sarawak X East Kalimantan	0
Sumatra X East Kalimantan	0

Appendix 7 Neutrality Test for the cyt *b* – CR and ND 6 – tRNA^{glu} – cyt *b* data

Table 1: Neutrality test using cyt *b* – CR data and samples of known origins only. Bold font indicates significance at $p < 0.05$. Value in brackets are excluding H4 lineage in Peninsular Malaysia.

	Fu and Li's D	Fu's Fs	Rozas R2
Total	**1.71719**	7.909	0.1181
Total Peninsular Malaysia	**1.50724**	6.563	0.1683
	(0.75202)	(0.297)	(0.1515)

Table 2: Neutrality test using ND 6 – tRNA^{glu} – cyt *b* data and samples of known origins only. Bold font indicates significance at $p < 0.05$. Value in brackets are excluding H4 lineage in Peninsular Malaysia.

	Fu and Li's D	Fu's Fs	Rozas R2
Total	**1.47748**	2.439	0.0872
Total Peninsular Malaysia	**1.39196**	6.658	0.1714
	(n.a)	(n.a)	(n.a)

Appendix 8 Multiple Sequence Alignment of the mtDNA repetitive region

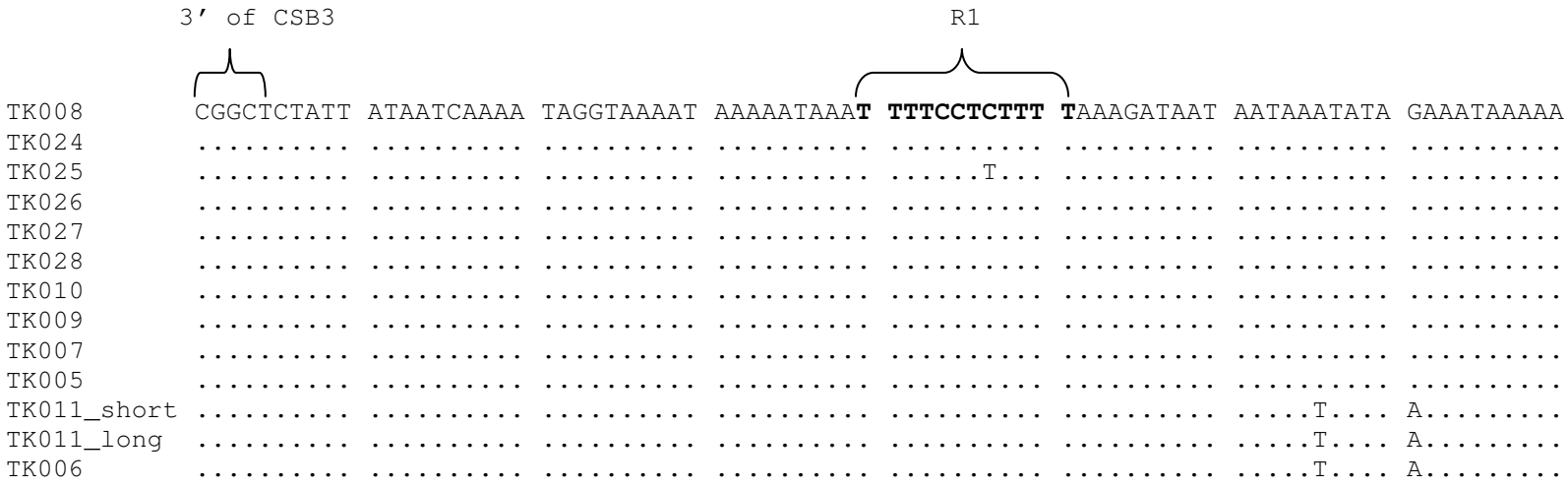
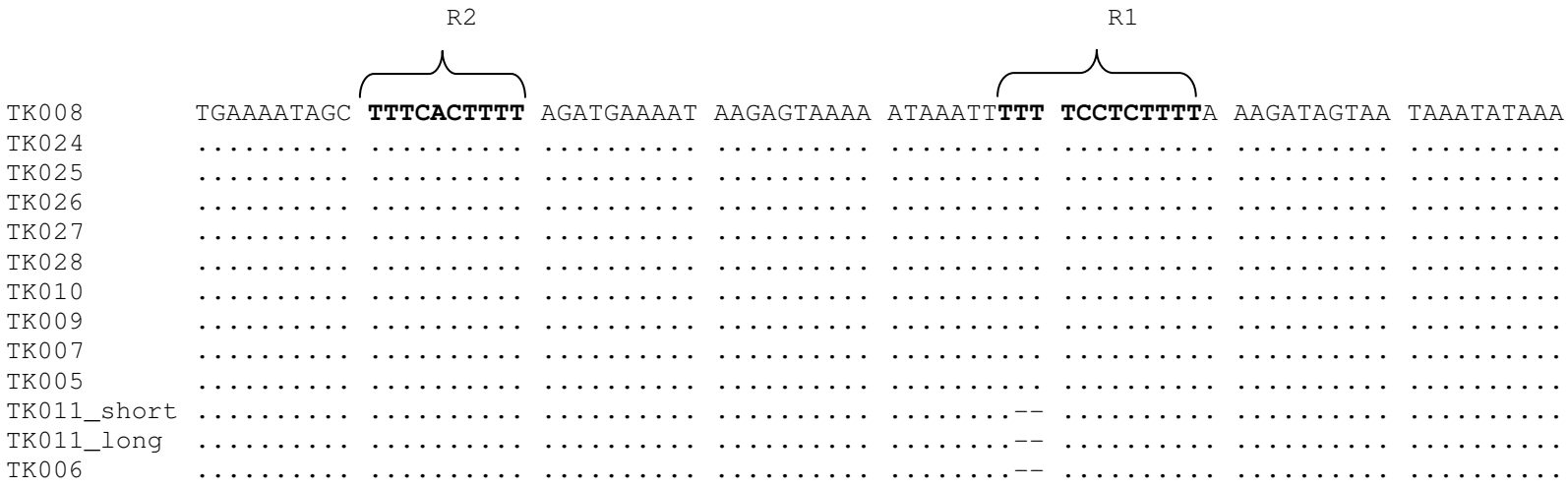
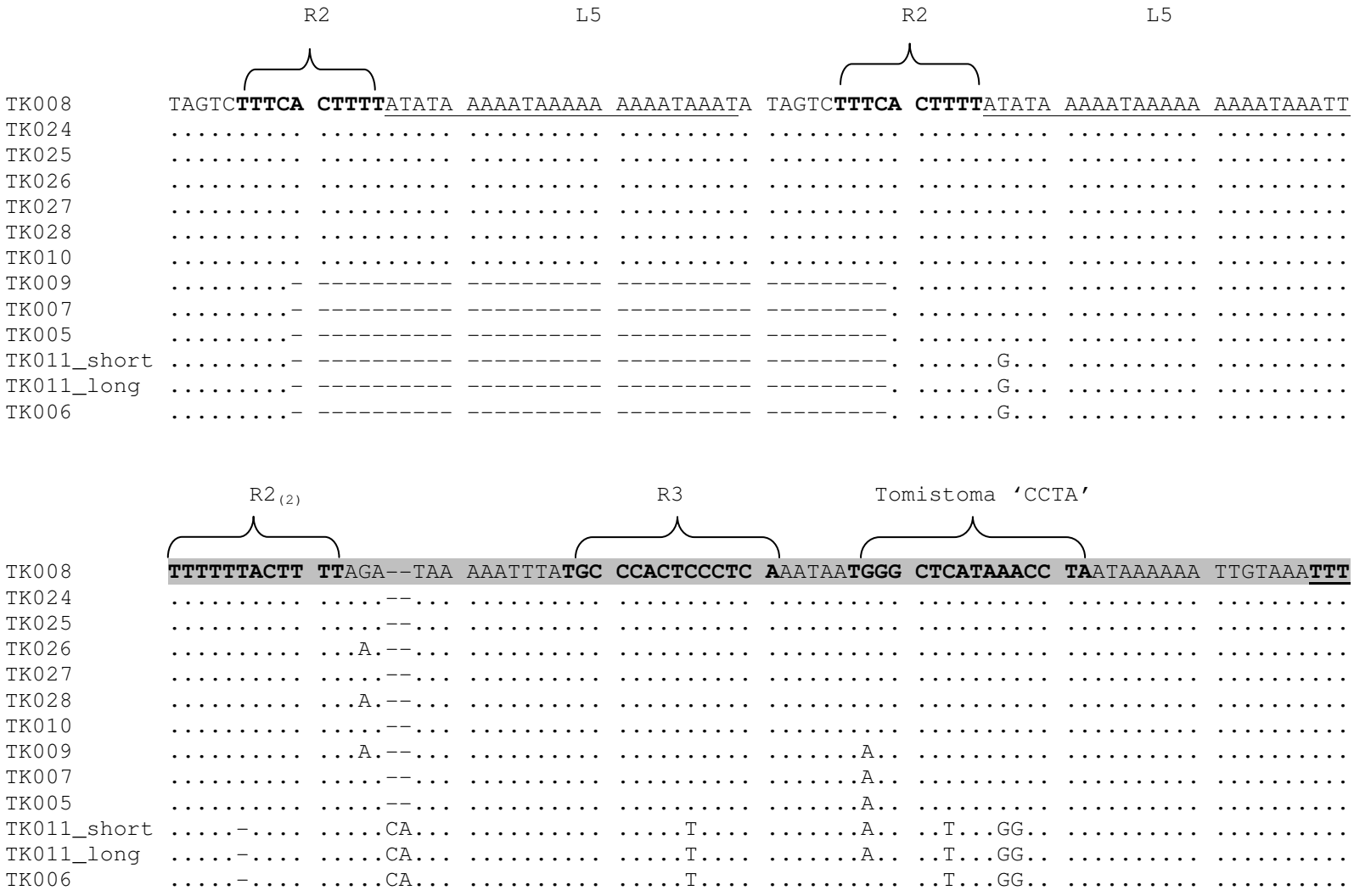
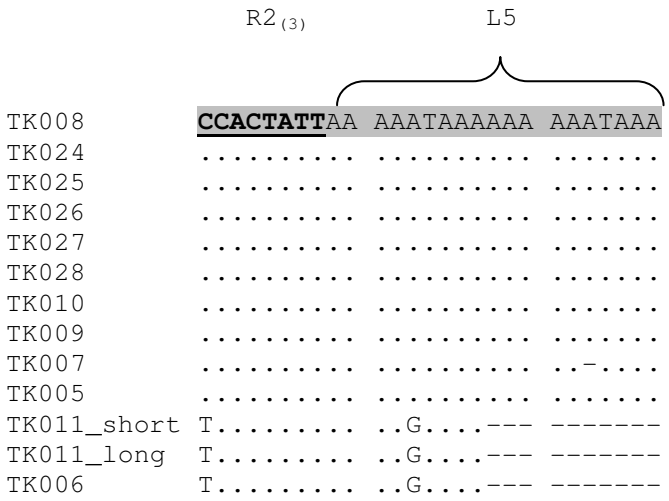


Figure 1. Sequence alignment of the repetitive region generated after the CSB III using primer L 15790. The 3' portion of the CSB III is indicated. Motifs R1 and R2 in the motif region are indicated in bold. The VNTR sequence is shaded grey and the motifs in the tandem repeats are in bold. The first 6 individuals are members of family unit, with TK008 being the maternal parent. The heteroplasmic bands of TK011 is indicated as 'short' for the 2.8 kb band and 'long' for the 3.0 kb band. TK006 is a homoplasmic individual which shared the same VNTRs sequence as the heteroplasmic individual.


R2
R1
TK008 TGAAAATAGC TTTCACTTTT AGATGAAAAT AAGAGTAAAA ATAAATTTTT TCCTCTTTTA AAGATAGTAA TAAATATAAA
TK024 ....
TK025 ....
TK026 ....
TK027 ....
TK028 ....
TK010 ....
TK009 ....
TK007 ....
TK005 ....
TK011_short ....-- ....
TK011_long ....-- ....
TK006 ....-- ....
R2
TK008 AATATAAATA AAAGTAAAAA TAAAAATGAA AATAAAAATA AAGATAATTT TTCACTTTTA AATAAAAATA AAAATAAATA
TK024 ....
TK025 ....
TK026 ....
TK027 ....
TK028 ....
TK010 ....
TK009 ....
TK007 ....
TK005 ....
TK011_short ....G... ....
TK011_long ....G... ....
TK006 ....G... ....


R2
L5
R2
L5
TK008 TAGTCTTTCA CTTTTATATA AAAATAAAAA AAAATAAATA TAGTCTTTCA CTTTTATATA AAAATAAAAA AAAATAAATT
TK024 ....
TK025 ....
TK026 ....
TK027 ....
TK028 ....
TK010 ....
TK009 ....- ---------- ---------- ---------- ---------. ....
TK007 ....- ---------- ---------- ---------- ---------. ....
TK005 ....- ---------- ---------- ---------- ---------. ....
TK011_short ....- ---------- ---------- ---------- ---------. ......G... ....
TK011_long ....- ---------- ---------- ---------- ---------. ......G... ....
TK006 ....- ---------- ---------- ---------- ---------. ......G... ....
R2(2)
R3
Tomistoma 'CCTA'
TK008 TTTTTTACTT TTAGA--TAA AAATTTATGC CCACTCCCTC AAATAATGGG CTCATAAACC TAATAAAAAA TTGTAAATTT
TK024 ....--... ....
TK025 ....--... ....
TK026 ....A.--... ....
TK027 ....--... ....
TK028 ....A.--... ....
TK010 ....--... ....
TK009 ....A.--... ....A.. ....
TK007 ....--... ....A.. ....
TK005 ....--... ....A.. ....
TK011_short .....-.... .....CA... ....T.... ....A.. ..T...GG.. ....
TK011_long .....-.... .....CA... ....T.... ....A.. ..T...GG.. ....
TK006 .....-.... .....CA... ....T.... ....T...GG.. ....


R2(3)
L5
TK008 CCACTATTAA AAATAAAAAA AAATAAA
TK024 ....
TK025 ....
TK026 ....
TK027 ....
TK028 ....
TK010 ....
TK009 ....
TK007 ....-....
TK005 ....
TK011_short T..... ..G....--- -------
TK011_long T..... ..G....--- -------
TK006 T..... ..G....--- -------