A MOLECULAR PERSPECTIVE ON THE FAMILY TESTUDINIDAE Batsch, 1788

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ABSTRACT

The Family Testudinidae have a diverse distribution and, although limited to tropical and sub-tropical latitudes, are present on all continents with the exception of Australia and Antartica. Their evolutionary history dates back to the late Paleogene at least, and periods of diversification and expansion appear to be closely associated with global climate change, particularly at the border of the Oligocene-Miocene transition.

To date, however, a revised and well-resolved taxonomy for the Testudinidae is lacking. Cranial characters, on which existing phylogenies for the terrestrial tortoises are based, are prone to environmental influences and display high levels of homoplasy, thereby limiting phylogenetic resolution. The molecular phylogeny presented in this thesis challenges current hypotheses of tortoise phylogeny based on non-molecular data, and provides the first robust estimate of inter-generic relationships within the Testudinidae.

All currently recognised species of terrestrial tortoises, encompassing all 11 genera were included in this study. Furthermore, a combination of mitochondrial markers, including Cyt *b* and ND4 gene fragments, were included and used in a combined approach with available morphological characters from published studies. To ensure a robust estimate was obtained, a combination of inference methods were utilised, including parsimony, maximum likelihood and Bayesian analysis.

Phylogenetic estimates obtained utilising ND4 sequence data and the combined data partitions provided resolution at all taxonomic levels. The Cyt *b* gene sequence, however, displayed high levels of homoplasy and saturation and, although it provided resolution at the terminal levels of the trees, was found to be unsuitable for resolution of deep level divergences within the Testudinidae.

Support for the status of *Gopherus* and *Manouria* spp. as the oldest representatives of extant tortoises is provided within this study. Furthermore, several unique relationships within the testudinids were resolved. The monophyly of the genus *Geochelone* could not be supported, and the elevation of several previously designated sub-genera to generic status is recommended, including *Stigmochelys, Chelonoidis, Asterochelys* and *Dipsochelys*. It is further recommended that the revised genus *Geochelone* be restricted to include the sub-genus *Centrochelys* and the previously assigned *Geochelone platynota* and *Geochelone*

elegans. The monophyly of the genera *Testudo* and *Homopus* could not be supported, and within *Homopus*, the reinstatement of the genus *Chersobius* is recommended. The large African endemic, *Stigmochelys pardalis* (*Geochelone pardalis*), was found to be closely associated with the genus *Psammobates* and other diminutive southern African forms, and not with the larger members of the previously recognised genus, *Geochelone*.

This revised taxonomy for the Testudinidae has implications for current theories regarding dispersal capability and the current biogeographical distribution of genera within the family, particularly in southern Africa and South America. The Testudinidae is characterised by oceanic waif dispersal, and there is support for a novel North African-South American connection. Furthermore, high levels of endemism and diversity of terrestrial tortoises within the southern African region, particularly the Western Cape province, appear to be associated with the aridification of this region, which commenced in the mid-Miocene. The conservation of this area, and the processes of evolution that define it, are of critical importance in the future conservation of the terrestrial tortoise species endemic to this area.

Given the current endangered or vulnerable status of all species of terrestrial tortoise, the application of DNA markers to address population level genetic structure within the group is advised. This is of particular importance in the development of effective conservation management strategies, which should be based on a combination of molecular, ecological and behavioral data. To this end, a panel of microsatellite DNA markers originally developed in the Galapagos Island endemic, *Geochelone* (*Chelonoidis*) *nigra*, was assessed for cross-species amplification of polymorphic product in all representatives of the Testudinidae. The suitability of these markers at the population level was assessed in a case study on the highly endangered western Cape Province endemic, *Psammobates geometricus*.

Utilising the molecular phylogeny obtained within this study, the evolution of microsatellite motifs and flanking sequences were assessed. Phylogenetically informative characters were identified both in the repeat units and flanking sequences of these loci. Despite the long periods of separate evolutionary history that characterise turtles and terrestrial tortoises, the conservation of microsatellite loci across the Order Chelonia emphasises the reduced rates of molecular evolution previously inferred for this group.

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CHAPTER 1

OVERVIEW

"Because they are still living, turtles are commonplace objects to us; were they entirely extinct, their shells – the most remarkable defensive armor ever assumed by a tetrapod – would be a cause for wonder"

Alfred Sherwood Romer

The Evolution of the Reptiles

Most land dwelling vertebrates alive today belong to the amniotes, a diverse clade with over 20 000 extant species (Gauthier et *al.* 1988). The major groups of amniotes include the mammals, birds, crocodylians, lizards, snakes and turtles[1]. The first fossil evidence for this diverse group appears in the Paleozoic, approximately 300 million years ago, with emergence of all the major groups by 250 million years ago (Laurin and Reisz 1995).

The early amniotes split into two evolutionary lineages, the Synapsida (mammals and their extinct relatives) and the Sauropsida (reptiles and their extinct relatives) (Gauthier et *al.* 1988). The Reptilia clade is characterised by a small tabular bone, the presence of a suborbital foramen and supraoccipital anterior cristae and a narrow supraoccipital plate (Gauthier et *al.* 1988). However, there is a major division within the group Reptilia based on the construction of the cheek region on the skull. In older representatives of the reptiles, the outer shell of the dermal bones has a continuous unbroken surface for attachment of jaw muscles. There appears to be a tendency for this outer shell to become perforated by one or more temporal openings or fenestra through which muscles can pass, presumably for increased muscle efficiency and action. The inter-relationships among Reptilia have been based on the number and position of these temporal openings (Zug 1993) and include:

1. The anapsids, which have the ancestral state, where the skull is completely roofed with no temporal openings, for example: the Captorhinidae and the Testudines

[1] 'Turtle' in this context is used to define all living members of the Order Testudines, including terrestrial and aquatic members

2. The diapsids, which have two openings in the temporal skull. The diapsids can be further classified into:
    - the Archaesauria including crocodiles (Order Pseudosuchia) and birds (Order Ornithosuchia)
    - the Lepidosauria including snakes (Order Squamata) and lizards (Order Sphenodontida) (Zug 1993).

The Evolution of the Testudines

Turtles (Order Testudines Linnaeus 1758; also referred to as Chelonia and Testudinata) have been considered to be a model system for the study of the evolution of physiology and organisation in the amniotes (Gauthier et *al.* 1988). They have retained the anapsid skull pattern and consequently have been regarded as basal reptiles (Frazer 1991). However, more recently several authors (Rieppel 1999, Rieppel and Reisz 1999) have questioned the placement of the Testudines in the anapsid class. Moreover, DNA sequence analysis places Testudines in the crown of the diapsids as opposed to the more traditional placement at the root of the reptile evolutionary tree. Although morphological characters have provided some support for this novel placement, there is incongruence with regards to the paleontological data as to where the turtles would have their origin in the diapsids (Rieppel 1999).

The major groups of turtles were in existence by the late Triassic, approximately 210 million years ago. One of the earliest known fossils, *Proganochelys* (Ernst and Barbour 1989), has a closed temporal region suggesting an anapsid branching (in Rieppel 1999). However, some of the details of the bone configuration in the temporal region of the skull do not match the pattern in the Paleozoic reptiles, which is representative of the anapsid condition. It has therefore been suggested that the anapsid skull developed secondarily in the Testudines (in Rieppel 1999). This hypothesis is supported when the Sauropterygia are included in the analysis. Sauropterygia are secondarily marine reptiles from the Mesozoic (commonly known as plesiosaurs) and increase the support for the placement of the turtles in the diapsid crown. There is some palaeontological support for the sister relationship between the two groups and limited support for an aquatic common ancestor, although the trees are characterised by high levels of homoplasy (Rieppel and Reisz 1999). Thus, the current debates revolve around where exactly in the diapsid crown the turtles should be placed.

The diapsids are subdivided into two major lineages, the Archosauramorphs and the Lepidosauramorphs, and the placement of the Sauropterygia-Turtle sister group with respect

to these two branches is conflicting depending on the type of evidence used (Hedges and Poling 1999). Morphological evidence supports a basal placement of the turtles to the Lepidosauramorphs (Figure 1.1 "B") but molecular data supports a placement with the Archosauramorphs (Figure 1.1 "C"). For both arrangements, however, statistical support is weak due to a high degree of independent evolution and morphological congruence between the anapsids and the diapsids. This suggests the need for a degree of caution when using morphological characters for deep level phylogenetic analysis.

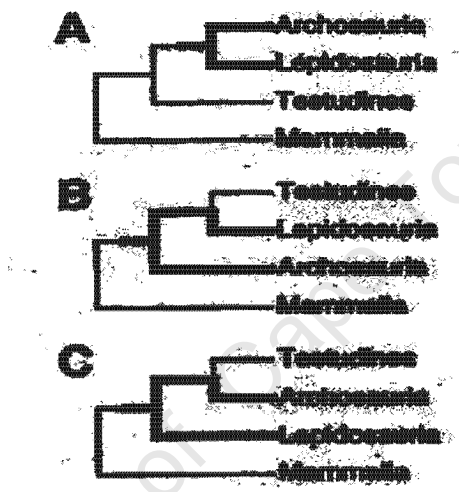


Figure 1.1: Diagrammatic representation showing the conflicting hypotheses for the placement of the Testudines in the Diapsid crown (modified from Kumazawa and Nishida 1999)

The Testudines are grouped into two suborders, the Cryptodira and the Pleurodira, based on how the neck and head are retracted into the body. The "hidden-necked" turtles (the Cryptodires) comprise the most abundant and geographically diverse group. They retract the neck vertically and posteriorly into the body cavity, producing a S-shaped curve in the neck. The majority of fossil and extant turtles belong to the Cryptodires, although the taxonomy of this assemblage remains poorly resolved (in Shaffer et *al.* 1997). The Pleurodires, more commonly known as side-necked turtles, lay their head and neck on their shoulders. This group is thought to represent the ancestral condition with members currently restricted to Australia, New Guinea and South America (in Shaffer et *al.* 1997). Both the Pleurodires and the Cryptodires are considered monophyletic assemblages (in Shaffer et *al.* 1997).

Extant turtles, although relatively conservative in their overall structure, inhabit a wide variety of habitats ranging from freshwater or marine, to semi-terrestrial, to completely terrestrial habitats (Harless and Morlock 1979). They are widely distributed across tropical and sub-tropical regions, as well as the more temperate zones in both the northern and southern hemispheres (Crumly 1984). This diverse order consists of 13 families, 87 genera and approximately 257 species (Iverson, 1997). Despite the fact that the relationships within the Cryptodira are relatively poorly resolved, the suborder can be divided into four superfamilies (in Shaffer et *al.* 1997): the Chelonioidea (sea turtles) represented by the Cheloniidae and Dermochelyidae; the Trionychoidea (soft shelled turtles) that include the Kinosternidae, Carettochelydae, Dermatemydidae and Trionychidae families; the Testudinoidea represented by the Testudinidae, Bataguridae and the Emydidae (terrestrial tortoises and pond turtles) and finally the Chelydridae (the snapping turtles), which is the sister group to the remaining clades (Gaffney and Meylan 1988). The Pleurodirans are represented by two families; the Pelomedusidae and the Chelidae and these have been restricted to the Southern Hemisphere since the Halocene (Ernst and Barbour 1989).

The Terrestrial Tortoises

Family Testudinidae

Within the Crypotidires, the family Testudinidae Batsch, 1788 comprises all known terrestrial tortoises. They are a monophyletic group and are thought to have originated from the ancestral and widely distributed members of the semi-terrestrial Emydidae (Crumly 1984), although closer affinities to the Bataguridae have been suggested (Gaffney 1979, Hirayama 1985). Members of the Testudinidae possess most of the following derived characters: two or less phalanges in the fingers and toes, coalesced trochanters of the femur, a wide ethmoid feature, lack of auxillary or inguinal glands, expansion of the coracoid blade and the lack of a cloacal bursa (Crumly 1984).

Distribution

Terrestrial tortoises have a wide distribution and are found on all continental landmasses (with the exception of Australia and Antartica) as well as several islands (the Galapagos, the Seychelles, Madagascar and Aldabra). This distribution lends support to the theory that terrestrial tortoises can disperse efficiently across marine barriers (Williams 1950). Members of this family are confined to warm, temperate zones and particularly the tropical and sub-tropical regions, a distribution perhaps limited by their ectothermic biology (Meylan and Auffenberg 1987). They occupy a diverse range of habitats, for example the larger members of the family, including the *Manouria* spp., are found in the tropical forests of south east Asia, whilst the smaller species, like *Homopus*, are found in the arid deserts of Namibia (Crumly 1984). This adaptation to a considerable range of ecological diversity is reflected in a broad spectrum of structural diversity. The American genus *Gopherus*, for example, is characterised by the specialisation of the front feet, which are flattened for burrowing (Harless and Morlock 1979). The range in shell size and shape amongst the members of the genus *Geochelone* is greater than that between most other families of turtles (Crumly 1982). For example, the giant tortoises of the Galapagos Islands, *Geochelone nigra*, are amongst the largest living completely terrestrial ectotherms with a carapace length up to one and a half meters. In comparison, *Geochelone elegans*, the Indian star tortoise, is sexually mature at about 20 centimeter shell length. In response to geographical isolation several of the populations on the Galapagos Islands have developed races that show a wide range of diversity within this genus and 15 sub-species are currently recognised (Caccone et *al.* 1999a, Harless and Morlock 1979). An example of a distinctive Galapagos race is the Saddleback tortoise. This species has developed longer front legs and an open fronted "saddleback" shell that allows it to browse on higher vegetation since it facilitates vertical

extension of the head and neck (Harless and Morlock 1979). *Malacochersus tornieri* is an African endemic that shows remarkable adaptations to its surrounding habitat - this tortoise has a highly flexible and depressed shell, which enables it to conceal itself efficiently between narrow boulders and beneath rocks (Ernst and Barbour 1989, Harless and Morlock 1979).

Conservation Issues

The destruction and degradation of natural habitats has been the major contributing factor to the accelerating decline of biodiversity worldwide (Klemens 2000). By the late 1980's, it was estimated that over 76% of the world's primary forests had been destroyed. This includes 50% of the tropical rainforests, 55% of the coastal temperate rainforests, 65% of the natural habitat in sub-Saharan Africa and 67% in Asia (Klemens 2000). This habitat loss has had an adverse impact on approximately 60% of the world's terrestrial tortoise species. These range from the effects of habitat degradation and habitat fragmentation to introduced species and predators, as well as the direct effects of human cultural and medicinal practices (Klemens 2000). One of the major problems facing conservationists is the paucity of baseline data regarding the distribution, ecology, behavior and levels of genetic variation within tortoise populations. This makes it difficult to assess the status of many of these populations and hinders the implementation of successful management policies. There are currently six species of terrestrial tortoises listed in the "endangered" category of CITES APPENDIX I, while all remaining species have been placed in CITES APPENDIX II, with the majority of them in the "vulnerable" category (www.cites.org). Critically endangered species include *Geochelone nigra*, *Geochelone radiata*, *Geochelone yniphora*, *Gopherus flavomarginatus*, *Testudo kleinmanni* and *Psammobates geometricus*.

Habitat loss can be defined as the destruction or elimination of natural vegetation to the extent that it is no longer able to support indigenous biota (Klemens 2000). Habitat loss is primarily the result of urbanisation and agricultural practice, and is one of the primary causes of population decline in terrestrial tortoises (Klemens 2000). This is particularly detrimental to endemic species that are localised and restricted to areas of specific habitat. An example of this problem is the status of the Geometric tortoise, *Psammobates geometricus*. This is a small South African species that is limited to a specific veldtype, the Renosterveld, which forms part of the highly endangered Cape Floral Kingdom (Baard 1992). Other species threatened by habitat loss include the South American Chaco tortoise, *Geochelone chilensis*, whose habitat has been irreversibly damaged by overgrazing and agriculture, and *Testudo*

horsfieldii, the Central Asian tortoise whose numbers have been declining due to irrigation of semi-desert steppe habitat in the Turkman Republic (Harless and Morlock 1979).

An additional adverse effect of this destruction of natural habitat is fragmentation of populations. This occurs when once continuous populations become separated from one another, and in the case of land tortoises, is of particular significance, as populations are known to be severely restricted by geographical barriers largely as a result of their limited potential for mobility (Highfield 1990). This in turn affects the genetic structure of the populations as it effectively limits gene flow, for example in the Malagasy endemic, *Geochelone yniphora*, where the remaining populations estimated at some 1000 individuals, occur in highly fragmented habitats (Klemens 2000).

A further threat to tortoise populations is the degradation of natural habitat. Although closely associated with habitat loss, processes associated with pollution, the introduction of alien invasives and the alteration of fire regimes reduce the ability of extant habitats to support natural populations. A number of species are known to have been particularly affected by this and include the Natal hinge-back tortoise, *Kinixys natalensis* and the South African bowsprit tortoise, *Chersina angulata* (Klemens 2000).

Introduced species are another major threat to tortoise populations. One of the best-studied cases is on the Galapagos Islands where young *Geochelone nigra* are known to fall prey to introduced species of dogs, cats and pigs. This high juvenile mortality has a detrimental impact on the population age structure and, as a result, on the reproductive parameters of this species (Caccone et *al.* 1999a, Klemens 2000).

Current Taxonomy

The family Testudinidae has an extensive fossil record that dates back to the early Eocene and it would appear that tortoises were once more diverse and more widely distributed geographically (Auffenberg 1974). Fossil forms have been found as far north as South Dakota, Mongolia and Moscow (Crumly 1984). With the families known preference for warmer climates, these fossil forms have been used as indicators of historically temperate climates particularly in the northern lattitudes that are today characterised by Arctic conditions (Holman 1971).

The classification of the Testudinidae has been modified several times during the last 50 years. Reasons for this include large amounts of parallelism and convergence, compounded by the retention of ancestral features, which has made the elucidation of the relationships between the genera difficult to assess (Auffenberg 1974, Crumly 1984). In addition much of the fossil evidence currently available is incomplete and highly fragmented with only a few of the specimens retaining the limbs, girdles and skulls making classification to the generic level difficult (Auffenberg 1974). Furthermore, most studies to date have not been taxonomically complete and have concentrated on a limited number of characters with many of the anatomical systems not studied at all (Crumly 1984). These factors have resulted in a poorly resolved phylogeny with the taxonomy often confusing and conflicting, particularly at the generic level.

Current taxonomy has identified 11 genera and approximately 40 species based on phylogenies produced from morphological data sets (Iverson 1997). The name Testudo was originally used for all terrestrial tortoises. However, a number of monophyletic genera were subsequently introduced and *Testudo* was given generic status (in Gerlach 2001). There were several genera introduced that recognised the smaller tortoise species, such as *Homopus, Pyxis* and *Psammobates*, while all the larger species were placed in the genus *Geochelone* (in Gerlach 2001). Following partial revisions within this genus, there were several subgenera that were eventually afforded full generic status, such as *Manouria* and *Indotestudo* (Crumly 1984). The relationships within *Geochelone*, however, remain clouded with the potential for several more subgenera to be raised to generic rank (Table 1.1).

Table 1.1: The genera represented in the Family Testudinidae from Iverson (1997). Values in parenthesis indicate the opinions of various authors as to the designations of some sub-species to full species status.

Genera	Species
Chersina	1
Gopherus	4
Homopus	5 (6)[A]
Indotestudo	2 (3)[B]
Kinixys	4 (6)[C]
Manouria	2
Malacochersus	1
Psammobates	3
Pyxis	2
Testudo	5
Geochelone	11

A - Hewitt (1937)

B – Harless and Morlock (1979), Iverson (1997)

C – Boycott and Bourquin (1988), Swingland and Clemens (1989), Iverson (1997)

Research Aims

In light of the poorly resolved taxonomy of Testudinidae and given the vulnerable status of many of the currently recognised tortoise species, the major objectives of this thesis are:

i) To elucidate the inter-generic relationships among extant tortoises using two mitochondrial gene fragments

ii) To compare molecular phylogenetic inferences obtained in this study with currently accepted phylogenetic estimates based on morphological characters

iii) To explore a number of hypotheses that could explain the enigmatic global distribution of the Family Testudinidae

iv) To assess the utility for cross-species amplification of a panel of microsatellite markers, originally developed in *Geochelone nigra,* in the Family Testudinidae

v) To utilise these microsatellite markers to assess the genetic structure of the highly endangered South African endemic, *Psammobates geometricus* - a conservation priority in the Western Cape Province.

Thesis Organisation

This thesis comprises eight chapters, and includes a chapter (Chapter Two) that covers the current literature on the Family Testudinidae and the limitations of presently accepted phylogenetic estimates, which for the most part have been based on morphological characters. Chapter Three is a comprehensive discussion on the state of the field of molecular systematics, including recent debates on the utility of parsimony and model based methods of inference, hypothesis testing and the recently implemented bayesian approach in the assessment of phylogenies. Chapters Four and Five are the Materials and Methods and the Results chapters respectively, whilst Chapter Six is a discussion of the major points representing the original research aims outlined for the phylogenetic aspect of the study. Because a diverse range of topics are covered in Chapter Six, it has been sub-divided into three parts. Part I deals with the phylogenetic utility of the selected genes that were utilised in the study and the effects of selected parameters, that describe sequence evolution, on model based approaches of inference. A revised taxonomy for the terrestrial tortoises, based on both molecular and morphological data sets, is addressed in Part II. Several hypotheses to explain the enigmatic distribution of the old and new world tortoises, based on the revised taxonomy, are presented in Part III of Chapter Six. Part IV deals specifically with the African genera of the Testudinidae, concentrating on the high levels of endemism observed on the continent, particularly in southern Africa.

Cross-species utilisation of microsatellite markers, originally developed in the Galapagos tortoise, was tested in all species of terrestrial Chelonians. This aspect of the study is introduced in Chapter Seven. The information obtained from the revised taxonomy for the Family Testudinidae was used to address the utility and the phylogenetic response of these markers in all members of this diverse group. Subsequently, a molecular genetic case study on the South African tortoise, *Psammobates geometricus,* was implemented. Polytypic microsatellite markers were used to assess population structure in this endemic tortoise to determine the effects that habitat fragmentation and the subsequent decrease in population numbers has had on the conservation genetic status of this endangered species.

Finally, Chapter Eight is a record of all references quoted during the course of this dissertation.

CHAPTER 2

CURRENT TAXONOMIC STATUS AND PHYLOGENY OF THE FAMILY TESTUDINIDAE Batsch, 1788

Introduction

Terrestrial tortoises have a global distribution and are present on all the continents, with the exception of Australia and Antartica (Auffenberg 1974) (Figure 2.1). They also occur or have historically been present on several Ocean Islands, including the Galapagos Islands, Bermuda (Meylan and Sterrer 2000), Madagascar, the Seychelles and the Mascarenes (Austin and Arnold 2001). Terrestrial tortoises are considered an ideal group for the study of ecological and climactic patterns due to their wide and diverse distribution, however the taxonomy of the family remains poorly resolved (Auffenberg 1974, Crumly 1984, Gerlach 2001).

Tortoises are known from the tropical[2] and sub-tropical regions of the world where they favour relatively mesic to xeric habitats. This distribution is linked to their ectothermic physiology. Ectothermic vertebrates can only survive in climates that provide sufficient heat for activity, growth and reproduction, whereas endotherms are not restricted by these limitations, and can supplement environmental heat with their own (Meylan and Sterrer 2000). It is this dependence on warmer climates that has made ectotherm fossils, for example crocodiles and tortoises, useful as paleoclimactic indicators, particularly as evidence for historically milder climates (Brattstrom 1961, Holman 1962). An example is the giant fossil tortoise (*Geochelone*) found in the Wood Mountain Formations, southern Saskatchewan, Canada. The presence of these fossil forms suggests that this area would have experienced a sub-tropical climate during the upper Miocene (Holman 1971). Today the climate is substantially cooler and there are no terrestrial tortoises currently represented.

The habitat types of the Family Testudinidae range from tropical decidious forests, thorn bush, savannah and steppes to near desert conditions (Harless and Morlock 1979). Their current distribution appears to have involved several dispersal events, local extinctions and reinvasions during their evolutionary history. Furthermore, explanations of their present

[2] The tropics are characterised by having relatively constant temperatures throughout the year (Brattstrom, 1961)

biogeography from morphological phylogenies are not strongly supported by the fossil record (Auffenberg 1974, Crumly 1984, Gerlach 2001). Iverson's taxonomic revision (1997) will be referred to for discussion on current distribution, while a detailed description of the conflicting taxonomies will be introduced in the overview of recent phylogenetic studies conducted on the Family Testudinidae.

Following Iverson's revisions (1997), the family currently comprises 11 genera and approximately 40 species and 40 sub-species (Table 2.1). The designation of a number of sub-species, several of which are still under consideration, remain contentious. For example *Testudo graeca ibera* (Gmira, 1993) and *Kinixys belliana nogueyi* are two such examples under review (Swingland and Clemens, 1989).

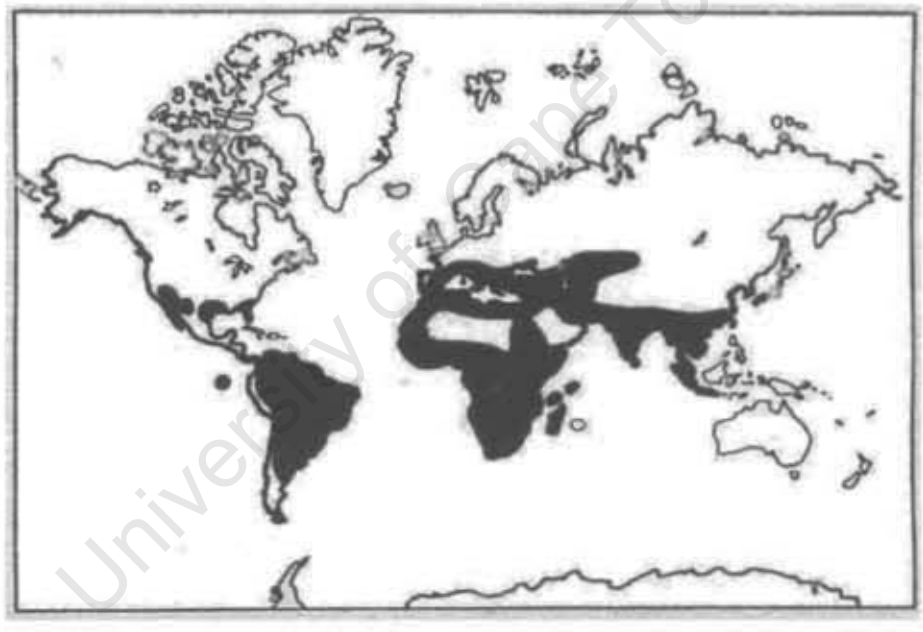


Figure 2.1: Map showing the current tropical and sub-tropical distribution of the terrestrial tortoises, Family Testudinidae (modified from Ernst and Barbour 1989).

Table 2.1: Conflicting taxonomic classifications of the Family Testudinidae based on morphological analysis (generic designations are in bold italics, sub-genera are underlined italics, with species designations in italics).

Auffenberg (1966, 1974)	Crumly(1982, 1984)	Iverson (1997)* and Gerlach (2001)
Kinixys	***Kinixys***	***Kinixys**** Bell, 1827
		K. belliana (K. speki)
		K. erosa
		K. homeana
		K. natalensis
Pyxis	***Pyxis***	***Pyxis**** Bell, 1827
Acinixys		*P. arachnoides*
		P. planicauda
Testudo	***Testudo***	***Testudo**** Linnaeus, 1758
		Testudo
		T. graeca
		T. hermanni
		T. kleinmanni (Pseudotestudo?)
		T. marginata
		Agrionemys Khozatsky and Mlynarskii, 1966
		A. (T.) horsfieldii
Malacochersus	***Malacochersus***	***Malacochersus**** Lindholm, 1929
		M. tornieri
Geochelone (6 sub-genera)	***Geochelone***	***Geochelone**** Fitzinger, 1835
Asterochelys Gray, 1873		*G. elegans*
G. radiata		*G. platynota*
G. yniphora		***Asterochelys*** Gray, 1873
Aldabrachelys Loveridge+Williams, 1957		*A. yniphora*
G. gigantea		*A. radiata*
Indotestudo Lindholm, 1929		***Dipsochelys*** Bour, 1982
G. elongata		*D. arnoldi*
G. forstenii		*D. dissumieri*
G. travancorica		*D. grandidieri*
Geochelone Fitzinger, 1835		*D. hololissa*
G. sulcata		*D. aprupta*
G. platynota		***Stigmochelys*** Gray, 1873
G. elegans		*S. pardalis*
G. pardalis		***Centrochelys*** Gray, 1872
Chelonoidis Fitzinger, 1835		*C. sulcata*
G. carbonaria		***Chelonoidis*** Fitzinger, 1835
G. chilensis		*C. carbonaria*
G. denticulata		*C. chilensis*
G. elephantopus		*C. denticulata*
Manouria Gray, 1852		*C. nigra*
M. emys		***Cylindraspis*** (Fossil)Fitzinger 1835
M. impressa		
Chersina	***Chersina***	***Chersina**** Gray, 1831
		C. angulata
Gopherus	***Gopherus***	***Gopherus*** Rafinesque, 1832
		Gopherus
		G. berlandieri
		G. polyphemus
		Xerobates Agassizi, 1857
		X. agassizii
		X. flavomarginatus
Not defined (see Geochelone)	***Indotestudo***	***Indotestudo**** Lindholm, 1929
		I. elongata
		I. forstenii
Not defined (see Geochelone)	***Manouria***	***Manouria**** Gray, 1852
		M. impressa
		M. emys
Psammobates	***Psammobates***	***Psammobates**** Fitzinger, 1835
		P. geometricus
		P. tentorius
		P. oculifer
Homopus	***Homopus***	***Homopus**** Duméril and Bibron, 1835
		H. areolatus
		H. boulengeri (Chersobius Hewitt, 1937*)*
		H. femoralis
		H. signatus (Chersobius Hewitt, 1937*)*

Current Distribution of the Testudinidae

African Distribution

Although the fossil record does suggest a historically diverse and broad representation in Europe, North America and Asia (Auffenberg 1974, Crumly 1984), extant members of the Testudinidae reflect their greatest diversity in sub-Saharan Africa. Ethiopian representatives include members of the widespread Old and New World genus *Geochelone*, including *G. sulcata* and *G. pardalis*. Also present, and confined to North Africa, are representatives of the genus *Testudo*. These include *T. graeca* and the highly endangered *T. kleinmanni* in Egypt (van der Kuyl et *al.* 2002). *Testudo kleinmanni* has also been placed in the sub-genus *Pseudotestudo* (Loveridge and Williams 1957). It has been suggested that *T. graeca* of Algeria is in fact a separate species, *T. whitei*, and could be elevated to a separate genus, *Furculachelys whitei* (Highfield and Martin, 1989).

Members of the Testudinidae that are endemic to Africa include the genus *Kinixys*, which include the only members of the order that have a hinge allowing for the posterior movement of the carapace (Ernst and Barbour 1989). This genus is represented by the forest dwelling species *K. erosa* and *K. homeana* in West Africa, the widespread savannah and scrub adapted species, *K. belliana* spp. and finally *K. b. spekii* and *K. natalensis* found further south. *Kinixys belliana spekii* has been referred to as one of four sub-species under *Kinixys belliana* (Boycott and Bourquin 1988, Iverson 1997) although this remains a matter of some contention. Unfortunately, the genus *Kinixys* was not sampled across its full range and therefore samples of potential sub-species taxa could not be obtained. A definitive taxonomic assessment for this genus will be required.

The genus *Malacochersus*, found in Tanzania and Kenya, is more commonly known as the pancake tortoise, and is characterised by a highly depressed shell. This is thought to be an adaptation that allows the species to conceal itself between boulders and under rocks, both of which typify the east African landscape (Harless and Morlock 1979). The smaller southern African tortoises included in the group of African endemics, include *Homopus* spp., *Psammobates* spp., *Kinixys* spp. and *Chersina* spp., none of which reach a carapace length of more than 20 centimeters (Harless and Morlock 1979). Despite being characterised by high levels of variation, *Chersina* spp. and *Malacochersus* spp. are considered monotypic genera (Harless and Morlock 1979). *Malacochersus* is considered the most divergent member of the Testudinidae (Ernst and Barbour 1989). Both *Psammobates* spp. and *Homopus* spp. are represented by several well-defined species that are endemic to South

Africa, and in particular to the Western and Eastern Cape regions of the country (Swingland and Clemens 1989). The high levels of endemism observed within the southern African region will be discussed in more detail in Chapter 6.

European Distribution

European genera include members of *Testudo* spp. and are found in particular abundance in the southern Palearctic regions. These include *T. graeca ibera* in the Balkans, Greece and Turkey; two sub-species of *T. hermanni*: *T.h. hermanni* in Italy, France and Spain and *T. h boettgeri* in the Balkans and Greece (Harless and Morlock 1979). The larger *T. marginata* and *T. weissingeri* are also found in Greece where they occupy restricted habitats (van der Kuyl et *al.* 2002) and it has been suggested that *T. weissingeri* is a dwarf form of the larger *T. marginata* (van der Kuyl et *al.* 2002). The distribution of *Testudo graeca* includes the Middle Eastern countries, Syria and Israel (*T. g. terrestris*), as well as Iran and Afghanistan (*T. g. zarudnyi*) and Libya (*T. g. terrestris*) (van der Kuyl et *al.* 2002). The Russian or Four-toed tortoise, *T. horsfieldii*, has a distribution that includes Pakistan, Afghanistan and parts of the Soviet Union. Khozatsky and Mlynarski (1966) consider *T. horsfieldii* divergent from the remaining *Testudo* spp. placing it in the sub-genus *Agrionemys.*

Indian And Asian Distribution

Tropical Southeast Asia and India have members of the genus *Geochelone*, *Manouria* and *Indotestudo. Geochelone elegans* occurs in Pakistan, India and Sri Lanka whilst *G. platynota* is confined to southern Burma. Both species are smaller than their African counterparts and inhabit grasslands and primary forests typical of the area (Harless and Morlock 1979). They are characterised by the presence of decorative starred patterns on their carapaces. The other two genera represented are *Manouria* spp. and *Indotestudo* spp. *Manouria* is currently recognised as the oldest of the extant tortoise genera, supported by plesiomorphic morphological characters (Crumly 1984, Ernst and Barbour 1989). This basal status is substantiated by their habitation of tropical evergreen forests (Auffenberg 1966, Crumly 1984) whilst derived tortoises appear to be more arid adapted in their ranges (Auffenberg 1974, Crumly 1984). The genus *Manouria* is represented by two well-defined species including *M. impressa* in Burma, Malaya to Vietnam and southern China, and its sister species, *M. emys*, in eastern India, Vietnam, China, Malaya, Sumatra, Borneo and other Indonesian Islands (sub-species *M. e. emys*).

Indotestudo spp. are endemic to Asia and India and are thought to show close affinities to the South American species of *Geochelone* (Harless and Morlock 1979). Three species are currently recognised; *I. elongata* and *I. travancorica*, that both support narrow elongated shells and are differentiated from each other by the presence of a nuchal scute in *I. elongata* (Harless and Morlock 1979). *Indotestudo forstenii* is distributed on Celebes and is probably derived from a long established population of *I. travancorica* (Harless and Morlock 1979). The group inhabits forested areas in Nepal, India, Burma and Vietnam (*I. elongata*) and the mesic evergreen forests in India (*I. travancorica*).

North American Distribution

The North American species are well-studied (Lamb and Lydeard 1994, Osentoski and Lamb 1995) and are represented by the genus *Gopherus*. Four distinct species are confined to the southern parts of the United States and Mexico. Although there is currently a limited representation of tortoises in North America, the fossil record for this area is rich in both diversity and density (Crumly 1984). It has been suggested that the reason for the current paucity in extant tortoises could be due to the cooling effects of climactic events in the Pleistocene, which may have caused local extinctions (Auffenberg 1974, Crumly 1984, Osentoski and Lamb 1995). *Gopherus* spp. are characterised by the morphological and behavioral adaptation of the front feet to burrowing. It is the only genus that displays this specialised feature and burrowing is thought to be an adaptation to the habitation of higher latitudes (Auffenberg 1974, Brattstrom 1961). Extensive burrow systems are excavated and the tortoises remain underground for a large part of the year (Harless and Morlock 1979). *Gopherus polyphemus*, found in the extreme south eastern United States, seems to be closely related to the endangered *G. flavomarginatus* (forming the Polyphemus species group), which is now limited to a remote drainage basin in north western Mexico (Harless and Morlock 1979). The other two species, *G. berlandieri* of south Texas and the Californian desert tortoise, *G. agassizii*, also appear to be closely related (the Agassizi group) (Osentoski and Lamb 1995). Some controversy exists surrounding the designation of new genera for these two species groups (Bramble 1982, Bour and Dubois 1984). However, Crumly (1987) has shown that the four species share a unique suite of characters not present in most other tortoises, and so for the purpose of this thesis all are considered united under the genus, *Gopherus*. *Gopherus* is considered an older assemblage (Auffenberg 1974, Crumly 1984) and shows close affinities with the extinct Eocene-Miocene genera, *Stylemus* (Ernst and Barbour 1989) and *Hesperotestudo* (Meylan and Sterrer 2000).

South American Distribution

Three species of *Geochelone* are endemic to South America. Despite its smaller size, *G. chilensis* is thought to be the closest extant relative of the giant tortoises of the Galapagos Islands, *Geochelone nigra* (Caccone et *al.* 1999a). *Geochelone chilensis* is limited to northern Argentina where it is adapted to dry lowland habitats (Ernst and Barbour 1989). The remaining two species, *G. denticulata* and *G. carbonaria*, show complex patterns of sympatry and overlapping ranges across much of their distribution in northern and central South America. Both these species are adapted to the more humid environments such as tropical evergreen and decidious forests and moist savannah (Caccone et *al.* 1999a). *Geochelone carbonaria* is also present on several West Indian Islands, however these populations are probably the result of an introduced population (Harless and Morlock 1979).

Ocean Island Distribution

Land tortoises are represented as fossils or extant species in several Ocean Islands. They appear to have the ability to disperse across marine barriers to colonise both offshore and marine Islands (Auffenberg 1974). Although poor swimmers, they float well and can survive long periods of time without water, possibly as a consequence of their ability to withstand relatively xeric conditions. Giant tortoises were once widespread on all continents except Australia and Antartica, as evidenced by the occurrence of giant fossil tortoises on the continental landmasses (Auffenberg 1974). However, since the Pleistocene they have only persisted on remote Oceanic Islands. These Islands include the Galapagos Islands, the Aldabra Atoll and Madagascar, and until very recently on the Seychelles, the Mascarenes and the Comores (Harless and Morlock 1979).

i. The Galapagos Islands

The present formation of the Galapagos Islands is a volcanic Archipelago less than five million years old (White et *al.* 1993). The unique evolutionary patterns of the flora and fauna of this remote chain of Islands have contributed significantly to Darwin's Theory of Natural Selection. In particular, the Galapagos Islands are famous for the giant tortoises that are native to them. The Galapagos tortoises, *Geochelone nigra*, survive as distinct populations in multiple localities within the Archipelago, and there are approximately 11 extant sub-species designated to this group, with an additional four represented by fossil evidence (Pritchard 1996). The Islands, being volcanic in origin, have never been connected to continental South America and tortoises probably reached the Islands by rafting, utilising the Humboldt Current, from the mainland approximately 1000km to the East (Caccone et *al.* 1999a).

ii. Aldabra Archipelago and the Seychelles

Geochelone gigantea, also referred to as *Aldabrachelys* and *Dipsochelys* (Palkovacs et *al.* 2002), were once widespread across the Islands of the Seychelles and the Aldabra Archipelago. However, since the turn of the 19th century, the effects of Island colonisations by man have severely impacted on these populations. The last remaining natural population of *Geochelone gigantea* occurs as an isolated population on the Aldabra Atoll (Palkovacs et *al.* 2002). In addition, there are several individuals maintained as captive populations in various institutions in the United States.

iii. Madagascar

The Island of Madagascar has a large percentage of endangered endemics, including four species of land tortoises. The two species of the genus *Geochelone*, *G. yniphora* and *G. radiata*, have been considered either as each others closest relatives (Auffenberg 1974), or not closely related at all (Crumly 1982). *Geochelone radiata* inhabits dry scrub and thorn scrub forests and is restricted to the extreme south and southwestern parts of the Island. *Geochelone yniphora* is an highly endangered species and is limited to fragmented patches of xeric bamboo scrub and savannah habitat around Baly Bay in western Madagascar (Caccone et *al.* 1999b). It has been estimated that there are less than 1000 individuals remaining in the wild (Curl et *al.* 1985). The other two species endemic to the Island have been placed either in monotypic genera (*Pyxis* and *Acinixys*) (Ernst and Barbour 1989), or in the same genus (*Pyxis*) (Bour 1981). Both species are listed as threatened. *Pyxis planicauda* inhabits dense, dry and tropical decidious forests in western Madagascar, while *P. arachnoides* is sympatric with *G. radiata* (Caccone et *al.* 1999b).

Current Taxonomy

Monophyly of the Family Testudinidae

The Family Testudinidae is considered a monophyletic group (Loveridge and Williams 1957). Several authors, however, have questioned this designation (Bramble 1971, Simpson 1942). This is largely a result of the complex biogeographic patterns of extant land tortoises, particularly between the New and Old World genera, and the high levels of parallelism and convergent evolution displayed within the family. These factors have prompted several researchers to question the monophyletic origin of the Family Testudinidae, and instead propose a paraphyletic or polyphyletic origin for the group (in Crumly 1984, Lamb and Lydeard 1994). A monophyletic group, using the definition advocated by Simpson (1961), "is very simply a taxon that derives its origin from any single taxonomic unit lower than itself" or "the most recent common ancestor of all those organisms and all the descendants of that common ancestor". Crumly (1984) although fully supporting the monophyletic status of the Testudinidae, suggested that it is not implausible that a wide-ranging species could independently give rise to separate evolutionary lineages, and used this theory to account for the enigmatic distribution of extant and recent fossil forms of land tortoises.

Terrestrial tortoises share several derived features with the semi-aquatic Emydidae and the Bataguridae turtles, originally placed as subfamilies within the Testudinidae (McDowell 1964). Recent systematic studies (Gaffney and Meylan 1988) suggest a closer affinity between the Testudinidae and the Bataguridae, either as sister taxa (Gaffney 1979) or a paraphyletic Bataguridae with respect to the Testudinidae, although support for this Testudinidae/ Bataguridae clade was poor (Hirayama 1985). The three sub-families were each raised to family level and assembled under the suborder Testudinoidea (Gaffney 1984, Gaffney and Meylan 1988). More recently Schaffer et *al.* (1997), using a combination of morphological and molecular data, have shown that the Testudinoidea do consist of two well-defined groups; (i) the Emydidae and (ii) a group that contains the Bataguridae and Testudinidae, for which they propose the name Testudinoidae. Within this newly defined group, there is strong support for the monophyly of the Bataguridae from the molecular data set but not from the morphology. The elucidation of the relationships within the Testudinoidea, particularly between the Testudinidae and the Bataguridae, which are still represented by conflicting hypotheses, remains uncertain and awaits further investigation (Hirayama 1985, Lamb and Lydeard 1994).

Overview of the Recent Taxonomy

The classification of the Family Testudinidae has been modified at least seven times in the last 30 years (in Crumly 1984). The frequent alterations and resulting star-like phylogeny is indicative of an inadequately understood and unresolved evolutionary history. Few, if any, of the current taxonomies have been based on taxonomically complete data sets and the anatomical systems that have been studied are comprised of characters that are prone to frequent character reversals and high levels of homoplasy even between closely associated species (Loveridge and Williams 1957, Auffenburg 1966, Crumly 1984). For example, Zug's 1966 paper on penial morphology included only three tortoise species (*G. carbonaria, G. berlandieri* and *G. polyphemus*), while a study by Petterer and Neuville in 1914 included only a single specimen of *G. gigantea* (in Crumly 1984). Loveridge and Williams (1957) used head scutellation patterns in an attempt to revise the taxonomy but concluded that they were too variable to be informative in defining the evolutionary history of the group.

The name Testudo (Linnaeus 1758) was originally used to encompass all members of the terrestrial Chelonians. During the 19th and 20th century several generic designations were introduced, particularly for the smaller African and Malagasy species, with sub-generic designations especially prominent within the genus *Geochelone* (Table 2.1). The name Testudo was discontinued and lowered to generic rank. The majority of the sub-generic assignments in *Geochelone* were later disregarded (Ernst and Barbour 1989). Subsequent morphological studies, although unresolved and often variable, seemed to converge on the assignment of supra-generic groups, for example Williams (1952) and Loveridge and Williams (1957). These groupings seemed to agree on the basal placement of the *Gopherus* clade, a *Geochelone* assemblage encompassing all the larger species (including *Manouria* and *Indotestudo*) and a clade recognising the smaller African and Malagasy species, defined by several distinct genera such as *Psammobates* and *Chersina*. More recently, several taxonomic studies have addressed the presumed monophyletic status of the *Geochelone* group that was assumed *a priori*, possibly on biogeographic evidence (Crumly 1982). There appears to be a consensus on the gradual move away from the broad concept of *Geochelone* and the recognition of most of the sub-genera at full generic rank (Bour 1982, Gerlach 2001). These studies also include hypotheses on the inter-generic relationships among the recognised genera of the land tortoises. Several of these taxonomic revisions will be discussed in some detail below and include Auffenberg (1966), Crumly (1982), Crumly (1984) and Gerlach (2001).

Taxonomic Revisions

The Family Testudinidae is characterised by clubshaped front feet and columnar hind feet with no independently moving digits (Harless and Morlock 1979). Auffenberg (1966) used the carpus of the land tortoises as a character to reassess the taxonomy of the family (Figure 2.2). The carpus shows high levels of individual variation with complex patterns of fusion of the carpal elements and in some cases complete loss of a digit, for example in *T. horsfieldii* (Auffenberg 1966). This structural variation in the degree of fusion of the carpal elements was attributed to a reduction in mobility. Despite the high levels of variation, several basic structural patterns were identified that characterised each of the major groups recognised at that time. There was limited support for affinity between the *Gopherus* and *Testudo* clades and the suggested relationships within the genus *Geochelone*, specifically between the sub-genera *Geochelone*, *Centrochelys* and *Chelonoidis*. The subsequent phylogeny agreed closely with the study of Loveridge and Williams (1957) (Table 2.1), which was based largely on shell structure. Both studies confirmed the basal status of *Gopherus* and the sub-genus *Manouria*, as well as the generic ranks afforded the derived African and Malagasy tortoises. However, elucidation of the relationships between the sub-genera of the genus *Geochelone* was poor. The main reasons for the lack of resolution included the fact that the level of homology in carpal elements has not been established for tortoises. These have not even been determined at the level of the Reptilia due to high levels of variation displayed in the early tetrapods (Romer 1956). Auffenberg further suggests that the range of specialisations represented within the group may be independently derived with major functional implications, and so few robust taxonomic conclusions could be drawn. This study was criticised by Bramble (1971) and he subsequently reinterpreted the carpal structure based on functional considerations. This re-evaluation, however, was limited to the North American genus, *Gopherus*.

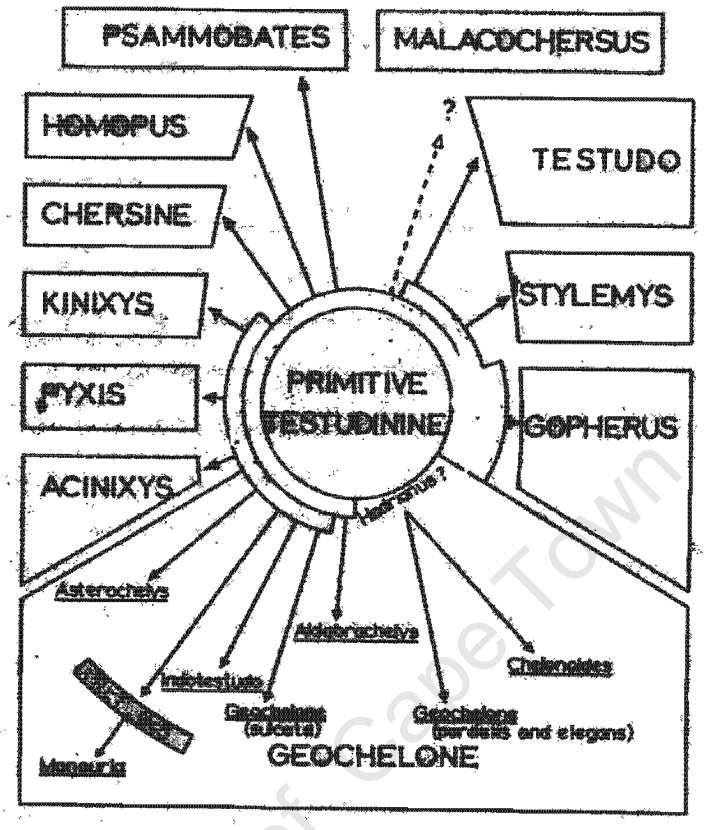


Figure 2.2: The taxonomic relationships in the land tortoises, Family Testudinidae, based on carpal architecture (modified from Auffenberg 1966)

Members of the genus *Geochelone* (sensu Auffenberg 1974), although widely distributed in the fossil record, are currently limited to Gondwanaland derivatives. Fossil species are found as far north as Moscow, South Dakota (USA) and Mongolia (Asia) (Crumly 1982). The genus exhibits large amounts of structural diversity, particularly in shell shape and size, for example the Galapagos tortoise, *Geochelone nigra*, and is found in a variety of ecological habitats (Caccone et *al.* 1999a). Following Loveridge and Williams (1957) and Auffenberg (1966, 1974), the genus was afforded a number of sub-generic ranks that included *Manouria* and the Indian representatives of the *Indotestudo*. Crumly (1982) used characters from the cranial osteology of tortoises to redefine the sub-genera allocated to the genus *Geochelone* (Table 2.1). He argued that the original descriptions of these six sub-genera were often brief or absent. Despite this, the monophyletic status of each of these was still accepted, eventhough the original designations appeared to be broadly based on biogeographic evidence and incompletely resolved cladograms (Crumly 1982). The results of this cladistic

analysis showed that relationships between the Neotropical *Geochelone* (sub-genus *Chelonoidis*), the Afro-Indian assemblage (sub-genus *Geochelone*), and the Malagasy tortoises (sub-genus *Asterochelys*) were not supported by shared derived cranial characters, and consequently there was no evidence for their assumed monophyly. He recommended that the sub-genera *Manouria* and *Indotestudo* be elevated to full generic status, and that until more conclusive evidence was presented, the remaining sub-genera within *Geochelone* be abandoned.

A subsequent study by Crumly (1984) included representatives of all extant tortoise genera and several fossil specimens, including *Stylemus* and *Hesperotestudo* from North America, which are closely affiliated with the extant *Gopherus*. The study was based on a combination of 60 morphological characters, including cranial characters, carpal elements and the carapace. The results of this study supported several of the observations from earlier studies, and culminated in a new classification for the Testudinidae (Figure 2.3). The oldest extant genus was reported to be *Manouria* and this status was supported by the presence of class II mental glands, that are present in Batagurines and other turtles, but not in other land tortoises (Winokur and Legler 1975). In addition, members of this genus lack surangular processes and show a split supracaudal scute and hexagonal neural bones, further supporting an older origin (Mlynarski 1976). *Gopherus* was also an older group with shared plesiomorphic features, including the lack of surangular processes and a broad cervical scute. The assemblage was monophyletic with the species exhibiting shared derived features including a median premaxillary ridge and a derived class I mental gland (Winokur and Legler 1975). *Gopherus* and *Manouria* were united under the sub-family Gopherinae. The remainder of the land tortoise genera were united by the absence of mental glands (sub-family Geocheloninae) and grouped into two tribes: the Geochelonini and the Testudini.

Crumly hypothesised that the next oldest group representing the land tortoises was the genus *Geochelone*, in the tribe Geochelonini, but the actual relationships among the species within this tribe remained unresolved. It was suggested that the Geochelonini might represent an intermediate form between the basal Gopherini tribe and the more derived members of the new tribe, Testudini. The tribe Testudini contained the remaining genera (except *Kinixys*), including the newly elevated *Indotestudo*, and was considered monophyletic. The genera were united by the presence of a short trachea with between 15 – 20 cartilaginous rings (Crumly 1984). The placement of *Kinixys* was contentious as the group showed several conflicting characters, complicating its assignment to either of the two tribes.

Kinixys does not share a short derived trachea with the members of the tribe Testudini, but instead has a long trachea shared with the Geochelonini and the Gopherinae. However, members of *Kinixys* do share several derived features with the genus *Indotestudo*. It had been suggested that the longer trachea that unites *Kinixys* with the Geochelonini, might actually be a secondary trait that is associated with its elongated shell elements (Williams 1950). More definitive evidence is required to elucidate the accurate placement of this genus. Crumly (1984), also showed a close relationship between *Testudo* and *Malacochersus*, and further suggested that there was insubstantial support for the perceived sister status relationship between *Testudo* and *Indotestudo*. Within the *Geochelone*, the cladograms showed little relationship between the two Malagasy Island endemics, *G. yniphora* and *G. radiata*; and no relationship between the two African endemics, *G. pardalis* and *G. sulcata*. Instead *G. sulcata* was placed in an unresolved trichotomy with two Asian endemics (*G. elegans* and *G. platynota*), with the next closest relatives being the South American assemblage, including *G. nigra*, *G. chilensis*, *G. denticulata* and *G. carbonaria*.

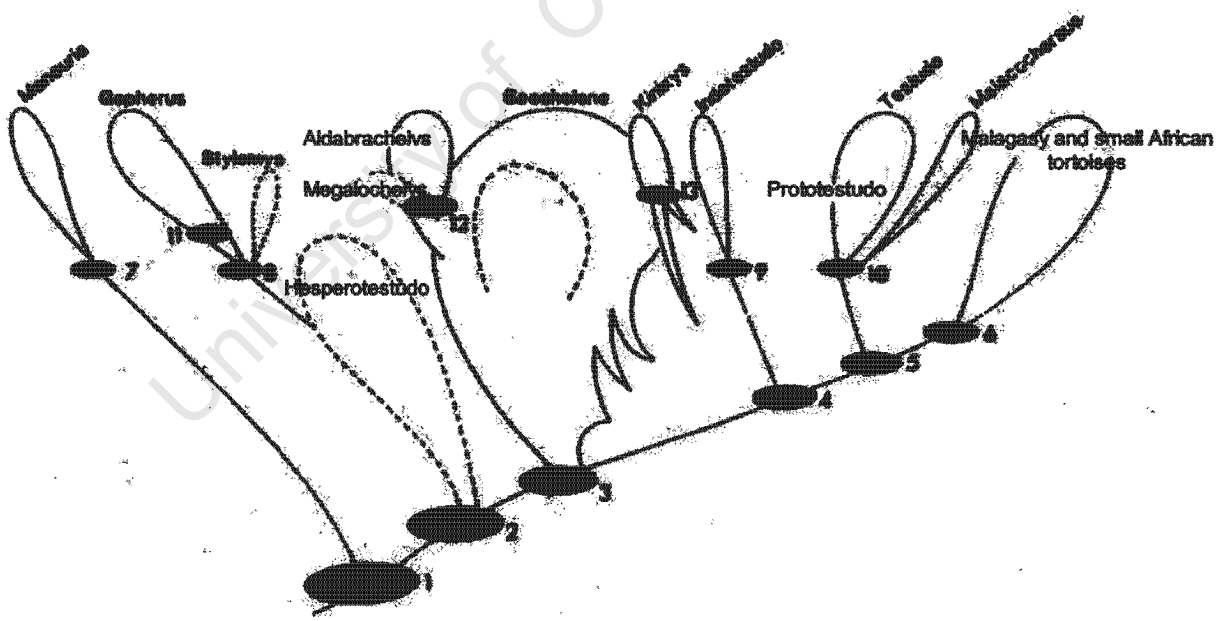


Figure 2.3: A phylogeny for the land tortoises, Family Testudinidae, based on derived morphological characters. The taxa outlined by a dotted line represent extinct genera (modified from Crumly 1984).

Following revisions by Gaffney and Meylan (1988) and Meylan and Sterrer (2000), there was widespread recognition for i) the basal placement of *Manouria*, ii) paraphyly in the genus *Geochelone* and iii) grouping of the African forms with the Malagasy species. The genera *Testudo* and *Indotestudo* were included in this larger clade. The taxonomy, however, still lacked resolution at the generic level and the specific relationships between the genera remained unresolved.

More recently Gerlach (2001), used 66 cranial osteological characters, excluding single species autapomorphies and individual or ontogenetically variable characters to revisit the taxonomy of the Testudinidae (Figure 2.4). This study represents the most taxonomically complete morphological study to date (Table 2.1). It supports the monophyly of the Testudinidae and places *Manouria* as the most basal genus in the family. Of particular interest is the suggestion that within the paraphyletic genus *"Geochelone"*[3], the majority of the previously sub-generic ranks should be raised to full generic status. The rankings are not supported by shared derived characters and in some instances the groups are united only by the lack of characters. Following this argument and taking into account the distinctiveness of other genera defined on morphological grounds (*Homopus, Chersina, Pyxis, Kinixys, Psammobates, Malac*ochersus and *Gopherus*), the recognition of several new monophyletic genera, previously included within the genus *Geochelone*, is indicated. These include *Stigmochelys (Geochelone pardalis), Dipsochelys (Geochelone gigantea), Asterochelys (Geochelone radiata and Geochelone yniphora), Chelonoidis (Geochelone denticulata, Geochelone chilensis, Geochelone carbonaria and Geochelone nigra), Cylandraspis, Centrochelys (Geochelone sulcata)* and the revised *Geochelone* represented by only two species, *G. platynota* and *G. elegans* (Table 2.1). In addition, *Testudo horsfieldii* is placed in the revised sub-genus *Agrionemys*. These suggestions agree closely with Bour (1981, 1984). Within the genus Gopherus, two sub-genera are recognised: *Gopherus sensu stricto* uniting *Gopherus polyphemus* and *Gopherus flavomarginatus*, and *Xerobates* (but see Ernst and Barbour 1989) containing *Gopherus agassizii* and *Gopherus berlandieri*. These groupings agree with previous evidence presented by Auffenberg (1976) and Bramble (1982) for two monophyletic assemblages within the *Gopherus*. The data set, however, was restricted by high amounts of variability and functional convergence in the cranial characters.

[3] "*Geochelone*" includes all sub-genera within the genus *Geochelone* as defined by Auffenberg (1974) with the exception of *Manouria* and *Indotestudo*, which have been raised to generic rank (Crumly 1982, 1984).

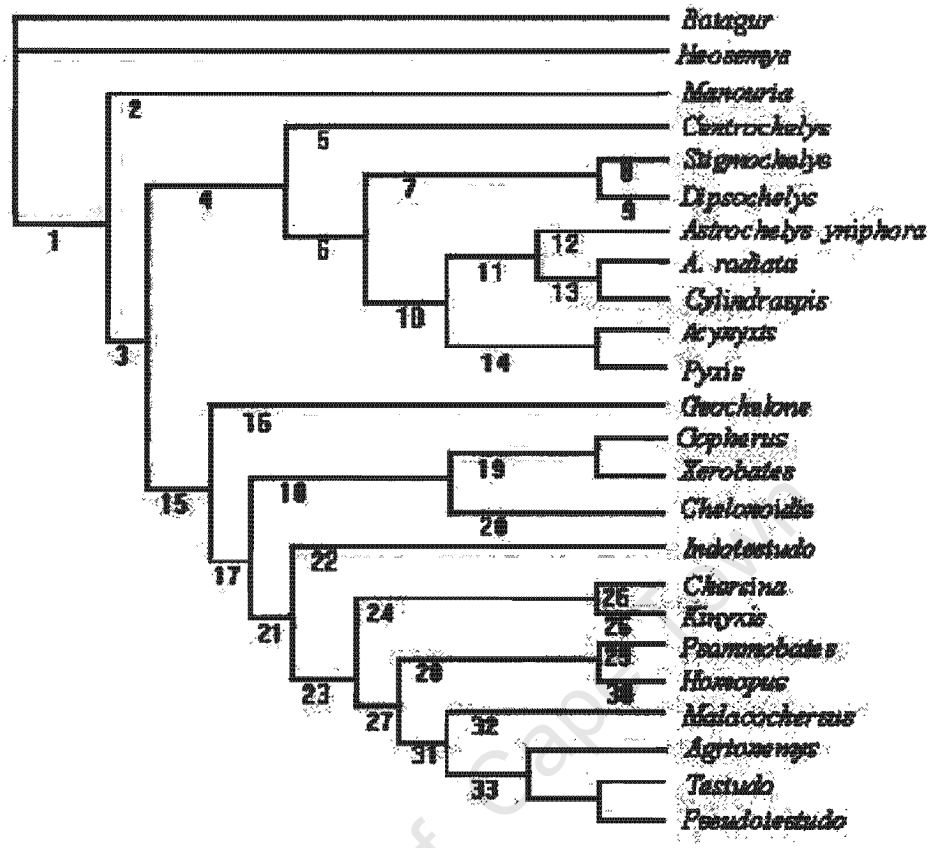


Figure 2.4: A phylogeny for the land tortoises, Family Testudinidae, based on 66 cranial characters (modified from Gerlach 2001). Based on the results of this study, it was recommended that several of the previously assigned sub-genera within the genus *Geochelone* be raised to generic level (Table 2.1).

Agreement in the Current Taxonomic Literature

Despite the extensive reclassifications of the taxonomic status of the Family Testudinidae over the last century, the phylogeny remains inadequately resolved. There is poor resolution within some of the genera, with various authors citing widely different interpretations of what species constitute these assemblages and at what level generic and sub-generic designations should be applied. Consequently, few, if any theories regarding the evolutionary history of the family can be proposed with any degree of confidence. However, within all the recent classifications there does appear to be some consensus relating to particular aspects of the phylogeny. These include:

1. The monophyly of the Family Testudinidae
2. The generic status of *Manouria* and *Indotestudo*
3. The basal placement of the genera *Manouria* and *Gopherus*
4. The presence of two distinct assemblages within the genus *Gopherus*
5. The paraphyletic status of the genus *Geochelone*
6. The presence of a derived clade containing the smaller African forms (except *Geochelone* (*Stigmochelys*) *pardalis*), the smaller Malagasy forms, as well as *Testudo* spp. and the newly elevated *Indotestudo*. These forms differ in their actual arrangements in the respective phylogenies and show higher levels of character reversal than is present in the other clades (Gaffney and Meylan 1988, Meylan and Sterrer 2000)
7. *Testudo* spp. and *Malacochersus* spp. appear to be closely related and share similar head scutellation patterns and basicranial morphology
8. *Testudo* does not appear to be the sister group to *Indotestudo*.

Limitations of the Current Taxonomies

It is evident that there are relatively few morphological characters that are informative enough for the elucidation of the phylogenetic relationships among the Testudinidae. Those that are available tend to be variable and often conflicting due to high levels of homoplasy. This morphological limitation is not a feature specific to the land tortoises and is becoming increasingly recognised as a general limitation in recent literature, especially since the introduction of alternative character sources for the inference of phylogenies, for example from molecular data. There has been significant conflict reported between the molecular and morphological data in the Family Iguanidae (Wiens and Hollingsworth 2000) and in the lizard family, Xantusiidae (Hedges et *al.* 1991), amongst others (Harris and Arnold 2000, Yu et *al.* 2000). A major source of this homoplasy is parallel evolution, where similar adaptive traits

evolve independently in two or more groups (Farris et *al.* 1982, Pearse and Pogson 2000). For example the hemi-penis in lizards has evolved independently on more than one occasion (Arnold 1986), whilst the phylogeny of the spiny lizard, *Sceloporus jarrovii*, has been complicated by the independent and parallel evolution of distinctive colour morphs (Wiens et *al.* 1999).

Morphologically, individual tortoises are very variable, for example the high levels of within population variation that exists in *G. chilensis* and *P. tentorius* (Harless and Morlock 1979, Boycott and Bourquin 1988). It is often difficult to assess the strength of many of the supposed synapomorphies within the group, which makes the assignment of overall affinities within the family weak. An increase in the number of pleisiomorphies and a concurrent decrease in the number of synapomorphies, due to evolutionary convergence, will lead to incorrect clustering of the groups and therefore incorrect inferences (Alvarez et *al.* 1999). In all studies done thus far, the levels of resolution attained for the phylogenetic relationships within the Testudinidae have been limited by the high levels of parallelism and convergence that characterise the group (Loveridge and Williams 1957; Auffenberg 1966, Crumly 1982, 1984; Gaffney and Meylan 1988 and Gerlach 2001). Reasons for this remain unclear although Auffenberg summarised potential factors in his 1974 review. These include the possibility that the groups are all closely related and assignments are often based on the lack of characters (see Emerson et *al.* 2000). It is also difficult to distinguish between convergent characters and those that represent natural groups, and this problem is further amplified by the fact that several of these groupings display various combinations of plesiomorphic features. This particular problem has also been shown to confound the inference of phylogenies for groups of lizards (Melado and Olmedo 1990) and fruit bats (Alvarez et *al.* 1999).

The magnitude of the problem was clearly illustrated in Crumly's 1984 study, where all the cladograms produced in the analysis were characterised by low consistency index scores (<0.5), and 25 out of the 57 characters employed in the study had C-ratios lower than 0.5. Despite the fact that these high levels of homoplasy are a problem when attempting to infer the evolutionary history within the Testudinidae, there has been limited effort to try and identify the phylogenetic level at which they occur. Until this problem is addressed in detail, phylogenies based on the morphology of the Testudinidae will not be efficiently resolved.

Fossil Record and Evolutionary Trends in the Testudinidae

The fossil record is an important aspect in the inference of the evolutionary history of a group. For example, fossil representatives may provide a practical root for phylogenetic analysis, enabling character polarities to be determined with a greater degree of confidence, or at least provide a more reliable morphological starting point (Smith 1997). Schaffer et *al.* (1997) included fossil representatives in their study to resolve the relationships between the Cryptodiran turtles. Although the inclusion of the fossil taxa did not dramatically alter tree topology, they did have a significant effect on the bootstrap proportions associated with the deeper level nodes. Fossils may aid in the elucidation of conflicting phylogenies by introducing unique sets of character combinations that may not be present in living species. These can i) redefine theories of character evolution and ii) clarify or allow for the reinterpretation of the sequence of character changes in complex morphologies (Novacek 1994, Smith 1997). However, one of the distinct disadvantages of incorporating fossils into taxonomies is that they invariably represent incompletely preserved organisms (Benton 1995)

The question of how representative the fossil record is of the evolutionary process and how thoroughly it is sampled is also open to debate (Benton 1995, 2001). For example, it has been suggested that the distribution of sedimentary rock may influence the preservation of fossils and therefore some organisms, or parts thereof, may be preferentially preserved over others (Benton 2001). This may result in several characters and even significant lineages being unavailable for analysis, which may contribute to a poorly resolved phylogeny (Benton 1995).

An example is the conflicting hypotheses to explain the evolution of Neornithe birds. The alternative theories of the evolution of the order are based on two bodies of evidence. On the one hand, paleontological evidence suggests that the birds diversified in Laurasia and had achieved worldwide distribution by the early Tertiary (Feduccia 1996). On the other hand, molecular evidence suggests an earlier Cretaceous origin for most avian orders (Cracraft 1973, 2001). The majority of the evidence to support the paleontological data is a result of the large amount of research that is carried out in North America and Eurasia (Cooper and Penny 1997) compared to limited research conducted in the southern continents, that could support an alternative hypothesis.

The Family Testudinidae have a diverse fossil record that indicates that they were more widespread in the past, occurring in northern Europe and as far north as southern Canada (Ernst and Barbour 1989). Despite the large number of fossil fragments available, many of the characters necessary for the accurate identification of the known fossil tortoises are not available for study. For example, many lack girdles and limbs and the skulls are often absent (Auffenberg 1974). For this reason, many of the larger fossils have been identified as belonging to the genus *Geochelone*, particularly sub-genus *Manouria*, although this may not be an accurate representation of the diversity of genera present. Fragmentary remains which have been assigned to the genus *Manouria* may in fact only be large, poorly defined fragments from Batagurid turtles to which they are closely affiliated, whilst *Indotestudo* fragments are equally poorly defined (Gerlach 2001). In addition, the paucity of smaller fossil specimens available for the family may be due to a bias either in the collection or preservation of the smaller fragments (Auffenberg 1974, Meylan and Auffenberg 1986). For this reason the evolutionary relationships between the extinct members of the genus and consequently the living members remains clouded.

The earliest Testudinid fossils date from the early Eocene of Asia, Europe and North America, although it is anticipated, particularly in light of the molecular evidence based on divergence estimates presented in this study, that members dating from the Upper Cretaceous could eventually be found (Auffenberg 1974). These early fossil specimens seem to show affiliation with the extant *Manouria* from Southeast Asia (Auffenberg 1974). For example, *Geochelone* (*Manouria*) *majusculus* from New Mexico deposits is currently the oldest known fossil, whilst another early Eocene representative is *Testudo (?) comptoni* from the Isle of Sheppey, England (Ernst and Barbour 1989). By the end of the Eocene several other species had evolved, including *Geochelone* (*Manouria) gilmorei* and *Stylemus uintensis* (closely affiliated to the extant *Gopherus*) in North America, along with the appearance of several *Testudo* species in Europe and the genus *Indotestudo* in Asia (Mongolia) (Ernst and Barbour 1989). *Manouria* is recognised as the oldest genus in the Family (Crumly 1984), although its distribution is currently restricted to Asia. However, there are a large number of *Manouria* fossils in North America, and the diversity of the group in both Asia and North America makes it difficult to determine a definitive centre of origin for the group and, as a result, for the Testudinidae as a whole (Crumly 1984).

By the middle Eocene there were several lineages that had differentiated in Laurasia and North America and it is from this stock that there appears to have been several invasions into

Africa between the Eocene and Miocene (de Lapparent de Broin 2000). It is the opinion of this author, based on information presented in this thesis, that there were subsequent dispersal events from North Africa to both India and Southeast Asia as well as to South America, and from there to the Pacific Islands.

Although, currently limited to the tropical and sub-tropical lattitudes, fossil representatives of the Testudinidae have been found as far north as Moscow and Mongolia, implying historically temperate climates for these regions (Figure 2.5). The range of the terrestrial tortoises seems to have been restricting since the Quaternary at least and this could be a result of the effects of the Pleistocene glaciation events (Auffenberg 1974). The interglacials, however, were characterised by periods of warming and it would appear from the fossil evidence that the northern ranges of the testudinids have been moving progressively further south with each of these events (Auffenberg 1974, Figure 2.5). These colder occurrences, in the northern lattitudes in particular, could explain the extinction of the giant land tortoises that are currently restricted to Ocean Islands, for example the Galapagos Islands and the Seychelles. Smaller species are known to burrow (Brattstrom 1961, Crumly 1984) and so could potentially escape these unfavorable conditions. Climate change does not fully explain the extinction of the giant landforms in the more temperate areas, for example on the African continent. On the Ocean Islands the extinction events have resulted from habitat modification and the introduction of predators since the colonisation by man in the late 18th century (Harless and Morlock 1979).

Crumly (1984) hypothesised a phyletic size decrease within the family, whereby the more primitive forest dwellers, for example *Manouria*, are represented by larger species whilst the more derived genera, including many of the southern African forms, are represented by the smaller, more arid adapted species, for example *Homopus* and *Psammobates*. The reasons for this remain unclear. There is, however, some evidence presented for major evolutionary changes in the Family that appear to be associated with the development of more xeric plant communities, and as a result with the more derived members of the family (Auffenberg 1974, Crumly 1982, 1984). These include morphological adaptations, the majority of which are associated with grazing in semi-arid and sub-tropical grasslands (reviewed in Auffenberg 1974). These evolving niche requirements could have favored the smaller species and could explain the high levels of diversity and endemism of tortoises on the African continent, particularly in southern Africa.

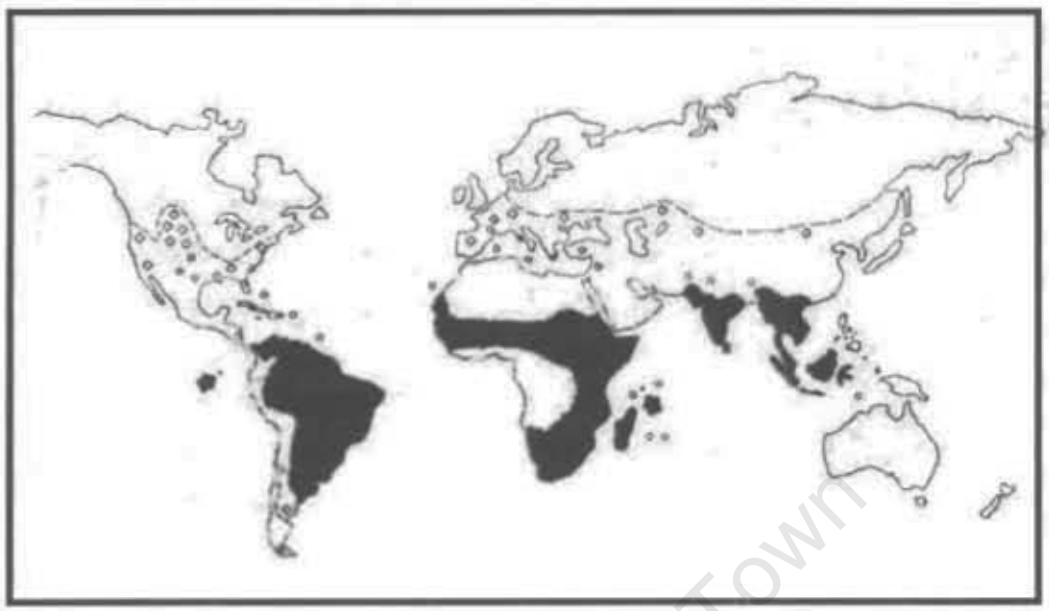


Figure 2.5: A map showing the historical and current distribution of the genus *Geochelone* (sensu Auffenberg 1974) and the reduction of its range with time. Diamonds represent the northern and southern limits in the Oligocene-Miocene, circles represent the Pliocene-Pleistocene limits, whilst the sold black and arrows represent the recent distribution (modified from Auffenberg 1974).

Research Aims

During the past decade, there has been a substantial increase in the number of molecular studies involving the Testudinidae, dealing with both the fossil and extant members of the family (Lamb and Lydeard 1994, Caccone et *al.* 1999a, 1999b, Austin and Arnold 2001, Palkovacs et *al.* 2002, van der Kuyl et *al.* 2002). However, these studies have largely concentrated on the Ocean Island endemics and the North American genus, *Gopherus*, and have been limited to a few taxa from each of these biogeographic assemblages of interest. In spite of the above mentioned limitations, these studies have contributed significantly to understanding the molecular mechanisms underlying the evolution of testudinid DNA, for example the decreased rate of mitochondrial DNA evolution that appears to characterise the group (Avise et *al.* 1992).

However, there have been no molecular studies that have encompassed all the extant genera in the Testudinidae, in an attempt to elucidate the phylogenetic and evolutionary relationships among members belonging to this family. Thus a major aim of this study is to address the complicated taxonomy of this diverse family of reptiles in order to gain insight into their enigmatic biogeographic distribution, which is thought to have involved several dispersal events, including rafting (Williams 1950). Previous hypotheses of evolution in the Testudinidae, particularly at the inter-generic level and the subsequent geographic distribution of the group have been limited as they are based on inadequately resolved morphological phylogenies.

With regards to this study, the phylogenetic positions of the monotypic Ethiopian endemics *Malacochersus* and *Chersina* are of particular interest, as well as the supposed sister relationship between the *Testudo* and the *Indotestudo* genera. The phyletic position of the *Geochelone* (*Stigmochelys*) *pardalis* species within the genus *Geochelone* is assessed, along with the proposed assignment of generic designations to previously recognised sub-genera within *"Geochelone"*. In addition, the high levels of variation within the southern African region, particularly in the *Psammobates* group and the *Homopus* group, with the potential revival of the genus *Chersobius* within the *Homopus*, will be addressed (Table 2.1).

All recognised extant species from the 11 recognised genera were represented in the study. Two mitochondrial genes were selected for analysis and included the Cytochrome *b* gene (Cyt *b*) and the Nicotinamide Adenine Dinucleotide Dehydrogenase subunit 4 gene (ND4). The phylogenetic utility of these genes in recovering a robust phylogeny for the Family

Testudinidae, which is characterised by an ancient but rapid radiation, particularly in the Southern Hemisphere, is assessed.

CHAPTER 3

THE THEORY OF PHYLOGENETIC INFERENCE

Introduction

As discussed in the earlier chapter, most of the current literature on tortoise systematics is based on morphological studies. Although these studies offer insight into the relationships among the extant genera of the Testudinidae, a more resolved phylogeny is required for the accurate elucidation of the taxonomy within the group. With the advent of the polymerase chain reaction (PCR) and the automation of DNA sequencing, molecular data in the form of DNA sequences are readily accessible for most groups under study. Molecular data offers an alternative supplement to morphological data for the inference of the historical or evolutionary relationships amongst lineages or groups of organisms. It is important to bear in mind that the information contained within a molecular data set is itself incomplete. The sequences under analysis are the end result of various evolutionary and biological processes that have occurred over time at a particular locus, and about which one has to make inferences or assumptions to extract the maximum amount of information. The selection of one or more best fit trees or estimates of phylogeny from amongst a large set of possibilities within the tree space requires the definition of criteria which can assess how well the data fits the candidate trees (Swofford et *al.* 1996). These criteria are increasingly based on models of evolutionary change and on implicit or explicit assumptions regarding the evolutionary process, which allow alternative phylogenies to be assessed and compared. They are referred to as optimality criteria and are described by an objective function i.e. a mathematical model that allows for the evaluation of a given tree (Swofford et *al.* 1996). Algorithms are used to compute the value of the objective function and for selecting trees that have the best value or score according to the selected criterion, given a model of evolution, topology, specific branch lengths and the observed data (Swofford et *al.* 1996).

The main optimality criteria include Parsimony (Fitch 1971), Distance methods (based on models of evolutionary change) and Maximum likelihood (Felsenstein 1981). Distance methods are based on a matrix of overall pairwise distance data, while parsimony and maximum likelihood are based on discrete character data. This means that they can be used to estimate ancestral character states as well as estimating tree topologies. Apart from the differences in the type of character data that they utilise, the methods also differ in the extent

to which they make assumptions about the evolutionary process and the models of evolution that they incorporate. Distance and maximum likelihood approaches are considered model-based as they make explicit assumptions about the process of evolution, while advocates of the parsimony method argue that the strength of this method lies in the fact that it does not rely on such assumptions (Farris et *al.* 1970, Farris 1973, Sober 1988).

Sequence Evolution

All phylogenetic analysis, whether molecular or morphological in nature, begins with the presumption that the characters being compared represent homologous states (Hennig 1966, Swofford et *al.* 1996) i.e. homology is assumed *a priori* and only the *a posteriori* determination of homoplasy will refute the hypothesis. Once alignment and homology have been presumed for the sequences under study, optimality criterion are selected and, in combination with tree building algorithms, can be used to infer a best estimate gene phylogeny. Gene phylogenies, in turn, may be accurate representations of a species or organismal phylogeny if the substitutions or changes within a particular gene sequence represent orthologous steps in the evolutionary history of a group of organisms (Li and Graur 1991).

When comparing DNA sequences for the purpose of phylogenetic inference, the similarities or differences within the sequences, and the processes by which these differences arose, are of particular importance. As sequences diverge from a common ancestor, the number of nucleotide changes between them will increase. These changes will define the evolutionary relationships that can be inferred from the phylogenetic tree. However, as divergence times increase, the potential for a substitution to occur at a site that has already undergone a change also increases. These unobserved substitutions or changes are called multiple hits. The number of multiple hits, in the form of parallelisms, convergence and superimposed changes, can become so numerous that the phylogenetic signal uniting the taxa becomes obscured. This can obscure the actual number of evolutionary events or changes that have occurred at a site, resulting in the inference of an incorrect or misleading phylogeny. There are, however, a variety of methods available to correct for these superimposed changes. They invoke a model of evolution to describe or explain the evolutionary processes that generate the observed data, and are incorporated into the optimality criterion.

Models of DNA Substitution and Evolution

In phylogenetic analysis, models of evolution or sets of assumptions about the process of nucleotide substitution are required to describe the probabilities of change from one nucleotide to another. The ultimate aim, particularly of model based phylogenetic approaches, is to correct for superimposed or unseen changes along the branches of an evolutionary tree (Felsenstein 1981). These models are becoming increasingly realistic as knowledge about the mechanism of DNA mutation and evolution increases. Inferences regarding the realistic description of models of DNA mutation, or evolution, are based on a parametric approach. This means that the variables that constitute the model and therefore represent features of the evolutionary process are not known *a priori*. Instead, each set of parameters or variables (representing the average rates of the occurrence of all possible substitutions) can be estimated or modeled directly from the sequence data under analysis, using a stochastic or mathematically modeled Markov Process (Lio and Goldman 1998), for example the Jukes-Cantor (JC) (1969) model. In Markov Chain models, the future state of a character depends only on it's current state and not on the history of how that state was produced. The models assume that the substitutions on a phylogenetic tree follow a Poisson distribution and can therefore produce a substitution matrix that is able to specify the instantaneous rate of change from one character state to another (Lio and Goldman 1998). Once the rates of change amongst different nucleotides have been specified, the probability of change from one nucleotide to another, or the probability of remaining the same, can be calculated over evolutionary time and for any site (Whelan et *al.* 2001).

There are three main parameters or variables incorporated into the development of rate matrices for defining a specified model of evolution:

i. Base Frequency Parameters

The base frequency parameter describes the frequency of each of the nucleotide bases A, G, C and T averaged over all the sequences. It was originally assumed that the genome contained equal proportions of A+T and G+C, but this has long since been shown to be incorrect (Sueoka 1964). Base composition has been shown to vary greatly between levels of taxonomical hierarchy as well as across different parts of the genome (Mooers and Holmes 2000). These differences, thought to be caused by selection or directional mutation pressures as a result of misincorporation errors during DNA repair (Sueoka 1992), result in compositional bias that can make the inference of evolutionary relationships difficult. This is particularly a problem if incorrect assumptions about base composition are made and

incorporated into models of evolution. In the vertebrates, G+C content ranges from 37% in the tuna, *Thunnus thunnus*, to 50% in the lamprey, *Lampetra fluviatilis*, (Jabbari et *al.* 1997). Birds and mammals have been shown to have higher G+C contents than poikilothermic vertebrates (Bernardi et *al.* 1997), while the proportion of G+C in bacteria ranges from 25% in a species of *Mycoplasma*, to over 75% in a gram-positive species, *Micrococcus luteus* (Li 1997). All mitochondrial genomes sequenced to date have been characterised by relatively low G+C contents with respect to the rest of the genome (in Mooers and Holmes 2000). This range of differences in the frequencies of the nucleotides, at both the taxonomic level and the gene level, may affect the rate of change from one nucleotide to another, and so should be taken into consideration in studies of phylogenetic inference.

The overall base composition for a set of DNA sequences can be represented by a vector F, where:

$$F = \{f_A\ f_C\ f_G\ f_T\}$$

and f_A is the equilibrium frequency of the nucleotide A. If the bases are present in equal proportions then $f_A = f_C = f_G = f_T$. Alternatively, the model can allow for differences in each of the relative base frequencies by adjusting the vector F (Page and Holmes 1998).

ii. Substitution Parameters

There are six possible substitution types among the four nucleotides, and the rate at which they occur describes the base substitution parameters (Figure 3.1). This rate may be dependent on the overall frequency of the base composition of the DNA (Lio and Goldman 1998).

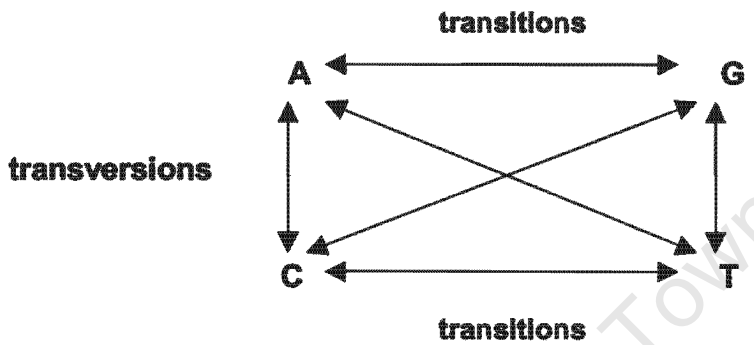


Figure 3.1: Diagrammatic representation of the possible substitution types (transitions and transversions) among the four nucleotides in DNA sequence data.

Transition substitutions (purine/purine A/G, and pyrimidine/pyrimidine C/T) usually occur with a higher frequency than transversion substitutions (purine/pyrimidine) in DNA (Brown and Simpson 1982). Within models of evolution, transitions are assigned a weight, *k*, relative to a rate of one for transversions, and *k* is usually significantly greater than one (Lio and Goldman 1998).

The probability of a nucleotide substitution is given by the matrix. P_t, where:

$$P_t = \begin{pmatrix} P_{AA} & P_{AC} & P_{AG} & P_{AT} \\ P_{CA} & P_{CC} & P_{CG} & P_{CT} \\ P_{GA} & P_{GC} & P_{GG} & P_{GT} \\ P_{TA} & P_{TC} & P_{TG} & P_{TT} \end{pmatrix}$$

where P_{AC} is the probability that a site with nucleotide A changed to the nucleotide C at the end of time interval, t (Page and Holmes 1998). The matrix specifies the substitution rates for all possible substitution types as represented by Figure 3.1.

The simplest evolutionary model incorporating the above two parameters is the Jukes and Cantor model (1969). This model was one of the first to be proposed and assumes that the frequencies of all the nucleotides in the sequences are equal and that all the substitution types are equally likely. However, since the implementation of this model several improvements have been made which reflect the biological process more closely and thereby provide more realistic assumptions about the evolutionary process. These include the Felsenstein (1981) model, which allows for unequal base frequencies, and the Kimura (1980) model, which allows for differences in the rate of change of nucleotides classes, such that transitions occur at a different rate to transversions. The model proposed by Hasegawa et *al.* (1985) incorporates the assumptions of the Felsenstein and Kimura models by allowing transitions and transversions to occur at different rates and simultaneously allowing for variation in the base frequencies.

This trend of increasing model complexity, by allowing more parameters to vary, culminated in the development of the General Time Reversible Model (GTR) (Rodrigues et *al.* 1990). This model allows for unequal base frequencies and allows for each of the six possible substitution types to occur at different rates. Most of the earlier models can therefore be assumed to be special or limited cases of the GTR model. Most models assume reversibility of the rate matrix (Yang 1994a), such that observing an A to T change in one sequence has the same probability as observing the T to A change. However, it is possible to make inferences about the direction of this evolution, or alternatively constrain evolution, if information particular to a set of sequences exists, for example information from the fossil record (Page and Holmes 1998).

iii. Site Rate Heterogeneity Parameters

a. The Gamma Distribution (Γ)

Substitution rates have been shown to vary between lineages and between sites within a gene (Fitch and Margoliash 1967). Variable rates of substitution at sites can have considerable impact on the determination of an accurate sequence divergence estimate. The most obvious solution is to model each site according to its own rate parameter, for example, in a sequence with k sites there would be k rate parameters. However, the disadvantage of this approach is that there would be many more parameters necessarily incorporated into the substitution model, which results in increased uncertainty in the estimate and increased computing time (Page and Holmes 1998). One approach to overcome this potential problem is to assume that the distribution of the relative rates across the sites (with four rate

categories) approximates a gamma distribution (Yang 1993, Yang 1994b), with a shape parameter defined by α, which determines the extent of the heterogeneity. The gamma density function can take on two qualitatively distinct shapes depending on the value of α and ranges from zero to infinity. In an L-shaped distribution, most of the sites in the sequence will have low rates of change but a few are represented by higher rates. Large values of α, on the other hand, are represented by a bell shaped curve and define low rates of variance (where variance is $1/\alpha$)(Lewis 2001), or similar rates of substitution at the majority of the sites. Estimates of α from nuclear and mitochondrial genomes range from 0.16 to 1.37 (Page and Holmes 1998). The advantage of assuming a discrete version of the gamma distribution is that it requires the addition of only a single parameter to the substitution model. Another method for describing rate heterogeneity includes assigning specific rates of substitution to different parts of the gene or to domains, for example, assigning rates to the three different codon positions of protein coding sequences (Voelker and Edwards 1998). Rate heterogeneity has also been shown to be affected by taxon sampling in the data set and may affect the inference of the phylogeny, as more data is added (Sullivan 1996).

b. Proportion Of Invariable Sites (*Pinv*)

Another method for dealing with among site rate heterogeneity is the invariable sites model, in which a proportion of the sites (*Pinv*) is assumed to be completely resistant to change (Hasegawa et *al.* 1985, Swofford 1999).

Combining these two models (Γ and *Pinv*), such that some sites are assumed invariable and rates at the remaining sites follow a gamma distribution, has been shown to significantly improve the fit of the data (Yang 1994b, Yang 1996a, Rogers 2001). The incorporation of these rate heterogeneity parameters allows for increased accuracy in the estimation of branch lengths in phylogenetic estimates (Gu et *al.* 1995), as well as greater consistency over a range of varying conditions (Huelsenbeck 1995). They can also be incorporated into any one of the Poisson Process Models discussed previously, for example GTR+Γ+*Pinv*. This model represents the most complex of the evolutionary models and has been shown to be of particular importance in the analysis of large data sets (Yang 1994a). The inclusion of all or a combination of these model parameters, specific to the data set, allows for improved accuracy in the estimation of reliable phylogenies (Yang 1996b).

Methods of Phylogenetic Inference

Optimality Criterion I: Model Based Methods

All of the models describing sequence evolution can be used to estimate a phylogenetic tree that represents the best fit to the observed data. The probability of achieving this best fit estimate depends on the method of phylogenetic inference selected. This should aim to: (i) maximise the amount of information extracted from the data, (ii) allow for the realistic determination of the parameters incorporated into the optimality criterion and (iii) select an algorithm that will maximise the efficient searching of the available tree space (Whelan et *al.* 2001). One of the criticisms of model-based approaches of inference (distance, maximum likelihood and bayesian) is that they require too many assumptions to be made about the evolutionary process. However, the quality of the estimation will ultimately depend on the quality of those assumptions and so should be based on what is known or can be inferred about the sequences or biological systems under study. Model based methods can, therefore, be flexible or restrictive depending on the assumptions being made and on the number of fixed and estimated parameters being incorporated (Soltis et *al.* 1998). For example adding a *k* ratio (transition: transversion ratio) to the model can account and subsequently correct for a bias that may otherwise have been introduced should a difference in these relative rates be present in the data set (Soltis et *al.* 1998). However, if this ratio is calculated incorrectly it will affect the quality of the best estimate of phylogeny, and may even result in the selection of the incorrect tree topology. The models should therefore include only those parameters that are required for defining the most important features of the evolutionary process being modeled.

i. Distance Methods

Distance methods use similar models of evolution as those implemented in a maximum likelihood framework of the maximum likelihood method, and are thought to approximate a full maximum likelihood approach under certain conditions (Swofford et *al.* 1996). Distance based methods calculate pairwise evolutionary distances between all sequences in a given data set and a pairwise distance matrix is constructed. Correction factors that can account for multiple hits, or superimposed changes, can then be incorporated to produce a "corrected" distance matrix. For example, correction factors based on the Jukes-Cantor Model (1969) of evolution can be incorporated into the equation used to calculate the observed dissimilarities between the pairs of aligned sequences. The value of this included variable is determined by the parameters in the particular model of evolution invoked for a specified set of aligned sequences. The model chosen, and the correction factor

subsequently included in the calculation of the corrected distance matrices, becomes increasingly important as the evolutionary distance between the sequences increases (Nei 1987). The corrected distance matrix is considered the new data set and can be used to estimate a phylogeny using several criteria, including Least Squares (Cavalli-Sforza and Edwards 1967) and Minimum Evolution (Rzhetsky and Nei 1992). In the Minimum Evolution approach, the best estimate of phylogeny is the tree with the lowest value for the sum of branch lengths. In the least squares method, the tree that shows the least difference when comparing the original distance matrix and the pairwise distances obtained when summing branch lengths across the tree, is considered the best estimate (Swofford et *al.* 1996).

However, the major disadvantage of the distance approach is that there is a loss of evolutionary information when the discrete sequence data is converted into a distance matrix (Steel 1988). The method is not able to efficiently deal with models that contain parameters for which the values are not known *a priori*, for example, *k* (Whelan et *al.* 2001).

While distance methods use models of evolution to create corrected distance matrices for the pairwise comparisons of sequences, maximum likelihood and bayesian approaches, will at some point during the estimation procedure, use these models to calculate the probability of observing the aligned sequences given the model of substitution invoked.

ii. Maximum Likelihood

The theory of likelihood is credited to the English statistician R.A Fisher, who first described it in 1922 (Page and Holmes 1998). Its specific application, within a phylogenetic context, was developed by Edwards and Cavalli-Sforza (1964) and Felsenstein (1981). The principal of likelihood states that the explanation that makes the observed data the most probable, or the most likely, is the one that should be selected. In a phylogenetic framework, maximum likelihood is described by the following function:

Like(Tree) = Pr[Data I Tree and Parameters] (modified from Huelsenbeck 2000a)

where Like(Tree) is the likelihood value for a specified tree, and Pr[Data I Tree and Parameters] is the probability of the observed data given a tree and a specific model of evolution represented by model parameters (Huelsenbeck 2000a). It is important to note that both branch lengths and topology specify the tree.

The tree that maximises this probability function is considered the best estimate of the phylogeny and is typically referred to as the maximum likelihood estimate (MLE). The exact probability of change for each nucleotide along a given branch of a particular tree is calculated and depends on the length of the branch and a specified model of evolution (Felsenstein 1981). All observations, therefore, contribute to the final likelihood score and it has been shown that increasing the number of characters available for analysis, increases the accuracy and consistency of phylogenetic estimates (Mitchell et *al.* 2000). The use of all available data is one of the strengths of the method, in contrast to parsimony where certain character states, for example autapomorphies, are ignored.

Another advantage of the maximum likelihood method of inference is that it incorporates explicit models of sequence evolution and allows for the robust statistical testing of evolutionary hypotheses. It permits the use of complex models of evolution, and allows for the estimation of model parameters during the course of the inference process (Lio and Goldman 1998). Maximum likelihood estimates are phylogenetically consistent i.e. given enough data and a realistic evolutionary hypothesis will converge on the correct topology (Chang 1996, Rogers 1997, Siddall 1998, Farris 1999, Sullivan and Swofford 2001). The exact parameter estimates have also been shown to be robust to violations in the assumed models of evolution. For example, Sullivan and Swofford (2001) have shown that although the incorporation of rate variation among sites significantly improves the fit of the data, it is

not required that these parameters be modeled precisely to obtain an optimum inference (but see Buckley et *al.* 2001b).

An inherent disadvantage, however, lies in the fact that maximum likelihood is computationally intensive, particularly when analysing large data sets. It is not plausible to estimate every potential tree in the tree space and so heuristic searches have to be used which do not guarantee selection of the optimal tree topology (Swofford et *al.* 1996).

iii. Bayesian Analysis

One of the criticisms of parsimony, maximum likelihood and distance methods of phylogenetic inference is that they only provide point estimates of the phylogeny in the form of a single "best" inference or tree. There are very few methods available, which allow for the robust statistical testing of the data, or give some estimate of the significance, or a direct estimate of the confidence in the selected tree (Buckley et *al.* 2002). Non-parametric bootstrapping, for example, is a method that is widely used and gives some indication of the level of sampling error for the phylogenetic estimate by random resampling of the original data set (Felsenstein 1985, Newton 1996). Despite its widespread use, the method is not always reliable and there is some confusion as to the actual interpretation of the bootstrap values produced (Felsenstein and Kishino 1993, Hillis and Bull 1993). More recently, methods have been developed that allow for more exact inference of phylogenies and address some of the above mentioned criticisms. An example of one of these methods is bayesian analysis (Rannala and Yang 1996, Yang and Rannala 1997).

Bayesian analysis is a statistical inference method based on models originally developed by Cavalli-Sforza and Edwards (1967), to model the process of genetic drift and model branching patterns in human populations, using gene frequency data. The model was later modified by Felsenstein (1973, 1981) to incorporate discrete character data and model character evolution by Markov Model Process that culminated in the development of maximum likelihood analysis. Although bayesian analysis uses likelihood models as a framework, where the maximum likelihood function is described as the probability of observing the data given the tree and a model of evolution, bayesian analysis is essentially the posterior probability of the tree, or hypothesis, given the observed data. The posterior probability of the tree is based on its joint probabilities (conditional and prior probabilities), branch lengths and a specified model of evolution. The tree with the largest posterior probability is then selected as the best estimate of the phylogeny (Rannala and Yang 1996). The best estimate is found by sampling the posterior probability distribution i.e. the tree

space with minimal computational time. The major advantages of the method include computational efficiency and speed if an MCMC is used, and easy interpretation of the results. The posterior probability also provides some estimate of the confidence that can be placed in the phylogenetic estimate, or the probability that the estimated tree is the true tree under the specified model of evolution (Rannala and Yang 1996). Bayesian analysis is becoming increasingly popular, particularly in the analysis of large data sets and for the purposes of rigorous hypothesis testing (Thorne et *al.* 1998, Huelsenbeck et *al.* 2000b, Huelsenbeck et *al.* 2000c, Korber et *al.* 2000, Succhard et *al.* 2001, Buckley et *al.* 2002, Polkavacs et *al.* 2002, Whittingham et *al.* 2002).

Bayesian analysis can be described by the following function, which for six or less taxa approximates Bayes formula (Cavalli-Sforza and Edwards, 1967):

$$Pr[\text{Tree} \mid \text{Data}] = \frac{Pr[\text{Data} \mid \text{Tree}] \times Pr[\text{Tree}]}{Pr[\text{Data}]}$$ (modified from Huelsenbeck 2000a)

where Pr[Tree I Data] is the posterior probability of the tree given the data, or more simply phrased the "new" probability of the tree after the likelihood estimate (the conditional probability) has incorporated any prior probabilities, based on a prior knowledge of the data.

Pr[Data I Tree] is the likelihood function or the conditional probability of observing the data, given a tree and model of evolution.

Pr[Tree] is the prior probability of the tree and represents what is known about the tree or phylogeny before any data is observed. Very often in phylogenetic analysis, a flat or "uninformative" prior is used. This is when there is no prior knowledge about the phylogeny and effectively allows for all trees to be treated with equal probability before any data is observed (Newton et *al.* 1997), and with the branch lengths drawn from a uniform distribution. The effect of the value of the prior on the posterior probability distribution has been shown to not be significant (Rannala and Yang 1996). Furthermore, the method is robust to changing values of the prior. It is thought that this is largely as a result of the fact that most of the information is drawn from the observed data and so values of the prior distributions will only have a minimal affect on the posterior distributions (Rannala and Yang 1996). The prior is

constantly updated based on the new posterior probabilities calculated and the revised parameters are incorporated into the estimation procedure. These priors should eventually converge on the estimated parameters for a given data set.

Pr[data] for six taxa or less can be calculated using standard numerical formulae. It represents the sum of all the posterior probabilities for the trees and the integration of the potentially different branch lengths from all the sampled probabilities (Rannala and Yang 1996).

When more than six taxa are analysed in a given data set, more complicated methods are required for the derivation of the posterior probabilities, particularly with respect to the integration of the branch lengths over all the available topologies. One of the methods available is the Markov Chain Monte Carlo (MCMC) method (Yang and Rannala 1997, Larget and Simon 1999). This method obviates the need to sum over all the available topologies and allows for the more efficient evaluation of the integral for the estimation of the ancestral branch lengths, using the specified joint (conditional and prior) probabilities and a model of evolution.

The MCMC (Larget and Simon 1999), therefore, allows for the efficient searching, or at least sampling, of trees and model parameters from the posterior distribution, particularly when there are a large number of taxa in the data set. To ensure adequate coverage of the distribution space, several chains are run simultaneously, each initiated from a randomly selected search point (Huelsenbeck and Ronquist 2001). Some of these initial trees or samples are not included in the final probability estimate and are disregarded as "burn-in" (Besag and Green 1993). This is because they represent random starting points and will bias the MCMC, as it has yet to converge on an optimum landscape for that particular data set (Mau et *al.* 1999).

The bayesian estimate provides a consensus tree with posterior probabilities for each clade. These probabilities represent all the trees sampled by the MCMC in the posterior distribution, which contained that particular clade. The number of times a particular clade is sampled will give some indication of the frequency of that reconstruction in the distribution space, and as a result will give some indication of the confidence that can be placed in that relationship.

Optimality Criterion II: Parsimony

The principal of parsimony is defined by a minimalist approach (also referred to as Ockham's Razor), where the simplest explanation, or the one requiring the fewest assumptions, is the one preferred. In a phylogenetic context, the method has been implemented by Fitch (1971) in the form of parsimony. Parsimony analysis is used extensively in combined analysis approaches where variable data sets, for example molecular and morphological characters, are combined. In this method of inference, the minimum number of changes that are required to explain the observed data are calculated for each character or nucleotide position in the sequence data set. The sum of these changes is totaled across all characters on the tree to obtain a parsimony score. The tree that requires the minimum number of changes across all characters, or the tree with the lowest score, is considered the optimal tree (Fitch 1971). If the number of changes across the observed sequences is small, represented by low levels of sequence divergence, then the parsimony method should approximate the maximum likelihood estimate of phylogeny in selecting the optimal tree (Steel and Penny 2000). However, as evolutionary distance increases, the number of superimposed changes also increases. Because parsimony does not invoke explicit models of evolution, it is not able to efficiently deal with this potential homoplasy and becomes more likely to converge on the incorrect tree. This is because, with increasing evolutionary distance, the correct tree becomes less likely to be the one that has the fewest changes. It is possible however to describe a set of conditions in which parsimony will be more likely to recover the true tree. For example, if the number of phylogenetically informative characters (shared derived characters or synapomorphies) is high and the number of homoplasies is low. These conditions, however, are rarely met with in real data sets (Stewart 1993).

There are a number of methods to deal with the problem of potential homoplasy in parsimony analysis that includes various weighting schemes. These weighting schemes approximate the evolutionary models used in model-based methods of inference to varying degrees (Goldman 1990 but see Brooks and McLennan 2002). For example, introducing step matrices to account for transition: transversion bias, or down weighting third codon positions. When selecting a weighting method, it is unlikely that a single set of weights, selected and assigned based on presumed differences, will be applicable to all characters in the particular data set (*a priori* weights). Several other options are available that do not make this assumption, for example successive weighting (Farris 1969) and implied weighting (Goloboff 1993). These methods, as with maximum likelihood, take character reliability into account and effectively downweight the influence of specific highly variable characters.

The question to what extent parsimony does require assumptions or invoke underlying models of evolution has been an area of some debate. The debate focuses, for the most part, on what actually constitutes a specific model of evolution (Farris 1973, Brooks and McLennan 2002). For example, parsimony makes assumptions about the process of sequence evolution, in that it requires constancy of substitution rates between and across nucleotides and similar substitution rates among lineages i.e. the existence of a molecular clock (Yang 1996b). Weighting schemes can account, in part, for violations of these assumptions. The major difference between the model-based approaches and the parsimony approach, is the role of the individual observations in the reconstruction of the phylogeny. In parsimony analysis only a subset of the characters are considered phylogenetically informative. The remaining characters, including constant sites and autapomorphies, have no effect on topology reconstruction. This stands in contrast to distance and maximum likelihood analysis, where all characters are utilised in phylogenetic inference.

Whilst there is no real certainty, with any of the optimality criterion, as to which method is more likely to recover the true tree, it is becoming increasingly obvious that there are several "zones" in which one method performs worse than the other (Steel and Penny 2000). It is widely recognised that under a too simplistic model of evolution, where methods systematically underestimate the amount of change, the incorrect estimate of phylogeny is more likely to be selected for as more data is added (Swofford et *al.* 2001). An example of this is long-branch attraction (Swofford et *al.* 1996). In this case, taxa with large amounts of evolutionary change (or long branches) are grouped together in the phylogenetic estimate, which is incorrect because of the increased likelihood of shared character states due to convergent or parallel evolution on the longer branches. These taxa appear more closely related than do taxa with shorter branches that are in actual fact more closely related by shared character states from a common ancestor (Felsenstein 1978). Parsimony appears to be particularly affected by this problem and the conditions under which it can occur are referred to as the "Felsenstein Zone" (Huelsenbeck 1997). Maximum likelihood incorporates explicit assumptions about underlying models of evolution that should, in essence, account for the superimposed changes that may be responsible for this phenomenon (Huelsenbeck 1998). It is, however, becoming evident that violation of these models of evolution will also make these methods susceptible to this inconsistency and in this case it is termed the "Farris Zone" (or the "anti-Felsenstein zone") (reviewed in Swofford et *al.* 2001). Failure to account for site-to-site rate heterogeneity (Gaut and Lewis 1995, Chang 1996) and non-

independence among sites within a sequence (Schoniger and von Haesler 1995), are conditions under which it can occur.

Phylogenetic Inference and Hypothesis Testing

The ability for statistical testing within the likelihood framework is one of the strengths of this method of phylogenetic inference. For example, statistical tests allow for the ability to test which model of evolution provides the best or optimal fit for a given data set – an essential step in maximising the probability for the inference of the optimal phylogeny given the observed sequences. All phylogenetic methods make assumptions about the models underlying the evolutionary process, although there is debate as to the extent that parsimony evokes models of evolution. These inference methods have been shown to be less accurate and less consistent when incorrect models of evolution are assumed (Huelsenbeck and Hillis 1993, Bruno and Halpern 1999), particularly in the estimation of branch lengths (Yang et *al.* 1994, Buckley et *al.* 2001b). It is important that only those assumptions that are essential to describe the most realistic model of evolution that is able to extract the maximum amount of information from the data are selected and incorporated into the optimality criterion. On the other hand, the rejection of models that are too simple too fully explain the observed data, is also important. It has been suggested that to prevent the use of overly simplistic models of evolution, the analysis should always begin with more complex or parameter rich models (Sullivan et *al.* 1999). However, complex models have increased levels of variance or error associated with them due to the necessity of estimating more parameters from the same amount of data (Sullivan et *al.* 1999). Another disadvantage of this approach is that there is an increase in computation time and computing power required for models of increasing complexity.

Statistical tests can be used to aid in the selection of optimal models of evolution for the specific data set under study, and allow for the justification of the selection of one model over another. They can also be used in an evolutionary framework to test hypotheses from a behavioral perspective, for example, host parasite relationships (Huelsenbeck and Rannala 1997).

Statistical Testing of Models of Evolution

It is becomingly increasingly obvious that there is no standard or single model of evolution appropriate for use in all data sets. Different lineages, genes and even parts of genes seem to evolve at distinct rates, vary in the complexity of the evolutionary processes that define them and subsequently, in the assumptions that are required to best explain them (Posada and Crandall 2001a). For a reversible model of DNA substitution, there are 10 parameters to explain the probability of change between each of the four nucleotides, eight of which can vary: five rate parameters and three nucleotide frequency parameters (Posada and Crandall 2001a). In addition to these, site rate heterogeneity parameters in the form of a gamma distribution and proportion of invariable sites, can be incorporated into the model to add further realistic assumptions about the evolutionary process. These parameters can be estimated for the entire tree, for particular branches of the tree and even for different classes of data (Huelsenbeck and Rannala 1997). There are, therefore, a large number of potential models available to explain any given data set. These models range in complexity from the simplest, the JC (Jukes-Cantor), to the most complicated, the GTR +Γ+P*inv* (General Time Reversible with a gamma distribution and proportion of invariable sites). Considering the adverse effects of incorrect model selection on phylogenetic estimates, it is important that optimal models are selected (Goldman 1993). There are two statistical approaches available to determine the best fit models of evolution for a given data set and include:

i. The Likelihood Ratio Test (Felsenstein 1981, Goldman 1993, Huelsenbeck and Crandall 1997, Posada and Crandall 2001a, 2001b)

The likelihood ratio statistic (LRT), δ, is defined as the ratio between the likelihoods (maximised) of an alternative (L_1) and a null hypothesis (L_0):

$$\delta = {}^{4}\text{Log}L_1 - \text{Log}L_0 \qquad \text{(modified from Page and Holmes 1998)}$$

where L_0 is the likelihood maximised under the null hypothesis

L_1 is the likelihood maximised under an alternative hypothesis

When comparing models of evolution that represent nested hypotheses, the significance associated with the LRT can be estimated by allowing 2δ to approximate a chi-Squared distribution, with a number of degrees of freedom, q, where q is the difference in the number

[4] The log likelihoods are calculated as the values of the likelihoods are generally extremely small

of parameters between the two competing hypotheses (Yang et *al.* 1995). Models are considered nested models when the simple model ($-\ln L_0$) is a special case of the more complex model or alternative hypothesis ($-\ln L_1$) (Posada and Crandall 2001a). The likelihoods for each hypothesis are estimated from the same starting tree topology. In maximum likelihood inference, it has been found that estimation of the likelihood parameters are relatively robust to alternative starting tree topologies (Posada and Crandall 2001b). Using the LRT statistic, a number of increasingly complex models of evolution can be tested and the simplest model, that best explains the data, can be selected (Posada and Crandall 2001b). When models that are being compared are not nested, for example when testing for gamma distributed rate heterogeneity (Goldman and Whelan 2000), the significance of the 2δ statistic no longer approximates a chi-Squared distribution. In this case, the significance estimate can be obtained by a Monte Carlo Simulation or parametric bootstrapping where data sets, each with optimized parameters, are simulated according to the hypothesis being tested (Goldman 1993).

The LRT statistic has been used extensively to test the Molecular Clock Hypothesis (Zhang 1999). Zuckerlandl and Pauling introduced the concept of a molecular clock in 1965 (in Posada and Crandall 2001a). This hypothesis states "that genes and their proteins evolve at more or less constant rates", and that evolutionary time can be correlated with the amount of molecular divergence that has accumulated between lineages as represented by their sequence divergence estimates.

Assuming or imposing a molecular clock with sequence data can provide a useful tool for estimating species duration (Walker and Avise 1998), and for the timing of divergence events (Thorpe et *al.* 1995), which can in turn give some indication of the time scale of evolution. However, it is becoming evident that there is a large amount of variability in the rate at which DNA mutates or accumulates changes, which violates the assumption of the "universal" molecular clock (Hasegawa and Kishino 1989, Hedges et *al.* 1991, Avise et *al.* 1992, Martin et *al.* 1992, Mindell et *al.* 1996). This rate variation occurs at all levels of the analysis, for example differences in rates across nucleotides or genes within phylogenetic lineages (Britten 1986), or differences in the rate or tempo of change across homologous sections of DNA between lineages (Hillis et *al.* 1996). It is important to bear in mind that the use of clock assumptions is more reliable when studying closely related taxa. The rates of evolution of particular genes are more likely to be stable in taxa with similar life history traits, metabolic rates and generation times. The estimation of these "local" rates is preferable over the

implementation of the "universal" rate across all taxa or groups of organisms (Hillis et *al.* 1996). When calculating divergence estimates, mitochondrial DNA does not show linear divergence values beyond ten million years due to the accumulation of multiple hits. Most divergence estimates beyond this period will therefore represent underestimates, unless these multiple hits are corrected for within the model calculation (Kumar and Hedges 1998).

The rates of evolution for mtDNA have been shown to be relatively consistent among vertebrates, for example ~2% for primates (Wilson et *al.* 1985), 0.69%/MY in frogs, and 0.65%/MY in the lizard genus, *Roudakia* (in Weisrock et *al.* 2001). It is important to be aware of the effects of incorrect estimations of the clocks on the divergence estimates (Hillis et *al.* 1996). For example, mitochondrial DNA evolution in the order Testudines has been calibrated at 0.4% per million years (Avise et *al.* 1992). This represents a substantially reduced rate (an eightfold decrease) relative to the conventional vertebrate mtDNA rate of ~2% per million years (Wilson et *al.* 1985), which would significantly influence the calculation of subsequent divergence estimates. This reduced rate in the Testudines has been attributed to the long generation times of these reptiles, coupled with low metabolic rates (Avise et *al.* 1992). One of the limitations with this study, however, was the fact that there are very few accurately timed cladogenic or vicariant events with which to accurately calibrate the clock for the Testudines, and as such there may be room for error associated with this estimate (Avise et *al.* 1992). The characteristics of an assumed molecular clock should be investigated and calibrated for each new gene and group studied, so as to avoid the systematic effects associated with an incorrect estimation (Hillis et *al.* 1996).

Whether or not the data conforms to a molecular clock can be tested in a variety of ways. For example, in the case of the Likelihood Ratio Test (LRT), the null hypothesis has no molecular clock imposed (the unconstrained tree) ($-\ln L_0$), while the alternative hypothesis has a clock imposed (the constrained tree) ($-\ln L_1$). The degrees of freedom, q, is equal to $n - 2$, where n is the number of taxa present in the data set.

Another test available to test whether or not the data conforms to a molecular clock is the relative rate test (Tajima 1993). The test calculates the number of substitutions between two closely related taxa (sequences) and compares the number of differences observed to a third, more distantly related outgroup taxon. There are, however, several disadvantages in applying this method to evaluate potential differences in rates of evolution. The most significant of these is the fact that the test is simultaneously applied to the same sequences

for multiple sets of taxa and consequently is not an independent measure. This makes the assignment of the subsequent significance values associated with each pairwise comparison more difficult to interpret (Page and Holmes 1998). In addition, the selection of an appropriate outgroup taxon is important. If the outgroup is too divergent from the ingroup taxa being compared it will become saturated relative to the ingroup taxa and so will not detect rate differences (Sherer 1989).

ii. The Aikake Information Criterion (AIC) (Aikake 1974)

The AIC is another test that can be used in the selection of best-fit models of evolution for use in phylogenetic inference. It is based on the principal of minimizing the number of free parameters used in the models chosen, by issuing a penalty for the addition of parameters with no concurrent increase in likelihood score.

Statistical Testing of Tree Topology

As mentioned previously, non-parametric bootstrapping is a common method to estimate the significance or confidence that can be placed in a phylogenetic tree, by the resampling of the original data set (Felsenstein 1985). However, the interpretation of these bootstrap proportions is not fully understood and it would seem that the this method may be more suitable for examining parts of a tree, for example, subtrees or clades, or even specific branches, and not necessarily comparing competing tree topologies (Swofford et *al.* 1996, Whelan et *al.* 2001).

There are other statistical tests available, based on the LRT statistic, which allow for the estimation of the significance value associated with the point estimates in competing phylogenetic hypotheses. In these methods, the hypotheses being tested would be the specified tree topologies and the test statistic is again based on the estimated maximum likelihood score for each of these trees.

One of these is the Kishino Hasegawa (K-H) test developed by Hasegawa and Kishino (1989) and Kishino and Hasegawa (1989). It allows for the estimation of a null distribution to test the significance of two competing hypotheses, where the required distribution is calculated by non-parametric bootstrapping of the data. The test was originally developed to test the significance between two topologically distinct trees, representing competing phylogenetic hypotheses, for a given set of aligned sequences. The most important feature of the test, however, is that the competing hypotheses are based on trees that have been

specified *a priori* or independently of the sequence data under study. The maximum likelihood value for each of these trees is calculated, given specific rate parameters and a model of evolution, which is estimated from the observed data. The null distribution is calculated using these parameters and the non-parametric bootstrapping of the original data set. This distribution can then be used to estimate how well each of these competing topologies is supported by the data, based on their likelihood scores and those generated in the null distribution. This test is used extensively in the current literature, although it has been noted that in many cases it is being applied in the incorrect context of hypothesis testing (reviewed in Goldman et *al.* 2000). The test is used to calculate the significance of competing topologies, one of which is specified *a priori* and the other, based on a maximum likelihood estimate derived from the observed data, is specified *a posteriori*. For the test to be validated both topologies or hypotheses should be specified independently of the observed data, and the observed data is used to produce the null distribution. An inherent bias is therefore introduced when one of the specified trees is already a maximum likelihood estimate of the observed data.

The *a posteriori* comparison of competing hypotheses is of great importance in evolutionary biology. To overcome the problem outlined above, Shimodaira and Hasegawa (1999) have described a non-parametric test statistic, the S-H test, that allows for the comparison of two competing hypotheses, one of which is specified *a priori* and the other which is a maximum likelihood estimate, specified *a posteriori*. This revised test takes into account all possible topologies specified. Furthermore, the test compensates for the multiple comparisons that could be introduced when one of the possible topologies is a maximum likelihood estimate derived from the observed data, which is also used to generate the null distribution (Shimodaira and Hasegawa 1999, Goldman et *al.* 2000).

Measures of Accuracy in Phylogenetic Estimates

The ultimate goal in the inference of phylogenies is to obtain an accurate or reliable estimate of the evolutionary history among organisms or groups of organisms. This information can be applied to a variety of biological applications, ranging from behavioral to epidemiological studies. However, an estimate of the reliability or accuracy of the phylogenies is required to ascertain how much confidence can be placed in the inference. To assess the accuracy of inferred trees a number of approaches can be used:

1. Simulated Evolutionary studies, for example generating known experimental phylogenies for the purpose of testing the accuracy of phylogenetic methods (Huelsenbeck 1995, Sanson et *al.* 2002)
2. Statistical evaluations to assess the confidence and reliability of the inferred trees, for example the resampling of data sets (Felsenstein 1985), bremer decay indices (Bremer 1988) and likelihood ratio tests (Goldman 1993, Yang et *al.* 1994)
3. Congruence studies, where the extent to which independent data sets support similar evolutionary histories, which is not due to chance alone, is a test of the confidence that can be placed in the data sets (Swofford 1991, Miyamoto et *al.* 1994).

Congruence

With the advent of molecular techniques, it is becoming increasingly common for researchers to have access to more than one data set for the group of organisms under study. These data sets can be molecular (two or more different genes), allozymes, proteins or morphological in nature, or any combination of these. The question then arises as to how best to analyse these multiple data sets to extract the maximum amount of information from them and improve the phylogenetic resolution. Should the data sets be combined to produce a single set for a "total evidence approach" (Kluge 1989), or should the data sets be analysed independently and the resulting estimates compared using measures of topological or taxonomic congruence (Miyamoto and Fitch 1995).

The main focus of this debate centres around the evolutionary processes underlying the respective data sets or partitions and what criteria defines these data partitions (reviewed in Hillis 1995). The extent to which these processes differ and the effect these differences will have on the assumptions made during the phylogenetic inference and consequently on the phylogenetic estimate, are an area of concern (Huelsenbeck et *al.* 1996). For example, using two genes that evolve at different rates could produce different phylogenetic reconstructions

from each of the data partitions analysed. This incongruence could be an indication of the significant differences in the two genes due to underlying evolutionary and biological factors (Huelsenbeck et *al.* 1996). Alternatively, it could be a result of artifacts related to the method of inference. For example, too few characters or high levels of homoplasy that have not been appropriately corrected for. These are generally represented by low levels of support in the conflicting clades i.e. "soft incongruence" (Seelanan et *al.* 1997).

Causes of Incongruence

There are several reasons cited for potential incongruence between data partitions that range from sampling errors (Hillis 1996) to rate heterogeneity between lineages, or between the nucleotide sites of the gene being studied (Sullivan 1996), to lineage sorting and horizontal gene transfer (Soltis et *al.* 1998)

One of the potential causes of data incongruence is a result of an insufficient amount of data, either in the number of characters being analysed or in the number of taxa sampled in the multiple data sets. In the case of character data, there may be an insufficient number of phylogenetically informative characters in one of the data sets for the sequences under study (Graybeal 1998). The extent of the resulting incongruence will depend on the amount and the level of heterogeneity displayed within the sequences being compared, as well as the level of divergence between the taxa being sampled, both of which will determine the extent of the homoplasy observed (Graybeal 1998). The other cause of incongruence is a result of insufficient taxon sampling in one or both data sets (Hillis 1996, Mitchell et *al.* 2000). Although insufficient taxon sampling may result in misleading estimates of phylogeny due to long-branch attraction (Felsenstein 1978, van der Kuyl et *al.* 2002), increasing this sampling density does not necessarily result in improved phylogenetic accuracy (Kim 1996, Mitchell et *al.* 2000). The long-branch attraction could rather be a result of rate heterogeneity among lineages, where increasing the taxon sampling will not correct for the effects of the underlying differences in the evolutionary processes (Takezaki et *al.* 1995). There is still some debate as to whether it is desirable to increase the number of characters in a data set, by incorporating characters from an alternate gene, or increase the number of taxa sampled to cope with these inconsistencies (Hillis 1995, Kim 1996). When analysing multiple data sets, there are methods available to deal with the problem of unequal taxon sampling (Miyamoto 1996) and missing data (Wiens and Reeder 1995). However, it is advisable to attempt to compare equivalent data sets both in the number of taxa in each set and to ensure that there is no missing character data.

When analysing a combination of morphological and molecular data sets, a potential cause of the resulting incongruence is the inherent difference in the rate of the evolutionary processes underlying the different character sources (Soltis et *al.* 1998). These decoupled morphological and molecular rates of evolution are common in nature, as demonstrated for Cichlid fishes (Sturmbauer and Meyer 1992) and bats (Alvarez et *al.* 1999). Small changes at the molecular level, for example in protein coding genes, can result in large concurrent differences becoming visible in the morphological characters. The elucidation of these differences can become further complicated by the effects of hybridisation and rapid diversification, as a result of changes in the ecological continuum, that are not reflected at the level of the gene (Roberts 1993, Verboom 2000). Another potential cause of incongruence is the effect of parallel or convergent evolution, for example the parallel evolution of gigantism in lizards (Carranza et *al.* 2001) or the repeated evolution of carnivory in plants (Albert et *al.* 1992). Morphological characters are particularly prone to these phenomena, and if not properly accounted for, will result in the inference of potentially misleading phylogenies when the two data partitions are subsequently combined.

Another cause of incongruence is differences in the distributions of among-site rate variation in the molecular data sets under study. Bull et *al.* (1993) showed that when combining simulated data sets that had underlying differences in the rate of evolution, the resulting phylogenetic estimate was less accurate than when the slower evolving data set was analysed separately. However, Sullivan (1996) argued that these simulated data sets represented extremes of the continuum of among-site variation that would not be present in more realistic data sets. Bull et *al.* (1993) used two single rate models, one assuming a uniformly high rate the other a uniformly low rate, to simulate the data sets. Most real data sets, however, will be represented by more or less continuously distributed overlapping evolutionary rates among sites (Yang et *al.* 1994, Sullivan et *al.* 1995).

Sullivan (1996) showed that combining two data sets with significantly different underlying rates of variation resulted in two different but each a well supported topology. The phylogenetic signal was additive and yielded a better estimate of phylogeny than the two data sets analysed separately. The study was based on ten taxa of deer mice (*Peromyscus*) and grasshoppper mice (*Onychomys*), and the analysis of the 12S ribosomal RNA (α = 0.281) and a portion of the Cytochrome *b* gene (α = 0.649) (Sullivan et *al.* 1995). The improved estimate could be a result of the resolution of homoplasy in the data set with particularly high rates of variation that is obscuring the signal from the alternative data set.

The individual effects of the two rate parameters are homogenised within the combined analysis (Barrett et *al.* 1991).

As in the case above, when two different data partitions give competing but well supported phylogenetic estimates for the same taxonomic group under study, there are several methods available that allow for the estimation of the significance of the differences observed between them and the extent of the incongruence observed. As aluded to earlier, there are three ways in which multiple data sets can be analysed: a combined approach (Kluge 1989), a consensus approach (Miyamoto and Fitch 1995) and a conditional combination approach (Bull et *al.* 1993).

i. The Combined Approach

The combined approach is also referred to as the "total evidence" approach. All available data partitions are combined *a priori* and analysed as a single data matrix. This approach is said to maximise the informativeness and explanatory power over all the characters in the data set (Kluge 1989). Combining different partitions or data sets that are evolving at different rates, represented by different underlying evolutionary processes, may in fact help to resolve different levels of the phylogenetic estimate (Hillis 1987). For example, adding more characters in the form of an alternate data set may prevent the random noise, caused by excessive amounts of homoplasy, from swamping the phylogenetic signal (Barret et *al.* 1991, Sullivan 1996). This may result in a reduction in character conflict and the establishment of new and strongly supported groups that would not be found in either of the inferences generated from the individual data sets (Barret et *al.* 1991)(reviewed in de Queiroz 1993).

In addition, phylogenetic methods are assumed to become more consistent as more character data is added, and the method is more likely to converge on the correct phylogeny or tree (Huelsenbeck et *al.* 1996). The increase in the available data from which to extract information is therefore one of the advantages of this method of data combination. It does, however, assume that the chosen method of phylogenetic inference accurately accounts for the underlying differences in the evolutionary processes in each partition or data set (Bull et *al.* 1993, Chippendale and Wiens 1994, Hillis 1995).

ii. The Consensus Or Separate Analysis Approach

This approach maintains that multiple data sets represent natural and independent partitions and so should always be analysed separately (Penny et *al.* 1982, Miyamoto and Fitch 1995). The resulting independent phylogenetic estimates can then be compared for areas of phylogenetic corroboration or taxonomic congruence using a number of topological indices, for example the Partition Metric Test (PMT)(Penny et *al.* 1982). Advocates of the consensus approach argue that one of the downfalls of the combined analysis is that by increasing the number of characters that are potentially weighting a misleading phylogeny, only serves to add support to the incorrect tree. This situation could arise when the two different data partitions or data sets give rise to strongly supported but conflicting trees (Shaffer et *al.* 1991). In the consensus approach, however, strongly supported branches of the phylogenetic estimates from each of the independent data sets should be used to produce a consensus tree, which will represent a best estimate of the true phylogeny (Penny and Hendy 1986, Shaffer et *al.* 1997). This is because areas of strongly supported congruence in two different data sets are unlikely to occur by chance alone and so these groups are supported by more than one independent source of evidence (de Queiroz 1993).

However, one of the disadvantages of the consensus approach is that by keeping the number of characters in a data set small, there is an increase in the stochastic error, which may result in the inference of different topologies, even if the data partitions are represented by homogenous evolutionary mechanisms. The method cannot distinguish between sampling error and *bone fide* differences in the branching histories between the partitions (Huelsenbeck et *al.* 1996, reviewed in Soltis et *al.* 1998)

iii. The Conditional Combination Approach

An alternative approach to dealing with multiple data sets is a combination of the two scenarios already presented. The conditional combination approach only advocates combination of data sets that do not show any significant heterogeneity between them i.e. data incongruence (determined *a priori*) and do not support strongly conflicting phylogenetic inferences i.e. topological incongruence (Swofford 1991, Bull et *al.* 1993, de Queiroz 1993, Huelsenbeck et *al.* 1996). The significance of the degree of data heterogeneity is determined by statistical testing. These tests gives some indication of whether the apparent incongruence may be a result of sampling error, or is reflecting real differences in the underlying evolutionary processes that can result in different branching histories (de Queiroz et *al.* 1995). For example, the incongruence length difference test (ILD) (Farris et *al.* 1994)

and the LRT (Huelsenbeck and Bull 1996) are commonly used. If there is no evidence of significant heterogeneity between the data partitions, the data can be combined and analysed as a single data matrix. If there is significant heterogeneity detected, the data can be analysed using a number of different options.

A consensus approach can be used to determine the areas of the phylogenies that are strongly supported; the phylogeny can be left unresolved or the data can be re-analysed using a revised method of phylogenetic inference. This could include revising the model parameters to more realistically explain both or one of the data partitions, the aim of which is to try and reduce the incongruence or conflict (Bull et *al.* 1993, Miyamoto et *al.* 1994, Sullivan 1996). For example, homogenising the differences in the rates of evolution between the two data sets if they are evolving at very different rates. Another approach is to determine which taxa in the matrix are showing significant heterogeneity, and to remove these taxa from the data set and then combine the partitions (Seelanan et *al.* 1997).

One of the major arguments against the conditional combining of data is that the statistical tests that evaluate the extent and significance of the heterogeneity between data partitions may be too conservative (Sullivan 1996, Cunningham 1997). This will result in partitions, which are in fact represented by homogenous processes, not being combined (Sullivan 1996). This error in the significance tests is due to the fact that the suspected heterogeneity may only be an artifact of stochastic sampling error and not a reflection of differences in the evolutionary histories underlying the partitions (Sullivan 1996). It is important, therefore, that there is some understanding of the biological and evolutionary processes that could be underlying each of the data partitions and to consider these when assessing potential areas of incongruence.

Research Aims

It is important to note that phylogenetic trees are hypotheses of the process of evolution based on the quality and quantity of data available. Phylogenetic inferences should not be used in isolation to infer evolutionary relationships among organisms. Instead they should be used in combination with alternate data to support of refute hypotheses of evolutionary history. The field of phylogenetic inference is a rapidly expanding discipline and the selection and implementation of different methods of analysis, for example the Cladistic based approach of parsimony over the model based method of maximum likelihood, remains a contentious issue. The effects of the implementation of different methodologies on the resulting phylogeny for the Family Testudinidae, and the level of divergence at which the differences in their utility are likely to become problematic, was tested by incorporating parsimony, maximum likelihood and bayesian approaches within the framework of the study.

Furthermore, congruencies between phylogenetic estimates that have been established using different methods of inference and different data partitions, for example molecular versus morphological, increase confidence that the topologies are in fact representative of the true evolutionary history underlying the data partitions and potentially the group under study. Consequently, the approach adopted within the framework of this study will elucidate organismal inter-relationships and evolution within the Testudinidae.

CHAPTER 4

MATERIALS AND METHODS FOR PHYLOGENETIC RECONSTRUCTION

Sample Collection

Samples consisting of approximately 100µl of blood were collected from 172 individuals that encompassed all currently recognised extant genera and species in the Family Testudinidae (Iverson 1997). This broad range sampling strategy was deemed necessary, as it has been shown that the effects of insufficient sampling schemes on estimates of phylogeny can be significant (Lecointre et *al.* 1993). Phylogenetic accuracy can increase with increased taxon sampling (Hillis 1996, 1998, Malone et *al.* 2000) provided that the additional taxa do not introduce new long branches as opposed to those that they should, in effect, be breaking up (see Kim 1998, Poe and Swofford 1999, Mitchell et *al.* 2000). Samples were obtained from a variety of field localities (including Mexico, Indonesia, Southern Africa, Madagascar, Cameroon and Tanzania), institutions (University Of Durban-Westville, Port Elizabeth Museum Collection) and a number of private collections in the United States (Table 4.1). Individual samples were only included once a positive identification to species level had been made. In all cases a minimum of two samples from each identified species category were collected. Samples were also obtained for individuals from the Emydidae (*T. ornata and T. coahuila*), the Cheloniidae (*L. kempii and C. caretta*), the Dermatemydidae (*D. mawii*) and the Carettochelydae (*C. insculpta*). These samples were used as outgroup taxa in the study (Table 4.1).

Table 4.1: Sample identification codes and localities for individuals used in this study (n=172)

Numerical Designation	Species Name	Catalogue Number	Locality/ Institution
1A	*Geochelone gigantea*	#980319	St Louis Zoological Park, USA
1B	*Geochelone gigantea*	#386029	St Louis Zoological Park, USA
1C	*Geochelone gigantea*	#980320	St Louis Zoological Park, USA
1D	*Rocket (G. gigantea)*		Omaha Henry Doorly Zoo, USA
1E	*Geochelone gigantea*	#372004	St Louis Zoological Park, USA
1F	*Dipsochelys arnoldi (G. gigantea)*		Omaha Henry Doorly Zoo, USA
2A	*Chersina angulata*	#13A	Tygerberg Zoo, South Africa
2B	*Chersina angulata*	#13B	Tygerberg Zoo, South Africa
2C	*Chersina angulata*	#13C	Tygerberg Zoo, South Africa
2D	*Chersina angulata*		Omaha Henry Doorly Zoo, USA
2E	*Chersina angulata*	#2	Omaha Henry Doorly Zoo. USA
2F	*Chersina angulata*	#3	Omaha Henry Doorly Zoo, USA
3A	*Geochelone carbonaria*	#2	Rotterdam Zoo, Netherlands
3B	*Geochelone carbonaria*	#930791	St Louis Zoological Park, USA
3C	*Geochelone carbonaria*	#387023	St Louis Zoological Park, USA
3D	*Geochelone carbonaria*	#387021	St Louis Zoological Park, USA
3E	*Geochelone carbonaria*	#387002	St Louis Zoological Park, USA
4A	*Geochelone chilensis*	#8137	Sedgewick County Zoo, USA
4B	*Geochelone chilensis*	#8138	Sedgewick County Zoo, USA
5A	*Geochelone denticulata*	#372001	St Louis Zoological Park, USA
5B	*Geochelone denticulata*	#376002	St Louis Zoological Park, USA
5C	*Geochelone denticulata*	#961121	St Louis Zoological Park, USA
5D	*Geochelone denticulata*	#372002	St Louis Zoological Park, USA
5E	*Geochelone denticulata*	#382004	St Louis Zoological Park, USA
5F	*Geochelone denticulata*	#961120	St Louis Zoological Park, USA
7A	*Geochelone elegans*	#1A	Tygerberg Zoo, South Africa
7B	*Geochelone elegans*	#1B	Tygerberg Zoo, South Africa
7C	*Geochelone elegans*	#1	Omaha Henry Doorly Zoo, USA
8	*Geochelone nigra*		Omaha Henry Doorly Zoo, USA
9A	*Geochelone nigra*	#20A	Omaha Henry Doorly Zoo, USA
9B	*Geochelone pardalis*	#20B	Tygerberg Zoo, South Africa
9C	*Geochelone pardalis*	#1	Laikipia District, Kenya
9D	*Geochelone pardalis*	#3	Laikipia District, Kenya
9E	*Geochelone pardalis*	#4	Laikipia District, Kenya
9F	*Geochelone pardalis*	#5	Laikipia District, Kenya
9G	*Geochelone pardalis babcocki*	#L7	Angelo Lambiris, UND, South Africa
9H	*Geochelone pardalis pardalis*	#L1	Angelo Lambiris, UND, South Africa
10A	*Geochelone platynota*	#985380	Los Angeles Zoo, USA
10B	*Geochelone platynota*	#3	Private Collection
10C	*Geochelone platynota*	#1	Private Collection
10D	*Geochelone. platynota*	#2	Private Collection
10E	*Geochelone platynota*	#4	Private Collection
11A	*Geochelone radiata*	#RT4	Gladys Porter Zoo, Brownsville, Texas
11C	*Geochelone radiata*	#950203	Gladys Porter Zoo, Brownsville, Texas
11D	*Geochelone radiata*	#49VO	Gladys Porter Zoo, Brownsville, Texas
11E	*Geochelone radiata*	#55MAT	Gladys Porter Zoo, Brownsville, Texas
11F	*Geochelone radiata*	#183BOA	Gladys Porter Zoo, Brownsville, Texas
11G	*Geochelone radiata*	#123SAT	Gladys Porter Zoo, Brownsville, Texas
12A	*Geochelone sulcata*	#990130	Houston Zoological Park, Texas
12B	*Geochelone sulcata*	#990131	Houston Zoological Park, Texas
13A	*Geochelone yniphora*	#75	Wildlife Conservation Society
13B	*Geochelone yniphora*	#89	Wildlife Conservation Society
14A	*Indotestudo elongata*	#921023	St Louis Zoological Park, USA
14B	*Indotestudo elongata*	#921022	St Louis Zoological Park, USA
14C	*Indotestudo elongata*	#78	Thailand
14D	*Indotestudo elongata*	#80	Thailand
14E	*Indotestudo elongata*	#81	Thailand
14F	*Indotestudo elongata*	#83	Thailand
15A	*Gopherus agassizii*	#9030	Henry Doorly Zoo, Nebraska, USA

15B	*Gopherus agassizii*	#9031	Henry Doorly Zoo, Nebraska, USA
16B	*Gopherus berlandieri*	#epzoo4	El Paso Zoo
16C	*Gopherus berlandieri*	#epzoo5	El Paso Zoo
17	*Gopherus flavomarginatus*		Mapimi Reserve, Mexico
17B	*Gopherus flavomarginatus*	#11	Mapimi Reserve, Mexico
18A	*Gopherus polyphemus*	#387024	St Louis Zoological Park, USA
18B	*Gopherus polyphemus*	#940866	St Louis Zoological Park, USA
19B	*Homopus areolatus*	#Elands1	Elandsberg Game Reserve, South Africa
19C	*Homopus areolatus*	#Woes2	Worcester Valley, South Africa
19F	*Homopus areolatus*	#H3	Elandsberg Game Reserve, South Africa
19G	*Homopus areolatus*	#H7	Elandsberg Game Reserve, South Africa
19H	*Homopus areolatus*	#H11	Elandsberg Game Reserve, South Africa
19I	*Homopus areolatus*	#H5	Elandsberg Game Reserve, South Africa
20A	*Homopus bergeri*	#6692	Windhoek, Namibia
20B	*Homopus bergeri*	#6693	Windhoek, Namibia
21A	*Homopus boulengeri*	#15	Aus Townlands, Namibia
22A	*Homopus femoralis*	#14	Tygerberg Zoo, South Africa
22B	*Homopus femoralis*	Omaha	Wildlife Conservation Society
23A	*Homopus signatus cafer*	#16	Tygerberg Zoo, South Africa
23B	*Homopus signatus cafer*	#3	Wildlife Conservation Society
23C	*Homopus signatus cafer*	#2	Wildlife Conservation Society
24A	*Homopus signatus signatus*	#17	Tygerberg Zoo, South Africa
24B	*Homopus signatus signatus*	#5	Wildlife Conservation Society
24C	*Homopus signatus signatus*	#6	Wildlife Conservation Society
25A	*Indotestudo travancore*	#800276	Private Collection
25C	*Indotestudo travancore*	#6873	Henry Doorly Zoo, Nebraska, USA
25D	*Indotestudo travancore*	#6874	Private Collection
26A	*Indotestudo foresteni*	1	Private Collection
26B	*Indotestudo foresteni*	2	Private Collection
26C	*Indotestudo foresteni*	#NP2	Private Collection
26D	*Indotestudo foresteni*	#NP3	Private Collection
26E	*Indotestudo foresteni*	#NM3	Private Collection
27B	*Kinixys belliana*	#RMH	Ryan Heubinger, Private Collection
27C	*Kinixys belliana*	#RMH2	Ryan Heubinger, Private Collection
27D	*Kinixys belliana*	#UWEC2	Uganda Wildlife Education Center
29A	*Kinixys belliana*	#L3	Angelo Lambiris, UND, South Africa
30A	*Kinixys belliana spekii*	#L6	Angelo Lambiris, UND, South Africa
31A	*Kinixys erosa*	#1542	Cameroon, SW Province
31B	*Kinixys erosa*	#2649	Cameroon, SW Province
32A	*Kinixys homeana*	#8048	Henry Doorly Zoo, Nebraska, USA
32B	*Kinixys homeana*	R31287	Cameroon, SW Province
33A	*Kinixys natalensis*	#L4	Angelo Lambiris, UND, South Africa
34A	*Malacochersus tornieri*	#6A	Tygerberg Zoo, South Africa
34B	*Malacochersus tornieri*	#6B	Tygerberg Zoo, South Africa
34C	*Malacochersus tornieri*	#1	Edward Louis, Nebraska, USA
34D	*Malacochersus tornieri*	#L5	Angelo Lambiris, UND, South Africa
36A	*Manouria emys emys*	#930169	Honolulu Zoo, Hawaii, USA
36B	*Manouria emys emys*	#930168	Honolulu Zoo, Hawaii, USA
36C	*Manouria emys emys*	#850100	Honolulu Zoo, Hawaii, USA
36D	*Manouria emys emys*	#870297	Honolulu Zoo, Hawaii, USA
36E	*Manouria emys emys*	#930167	Honolulu Zoo, Hawaii, USA
36F	*Manouria emys emys*	#930176	Honolulu Zoo, Hawaii, USA
36G	*Manouria emys emys*	#930177	Honolulu Zoo, Hawaii, USA
37A	*Psammobates geometricus*	#G4	Elandsberg Nature Reserve, South Africa
37B	*Psammobates geometricus*	#G7	Elandsberg Nature Reserve, South Africa
37C	*Psammobates geometricus*	#G73	Worcester Valley, South Africa
37D	*Psammobates geometricus*	#G36	Ceres Valley, South Africa
37E	*Psammobates geometricus*	#G32	Ceres Valley, South Africa
37F	*Psammobates geometricus*	#G76	Worcester Valley, South Africa
37G	*Psammobates geometricus*	#eensamm	Eensaamheid, South Africa
37H	*Psammobates geometricus*	#harmony	Harmony Nature Reserve, South Africa
38A	*Psammobates oculiferus*	#5	Tygerberg Zoo, South Africa
38B	*Psammobates oculiferus*	#11	Tygerberg Zoo, South Africa

39A	*Psammobates tentorius tentorius*	#10	Tygerberg Zoo, South Africa
39B	*Psammobates tentorius*	#6109	Chaffee Zoo Gardens of Fresno, USA
39C	*Psammobates tentorius*	#6108	Chaffee Zoo Gardens of Fresno, USA
39D	*Psammobates tentorius*	#6356	Chaffee Zoo Gardens of Fresno, USA
39E	*Psammobates tentorius*	#6366	Chaffee Zoo Gardens of Fresno, USA
40A	*Psammobates tentorius verroxii*	#9A	Tygerberg Zoo, South Africa
40B	*Psammobates tentorius verroxii*	#9B	Tygerberg Zoo, South Africa
40C	*Psammobates tentorius trimenii*		Tygerberg Zoo, South Africa
41	*Psammobates tentorius trimenii*		Tygerberg Zoo, South Africa
42	*Pyxis arachnoides*		Private Collection
42A	*Pyxis arachnoides*	#pabr2	Private Collection
42B	*Pyxis arachnoides*	#paar1	Private Collection
42C	*Pyxis arachnoides*	#pabr3	Private Collection
42D	*Pyxis arachnoides*	#pabr4	Private Collection
42E	*Pyxis arachnoides*	#pabr5	Private Collection
42F	*Pyxis arachnoides*	#pabr1	Private Collection
42G	*Pyxis arachnoides*	#paob1	Private Collection
42H	*Pyxis arachnoides*	#paob2	Private Collection
42I	*Pyxis arachnoides*	#paar3	Private Collection
42J	*Pyxis arachnoides*	#paar4	Private Collection
42K	*Pyxis arachnoides*	#paar2	Private Collection
43	*Pyxis planicauda*		Private Collection
43A	*Pyxis planicauda*	#69	Private Collection
43B	*Pyxis planicauda*	#74	Private Collection
43C	*Pyxis planicauda*	#MOR4	Analiava, Madagascar
43D	*Pyxis planicauda*	#3	Private Collection
43E	*Pyxis planicauda*	#MOR21	Mosoarivo, Madagascar
44A	*Testudo graeca graeca*	#1	Edward Louis, Nebraska, USA
46A	*Testudo graeca terristris*	#1	Edward Louis, Nebraska, USA
47A	*Testudo hermanni*	#3A	Tygerberg Zoo, South Africa
47B	*Testudo hermanni*	#3B	Tygerberg Zoo, South Africa
47C	*Testudo hermanni*	#1	Henry Doorly Zoo, Nebraska, USA
47D	*Testudo hermanni*	#3C	Tygerberg Zoo, South Africa
48A	*Testudo horsfieldo*	#RMH`	Ryan Heubinger, Private Collection
48B	*Testudo horsfieldi*	#RMH2	Ryan Heubinger, Private Collection
48C	*Testudo horsfieldi*	#PET2	Ryan Heubinger, Private Collection
49A	*Testudo kleinmanii*	#TK1	Studbook Breeding Programme, Netherlands
49B	*Testudo kleinmanii*	#TK2	Studbook Breeding Programme, Netherlands
49C	*Testudo kleinmanii*	#8626	Studbook Breeding Programme, Netherlands
50A	*Testudo marginata*	#4A	Tygerberg Zoo, South Africa
50B	*Testudo marginata*	#4B	Tygerberg Zoo, South Africa
50C	*Testudo marginata*	#1	Gladys Porter Zoo, Brownsville, Texas
51A	*Carettochlys insculpta*	#1515	Black Hills Reptile Garden, South Dakota
52A	*Dermatemys mawii*	#300809	Zoological Society of Philadelphia, USA
53A	*Lepidochelys kempi*	#NNK779	Sea Arama Marine World, Galveston, Texas
54A	*Terrapene ornata*	#1	Gladys Porter Zoo, USA
55A	*Terrapene coahuila*	#TOO230	Gladys Porter Zoo, USA
60A	*Manouria impressa*	#954302	Fort Worth Zoo, Texas, USA
60B	*Manouria impressa*	#994308	Fort Worth Zoo, Texas, USA
61A	*Caretta caretta*	#1	Cape point, Western Province, South Africa

DNA Extraction

Total DNA was extracted from red blood cells by proteinase K digestion (0.1mg/ml) at 65°C for 1 hour in lysis buffer (50mM Tris-HCl pH 7.5; 400mM NaCl; 5mM EDTA pH 7.4; 0.5% SDS). This was followed by precipitation of the DNA in the presence of 2M ammonium acetate after the addition of 2 volumes of 100% ethanol (Sambrook et *al.* 1989). The resulting DNA pellets were air dried and resuspended in 100µl TE (100mM Tris, 10mM EDTA, pH 8). The genomic DNA was then diluted in sterile distilled water to a final concentration of between 10 - 50ng/µl and stored at -20°C until use. Blank extractions were carried out simultaneously with all batches of blood extractions to ensure that there was no contamination introduced into the DNA samples during the course of the extraction procedure.

Primers Selected for the Polymerase Chain Reaction (PCR)

Two mitochondrial DNA gene fragments were utilised in the study: the Cytochrome *b* gene (Cyt *b*) and the Nicotinamide Adenine Dinucleotide Dehydrogenase subunit 4 gene (ND4) (Table 4.2). Universal primers were used to amplify 429 base pairs (bp) of the Cyt *b* gene and were obtained from Kocher et *al.* (1989) and Paabo et *al.* (1990) (Table 4.2). Primers used to amplify a 742 bp fragment of the ND4 gene, and 240 bp of the Histidine, Serine and Leucine transfer RNA genes, were obtained from Arevalo et *al.* (1994). This produced a total fragment size of approximately 982 bp, commencing at the reference position 11165 (ND4 primer) of the aligned Bovine sequence of Anderson et *al.* (1982) (Table 4.2). The Cyt *b* gene and the ND4 gene were selected because both have been used extensively in phylogenetic studies to trace the evolutionary relationships in a wide variety of taxa, with varying amounts of success (Graybeal 1993, Sites et *al.* 1996, Wiens et *al.* 1999, Malone et *al.* 2000, Wiens and Hollingsworth 2000, Yu et *al.* 2000,). In the Order Testudines, the Cyt *b* gene has been used widely in both turtles (Bowen et *al.* 1993) and tortoises (Caccone et *al.* 1999a, 1999b, Austin and Arnold 2001), whilst the ND4 gene has, to date, only been used in studies on marine turtles (Dutton et *al.* 1996).

For each individual sample (172 in total), approximately 20 – 40ng of purified total genomic DNA was used in a 100µl PCR reaction containing a final concentration of 0.1pmol/µl of each of the forward and reverse primer for each gene pair, 1.5mM – 4mM $MgCl_2$, 0.2mM of each dNTP, 0.025 units of BIOTAQ DNA polymerase (Bioline, Whitehead Scientific) and 1X magnesium-free reaction buffer (Whitehead Scientific).

Annealing temperatures were optimised at 50°C for both gene fragments, and subsequent PCR reactions were performed using the following cycling parameters: 94°C denaturation (4 minutes) for one cycle; 94°C denaturation (30 seconds), 50°C annealing (30 seconds), 72°C extension (1 minute) for a total of 35 cycles, and a final 10 minute extension step at 72°C. Blanks were incorporated into each PCR batch to ensure there was no contamination of the PCR reagents or primers. The success of the PCR reactions were assessed by checking 10μl of the amplified product on a 1.5% agarose gel (Type D1-LE, Whitehead Scientific). The amplified product was visualised in agarose by staining the gel with Ethidium bromide ([EtBr] = 10μg/ml).

Automated Sequencing

The remaining PCR product (approximately 90μl) was purified using QIAquick® PCR Purification Columns (Qiagen Inc., Chatsworth, CA), following manufacturer's instructions, and eluted in 50μl of sterile distilled water, pH 8. This process removes any excess primers, nucleotides, polymerases and mineral oil that could interfere with subsequent enzymatic reactions. Following purification, 5μl of the product was run on a 1.2% agarose gel (Type D1-LE, Whitehead Scientific) and compared with the intensity of 5μl of a PGEM® - 3ZF(+) double stranded DNA control template (0.2g/L) (the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction kit v1.0, Applied Biosystems, Perkin Elmer, Forster City, USA) in order to estimate the concentration of the PCR fragments. All cycle sequencing reactions were performed using an ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction kit v1.0 (Applied Biosystems, Perkin Elmer, Forster City, USA). Reactions were performed in 10μl volumes that contained approximately 100ng of the purified PCR product, 4μl of the termination mix, 10pmol/μl of the specified sequencing primer and made up to a final volume with sterile distilled water, pH 8.

The sequencing primers were a combination of the original PCR amplification primers for Cyt *b* and ND4 respectively (Table 4.2), and a panel of eight additional sequencing primers for the ND4 gene, either designed specifically for this study (R. Heubinger) or obtained from published studies (Table 4.2). The sequencing reaction had the following cycling parameters: 96°C denaturation (30 seconds), 50°C annealing (15 seconds) and 60°C extension (4 minutes), for a total of 25 cycles. Forward and reverse sequences were obtained for each sample. Cycle sequencing products were purified of salts, polymerases and excess dye terminators using a G-50 Sephadex matrix (0.05g in 800μl sterile water) in Centri-Step spin

columns (Princeton Separations, Inc., Adelphia, NJ). Samples were dried in a Speed-Vac and stored at -70°C. Sequenced products were run on a 96 well ABI Prism 377 Automated Sequencer (Applied Biosystems, Perkin Elmer, Forster, CA) as per manufacturer's instructions.

Table 4.2: ND4 and Cytochrome *b* primer sequences and associated annealing and sequencing temperatures used in this study.

Primer name	Primer sequence 5' – 3'	*TA	**TS	Reference
ND4	CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC	50	50	Arevalo et *al.* 1994
Leu-tRNA	CAT TAC TTT TAC TTG GAT TTG CAC C	50	50	Arevalo et *al.* 1994
GeoFor	CAA CAC TAA CAC AAA CYC AAT G	-	50	#R. Heubinger
GeoEnd	ACA CAA TGA GGA GAA ACC CC	-	50	#R. Heubinger
GeoRev	TRT GAT CAT TGR GTT TGT GTT AGT G	-	50	#R. Heubinger
ND4-Rev	TAT TAG GAG AGT TTC TCG	-	50	Arevalo et *al.* 1994
Turt-Rev	TAA TAA TAG TGT TCG GCT ATG	-	50	Davis et *al.* (*pers. comm.*)
Turt-For	TTA TCC ACA ACA CAA TGA GG	-	50	Davis et *al.* (*pers. comm.*)
ND4#2	TAC GAC AAA CAG ACC TAA AAT C	-	50	Sites et *al.* 1996
MR#2	AGT GAG GCC GTG GGC RAT TAT TAG	-	50	Arevalo et *al.* 1994
Cyt *b*H15149	AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A	50	50	Kocher et *al.* 1989
Cyt *b*-Glu	TGA CAT GAA AAA YCA YCG TTG	50	50	Paabo 1990

*Ta = PCR primers and associated annealing temperature in ^{0}C

**Ts = Sequencing primers and associated sequencing temperature in ^{0}C

= primers developed in this study

Sequence Alignment

All data analysis was performed either on a Macintosh PowerPC G4 Cube (running MacOs 9.1) or a Flexii PII/Zxi PC (running Windows 98), using a variety of current molecular software packages.

Sequences were edited, aligned and confirmed using the programme Sequencher v3.0™ (Gene Codes Corp.). Cyt *b* sequences were aligned with reference to published sequence data from *Geochelone denticulata* (accession number: AFI192941), *Geochelone yniphora* (accession number: AFA020896), *Pyxis arachnoides*, *Pyxis planicauda* and *Testudo hermanni*, all of which were downloaded from GENBANK (NCBI web site). ND4 sequences were aligned with a published sequence of the African side-necked turtle, *Pelomedusa subrufa* (accession number: NC_001947).

There were no gaps present in the 429 bp segment of the Cyt *b* sequence for 156 individuals, including the outgroup taxa. For the ND4 sequence, however, there were several insertion and deletion events, particularly in the last 150 bp, which correspond with the tRNA regions of the gene product. This portion of the gene could not be unambiguously aligned across all the sequenced taxa due to the high levels of variability. Consequently, the homology of these regions could not be inferred with any degree of certainty, thus these regions were removed from further analysis (244 bp were excised). The final aligned product of the ND4 gene consisted of 738 bp.

The sequence alignment file was imported into MacClade 4.0 (Maddison and Maddison 2000) and converted into a Nexus file format (Maddison et *al.* 1997) for subsequent implementation in the phylogenetic inference package, PAUP* v4.0b2 (Swofford 1999). The final alignment file is not reported in this thesis but is available on request.

Both the Cyt *b* sequence data and the ND4 sequence data were translated into the corresponding amino acid sequence in MacClade v4.0 (Maddison and Maddison 2000), using the vertebrate mitochondrial code. The sequences were checked for stop codons. This translation procedure ensures the accuracy of the original alignment, as any incorrectly assigned insertion or deletion events are likely to interrupt the reading frames of these protein-coding genes. The Cyt *b* fragment contained 143 amino acids and the ND4 fragment contained 246 amino acids.

Data partitions

A combined data set was created by concatenating the 429 bp of the Cyt *b* sequence to the end of the 738 bp of ND4 sequence in MacClade v4.0 (Maddison and Maddison 2000), producing a new segment consisting of a total of 1167 bp (389 amino acids) for a total of 167 individuals, including the six outgroup taxa (Table 4.1). It has been shown that bias in the number of taxa present in different data partitions may affect the outcome of the phylogenetic estimate (Wiens and Hollingsworth 2000, Johnson 2001). For this reason only taxa that had entire sequence lengths for both the Cyt *b* and ND4 gene were combined in the formation of the combined data set.

Gene Statistics and the Phylogenetic Utility of the Selected Genes

Basic statistical summaries for both the gene segments utilised in this study were calculated using MEGA 2.1 (Kumar et *al.* 2001). These included base composition, base compositional bias (Irwin et *al.* 1991), number of transitions and transversions, and the percentage of variable and informative sites.

It has been established that variation in the base composition or nucleotide frequency between species or lineages can induce systematic error, and consequently affect the topology of the phylogenetic estimate (Lockhardt et *al.* 1994). This is of particular significance if a model that assumes these base frequencies to be equal across all lineages is invoked (Kirsch and Pettigrew 1998, Foster and Hickey 1999). For this reason base frequency stationarity among the sequences was evaluated for each data set using the chi-squared heterogeneity test implemented in PAUP* v4.0b2 (Swofford 1999). This was carried out using all sites, excluding constant sites and 1st, 2nd and 3rd positions respectively. The test was repeated with and without outgroup taxa to ensure that the presence of the more divergent outgroups did not bias the results (Cameron and Mardulyn 2001). Because this test has been shown to be phylogenetically biased i.e. it treats each taxon as an independent data point, minimum distance LogDet/paralinear transformed distance trees were included in the analysis to test for the effects of possible non-stationarity amongst the base frequencies (Fishbein et *al.* 2001).

In order to extract the maximum amount of information from a data set, it is important that there is sufficient signal in the gene under study to allow for the determination of relationships between lineages. Furthermore, it is important that the rate of evolution, or accumulation of phylogenetic signal in the gene, is closely matched to the depth of

evolutionary divergence required for the study (Graybeal 1993). The presence of phylogenetic signal, or signal that is significantly different from that expected from random, was evaluated using the g1 skew test (Hillis and Huelsenbeck 1992) implemented in PAUP* v4.0b2 (Swofford 1999). The tree length distributions were generated from 10000 trees drawn at random for each data set and included all sites and 1st, 2nd and 3rd positions respectively. Data sets with significant phylogenetic signal display left-skewed tree distributions as measured by the g1 test statistic (Hillis and Huelsenbeck 1992).

Although mitochondrial DNA is considered a single evolutionary unit, the genes that it encodes do not evolve at the same rate. The types and rates of the mutations that do occur vary within and between the protein and non-protein coding regions (Kumar 1996). This results in variation in the performance of various genes at different phylogenetic levels (Graybeal 1993, Zhardoya and Meyer 1996). In addition, as divergence time between lineages increase so does the number of sites that may become saturated due to the occurrence of multiple hits. The homoplasy that results may then affect the phylogenetic utility of the genes under study by obscuring the phylogenetic signal and in some cases converging on an incorrect phylogenetic inference. This is a particular problem when studying deep level divergences (Graybeal 1993). In order to determine the amount of saturation, if any, at the 1st, 2nd and 3rd codon positions of the Cyt *b* and ND4 genes, saturation plots were constructed in DAMBE (Xia and Xie 2001). The uncorrected sequence divergence (total number of observed transitions and transversions) is plotted against a corrected distance measure (F84) for each of the 1st, 2nd and 3rd codon positions respectively. Initially, the relationship should be linear i.e. the number of observed changes is equal to the number of expected changes under the corrected model, but as the evolutionary distance increases so to do the number of multiple hits. Saturation of the gene is then reached when the plot plateaus, even as evolutionary distance is theoretically still increasing (Zamudio et *al.* 1997).

A further measure of potential homoplasy in a data set can be measured by plotting the number of steps or changes observed for each site in the sequence under study. The greater the proportion of sites that have a large number of state changes, the greater the potential homoplasy present and the lower the phylogenetic utility of that gene. Frequency distributions were generated using MacClade v4.0 (Maddison and Maddison 2000) and calculated for 1st, 2nd and 3rd codon positions respectively for each gene, using the most

parsimonious tree constructed for each data set (Voelker and Edwards 1998, Mardulyn and Cameron 1999).

Genetic Distance Measures

Pairwise distance matrices, displaying uncorrected sequence divergence values, were constructed in MEGA v2.1 (Kimura et *al.* 2001). Pairwise distance matrices incorporating Kimura-2-Parameter corrections, Tamura-Nei corrections with and without gamma distributions and maximum likelihood (Waddle and Steel 1997) distance estimates were calculated using PAUP* v4.0b2 (Swofford 1999). Matrices were constructed for the Cyt *b* and ND4 sequences individually, with all the available sequence data (160 and 167 individuals respectively), and for the combined data matrix (167 individuals in total). Individuals within a species complex that differed by 0.1% - 0.5% sequence divergence were excluded from further analysis and species monophyly was therefore assumed (Danforth and Ji 2001). The final data sets consisted of 50 individuals (including two outgroups) for the Cyt *b* gene, 55 individuals (including three outgroups) for the ND4 gene and 55 individuals (including three outgroups) for the combined data set. Outgroup selection can have a profound influence on the topology of the phylogenetic inference (Milinkovitch and Lyons-Wheiler 1998) and for this reason a range of evolutionarily related taxa were selected (Sites et *al.* 1996, Matthee and Davis 2001). All inference methods were implemented both with and without outgroup taxa in order to determine their influence on the resulting tree topologies. *T. coahuila, T. ornata* and *L. kempii* were selected as outgroup taxa in the reduced data sets. The former two species are members of the Emydidae and are closely related to the Family Testudinidae (see Chapter 2), whilst *L. kempii* is an older lineage representing the Cheloniidae.

The Cyt *b* sequence data set contained fewer taxa than the ND4 data set. This was because *D. arnoldi* (1F), Rocket (1D), *G. pardalis babcocki* (9G) and *P. tentorius verroxii* (40A) failed to amplify sequences that were of sufficient quality to merit inclusion, despite repeated attempts at amplification. The Cyt *b* amplification products for these four species were subsequently cloned into an A-T Cloning Vector (Promega) but failed to produce quality sequence data.

An initial unweighted parsimony analysis of the Cyt *b* data set failed to establish *L. kempii* as an outgroup taxon. It was therefore excluded from the Cyt *b* data set but retained in both the ND4 and the combined data set. Both *Terrapene* samples were retained as outgroup taxa for the Cyt *b* data set.

Combining Data Partitions

Combining sequence data for a total evidence approach is highly recommended for phylogenetic reconstruction (see Chapter 3). The phylogenetic information contained within the different partitions may be cumulative, and by combining the data will result in the concurrent dispersion of backround noise that may otherwise limit phylogenetic resolution (Miyamoto et *al.* 1994). Although this is the general consensus in current phylogenetic literature there have been some exceptions where the combining of heterogenous data partitions has not necessarily resulted in a more strongly supported phylogenetic estimate (Funk et *al.* 1995). Despite these examples, it has generally been shown that by increasing the number of characters available for analysis, the probability that the resulting inference will converge on the true topology will also increase. This is a phylogenetic property known as consistency (Slowinski and Page 1999). The addition of characters from an alternative gene has been shown to improve the resolution of phylogenetic estimates, even more so than the addition of further taxa or more characters from the same gene (Mitchell et *al.* 2000). For this reason the Cyt *b* gene sequences and the ND4 gene sequences were combined to create a combined data partition of 1167 bp (see Data Partitions above). The Conditional Combination Approach advocated by Bull et *al.* (1993) was used in the analysis.

Despite the fact that both genes are mitochondrial genes, and are therefore inherited as a single, non-recombining locus (Avise et *al.* 1987), they may yield incongruent phylogenetic estimates due to the different evolutionary processes that define them (Cameron et *al.* 1992). There are several tests available that are able to assess the level of heterogeneity (if any) in the different data partitions and this ultimately determines whether the data sets should be combined in the phylogenetic inference (but see caveats in Sullivan 1996).

Each of the data sets were initially analysed separately and support for conflicting nodes was assessed using the non-parametric bootstrap (Felsenstein 1985) (see Phylogenetic Analysis for the implementation of the heuristic searches). This allows for the identification of nodes that are in strong disagreement between the two data partitions. Although it is not a statistical measure of heterogeneity, it does give an initial indication of the potential incongruence displayed.

The Incongruence Length Difference test (ILD), proposed by Farris et *al.* (1994), was used to test for underlying heterogeneity between the Cyt *b* and the ND4 data partitions. The test is based on the significance of observed differences in the tree lengths obtained when analysing the data sets i) individually, and then ii) randomly repartitioning the combined data

set into partitions that are of equal size to the original partitions, so as to generate a null distribution. The test evaluates whether or not the phylogenetic signal underlying the characters in each of the data partitions are in significant conflict. If the two data partitions are found to strongly support alternative trees, the combined analysis will show significantly longer tree lengths than the sum of the individual trees. In this case the combined set will display more homoplasy and the individual data partitions would be considered representative of significant incongruence or heterogeneity (but see Yoder et *al.* 2001).

The ILD test was also used to determine whether any significant heterogeneity existed in the phylogenetic signal displayed between the 1st, 2nd and 3rd codon positions of the Cyt *b* and ND4 genes respectively. Each gene was "partitioned" into three data sets representing each of the codon positions.

The ILD test was carried out in PAUP* v4.0b2 (Swofford 1999) under the "partition homogeneity test". All parsimony uninformative characters were excluded before analysis, as recommended by Cunningham (1997). The data sets were analysed with 1000 bootstrap replicates, five random addition replicates for each pseudoreplicate and with tree bisection and reconnection (TBR) branch swapping in effect.

Phylogenetic Analysis

Three methods of phylogenetic inference were utilised: Parsimony (Fitch 1971), Maximum likelihood (Felsenstein 1981) and Bayesian analysis (Rannala and Young 1996). The comparison of topologies obtained under different optimality criteria allows for the elucidation of confounding artifacts in the sequence data that may be more susceptible to the models invoked under one or the other of these methods of inference. Furthermore, all analyses were repeated with and without the outgroup taxa to determine the effects, if any, of the outgroup selection on the tree topologies. Each nucleotide was treated as an unordered character with four alternative states in all the data sets.

All analyses were implemented in PAUP* v4.0b2 (Swofford 1999) with the exception of the bayesian analysis, which was implemented in the programme MrBAYES v2.01 (Huelsenbeck and Ronquist 2001).

Optimality Criterion

i. Parsimony

a. A priori weighting schemes

All sequence data sets were initially analysed using unweighted parsimony with all nucleotide positions and substitution types weighted equally. The heuristic search strategy was conducted with TBR branch swapping, MulTrees option in effect and zero branch lengths collapsed. Support for individual clades was assessed using non-parametric bootstrapping (Felsenstein 1985), with 1000 pseudoreplicates and ten random addition replicates for each pseudoreplicate. Random addition replicates were included in all phylogenetic analyses to compensate for the potential effects of entry sequences on the resulting tree topologies (Wiens et *al.* 1999). All parsimony analyses were conducted with non-informative characters excluded, as these are known to inflate tree statistics (Sanderson ad Donoghue 1989). Several tree statistics were recorded for each tree analysed, and these included consistency indices (CI) (Kluge and Farris 1969), retention indices (RI) (Farris 1989) and tree length scores. These statistical measures provide an indication of the goodness of fit between the data and the topology. Consistency Index values provide an indication of the amount of homoplasy present in the data set, whilst RI values measure the amount of similarity present in the phylogenetic signal of the informative characters.

Within the parsimony framework, there are several methods available that allow for the modeling of evolutionary processes. These include the differential weighting of codons and

the differential weighting of substitution types (see Chapter 3). These methods are based on knowledge of the mutational processes underlying the sequences of interest. Estimates are incorporated into the inference procedure to compensate for the different constraints under which different classes of codons or substitution types are known to occur relative to other classes (Funk et *al.* 1995). The use of these weighting schemes has been shown to decrease the zone of inconsistency in simulation studies (Hillis et *al.* 1996). For example, transitions are known to occur more frequently than transversions and are therefore more likely to determine the levels of homoplasy that can ultimately affect the phylogenetic inference. To account for this, transversions can be afforded a higher weight than transitions in the parsimony analysis (Swofford and Olsen 1990). These methods have been shown to improve the level of support for clades as shown by non-parametric bootstrapping (Allard and Carpenter 1996, Yoder et *al.* 1996). Transition biases were calculated for each gene and for the combined data set and subsequently for 1st, 2nd and 3rd codon positions for each gene and the combined data set. One of the criticisms of differential *a priori* weighting is the assignment of arbitrary values or weighting schemes that are based on parallel studies of closely related groups or organisms and not derived directly from the data set under study (Voelker and Edwards 1998). To account for this, all values pertaining to the transition bias for each data set and codon position were calculated using information obtained directly from the sequence data and calculated in MacClade v4.0 (Maddison and Maddison 2000) or estimated from ModelTest v3.06 (http://bioag.edu/zoology/crandall_lab/modeltest.html, Posada and Crandall 1998).

Several different weighting schemes were implemented in each analysis to account for the observed transition bias and to assess the effects of this bias on the tree topology. Weightings ranged from a transversion: transition ration (Ti/ Tv) of 3:1 to 12:1. In addition, step matrices were generated for the codon specific transition biases and each codon position was assigned a codon-specific step matrix. The search strategy was identical to the one implemented for the unweighted analysis with 1000 pseudoreplicates and ten random addition replicates for each pseudoreplicate and TBR branch swapping to completion.

The downweighting of 3rd codon positions in parsimony analysis is especially recommended as these positions are known to accumulate transitions more rapidly due to the degenerate nature of the genetic code. Furthermore, it is recommended that these positions be removed entirely from the analysis if they are found to display high levels of homoplasy. However, a proportion of these sites may not actually be saturated and, as such, could still provide

information at a particular level of phylogenetic inference. The removal of such sites would therefore represent an unjustified loss of phylogenetic information (Yoder et *al.* 1996, Alvarez et *al.* 1999). The downweighting of 3rd position codons was therefore selected as opposed to their complete removal from the data sets.

b. A posteriori weighting schemes

The data sets were also analysed using successive weighting, a method advocated by Farris (1969). The characters are iteratively weighted based on their rescaled consistency indices, as calculated from an initial unweighted parsimony tree. Each character is weighted according to the amount of homoplasy it exhibits, such that characters that display high levels of homoplasy are given lower weights, whilst those displaying low levels of homoplasy are given higher weights.

c. Measures of Clade Robustness

Non-parametric bootstrapping was used to determine the levels of confidence that could be placed in each of the branches of the resulting phylogenetic inference (Felsenstein 1985). Bootstrap proportions are used extensively in the phylogenetic framework, despite the fact that there is debate as to the significance of the values associated with them (Hillis and Bull 1993, Zharkikh and Li 1992a, 1992b). The significance of the bootstrap values were classified as follows 50% - 69% weak support, 70% – 89% medium support and 90% – 100% strong support (Hillis and Bull 1993).

Decay Indices (DI) or Bremer support, based on the number of extra steps required before the monophyletic clade in question collapses (Bremer 1988, Donoghue et *al.* 1992), offers a further indication of the strength associated with particular nodes in a phylogenetic estimate. However, DI values are particularly difficult to interpret, even among nodes on the same tree derived from the same data, largely due to the fact that nodes can differ in the number of parsimony informative characters that influence or support a particular relationship (DeBry 2001). For this reason, only bootstrap proportions were used in the framework of this thesis for the assessment of the strength of clade support.

ii. Maximum likelihood

Maximum likelihood was used to determine the best-fit models of sequence evolution for each of the data sets and subsequently search for the optimal tree topologies represented by these model parameters. In addition, maximum likelihood was used to compare the relative natural log likelihoods of the tree topologies obtained under parsimony analyses and from bayesian estimates. This is because natural log likelihood scores provide a common denominator with which to compare different tree topologies, whereas the parsimony tree score depends on the specific weighting schemes employed and is therefore difficult to compare across trees (Sullivan and Swofford 2001). All maximum likelihood inferences were calculated using PAUP* v4.0b2 (Swofford 1999).

ModelTest v3.06 (Posada and Crandall 1998), implemented in PAUP* v4.0b2 (Swofford 1999), was used to assess each of the sequence data sets. ModelTest uses a hierarchical likelihood ratio test (hLRT) to determine the best-fit model of sequence evolution that best explains the substitution processes underlying each of the data sets. A total of 56 possible models can potentially explain the underlying data and these are compared sequentially and within a nested hierarchy. The programme calculates the likelihood score (reported as a natural log likelihood) for a specified topology using each of the available models, with all parameters estimated directly from the sequence data e.g. base frequencies. The natural log likelihood scores for each tree are then compared using the hLRT (Huelsenbeck and Crandall 1997) and the AIC (Aikake Information Criterion) (see Chapter 3, Aikake 1974). When the addition of a model parameter no longer significantly increases the likelihood score, as measured by the hLRT or AIC (see Chapter 3), that model is then taken as the putative best–fit model of evolution for that data set. The models tested range from the simplest, Jukes-Cantor (JC) (Jukes and Cantor 1969), which assumes equal rates of change for all substitution types and equal base frequencies, to the most complex, that of the General Time Reversible model (GTR) (Yang 1994), which assumes all substitution types occur at a specific rate and that base frequencies are not necessarily equal.

Rate heterogeneity among sites was accommodated by including one or both of the following parameters in each model tested: the proportion of invariant sites (P*inv*) (Hasegawa et *al.* 1985) and the discrete gamma distribution, Γ (Yang 1994) e.g. JC+Γ+P*inv*, HKY85+Γ or GTR+Γ+P*inv*.

The starting trees from which all parameters were estimated, were obtained from either a neighbour joining (NJ) analysis or the best estimate parsimony topology. The estimated parameters are thought to be relatively robust to starting topology (Yang 1994, Sullivan et *al.* 1995, Frati et *al.* 1997). To ensure that there was no bias introduced from alternative starting topologies, all parameters were estimated from both a NJ tree and a parsimony topology for each data set.

The parameters obtained from ModelTest v3.06 were used in a maximum likelihood analysis with TBR branch swapping, five random addition replicates, zero branch lengths collapsed and the MulTrees option in effect. If an alternate model had been selected with the AIC (Aikake Information Criterion), the maximum likelihood inference was repeated with these parameters to determine what effect the selected models would have on the subsequent topology.

The model parameters were re-estimated from the subsequent inference obtained for each data set and compared with the original values to ensure that the maximum likelihood score had converged on its maximum value, with optimal parameter estimation (Swofford et *al.* 1996). Clade support for maximum likelihood inferences was estimated using non-parametric bootstrapping with 100 pseudoreplicates and one random addition replicate for each pseudoreplicate.

Each analysis was repeated with and without the outgroup taxa to determine what effect these more divergent sequences had on parameter estimation and subsequent topology.

iii. Bayesian Inference

Bayesian methods produce probability estimates associated with both individual branches and entire tree topologies, and provide a statistical measure of the reliability of the inferred estimate of phylogeny, using a maximum likelihood framework. This holds an advantage over other methods of assessing robustness, for example non-parametric bootstrapping used in conventional maximum likelihood analysis and parsimony inference, which is time consuming and the interpretation of which remains contentious (see Chapter 3).

The programme MrBAYES 2.01 (Huelsenbeck and Ronquist 2001, Http://brahms.biology.rochester.edu/software.html) was used to infer bayesian estimates of phylogeny for each of the data sets. The programme uses a variant of the Markov Chain

Monte Carlo procedure to approximate tree topologies and parameters for maximum likelihood models of sequence evolution and periodically samples the posterior probability distribution. The frequency with which particular topologies or branches are sampled indicates their probability in the final estimate. The posterior probability of any clade in the final phylogenetic inference is the sum of the posterior probabilities of all the trees sampled that contained that clade (Huelsenbeck and Ronquist 2001).

The best-fit models of evolution for each of the data sets were shown to consist of the GTR+Γ+P*inv* models, as defined by ModelTest v3.06. As a result, this parameter-rich model was used as the specified model of evolution for all bayesian runs. The following starting parameters were used in MrBAYES: lset nst=st (the GTR model), rates=invgamma (site specific variation such that some sites are invariant and the rest are drawn from the gamma distribution i.e. +Γ+P*inv*) and base frequencies=estimate (base frequency proportions were estimated from the data set). The default settings for the priors (see Chapter 3) were set as follows: rate matrix = 1 (rGT=1, rCT=1, rCG=1, rAT=1, rAG=1, rAC=1), base frequencies = 0.25, gamma distribution = 0.5 and the proportion of invariant sites = 0.1. A "flat" or "uninformative" prior was used as a starting topology.

Four Metropolis Coupled Markov Chains were run simultaneously. Each chain was initiated from a randomly generated starting topology, which acts to increase the probability of finding alternative peaks in the tree space. Each chain was run for two million generations, with probabilities sampled every 50 generations. This gave 40 000 trees in the initial sample. The value of the likelihood scores generated for each tree was monitored graphically and stationarity or "burn-in" was shown to have occurred by the 1500th tree i.e. the parameters estimated from the posterior probability space were converging on stable estimates by the 1500th tree. After the 1500th tree, there was no indication of a significant increase in the likelihood scores. The initial 4000 trees were discarded and were not used in the calculation of the final probability estimate (Huelsenbeck and Ronquist 2001). The posterior probabilities of the clades represented in the final phylogenetic estimate were determined from the remaining 36000 trees, using the 50% majority rule consensus option in PAUP* v4.0b2 (Swofford 1999). Each run was repeated for each data set to ensure that they converged on the same topologies.

Sub-tree Analysis and the Construction of Reduced Data Sets

Initial analysis of the data sets, particularly the ND4 data and the combined data, resolved two assemblages of interest within the trees. The first included the grouping of the majority of the African taxa within a large derived clade that also contained the Asian, South American and Ocean Island representatives of the *Geochelone*. The second clade consisted of the North American genus *Gopherus*, the Southeast Asian *Manouria* spp., the Indian *Indotestudo* spp. and the European representatives of the *Testudo* spp., including the genus *Malacochersus*. To further investigate the relationships within these assemblages and potentially improve computational efficiency and resolution, additional data sets were constructed and analysed according to the protocol outlined above for parsimony analysis, maximum likelihood analysis and bayesian inference.

The first additional data set (the African genera) consisted of all the African endemics (including *Malacochersus*), with *G. flavomarg*inatus and *M. impressa* selected as outgroup taxa. There were 25 individuals analysed for the Cyt *b* gene, the ND4 gene and the combined data set. The second assemblage (the *Manouria-Gopherus* clade) consisted of the genera *Manouria, Gopherus, Testudo, Indotestudo* and *Malacochersus*, with *L. kempii, T. coahuila* and *T. ornata* as outgroup taxa. This data set consisted of 20 individuals for the Cyt *b* gene, the ND4 gene and the combined data set.

Furthermore, initial analyses indicated longer branches associated with *Psammobates oculifer* and *P. tentorius*. Data sets containing all the sampled individuals for the genus *Psammobates* were constructed for the Cyt *b* gene, the ND4 gene and the combined data set and again rigorously analysed. The data sets contained 20 individuals, *with I. elongata* and *T. hermanni* as outgroup taxa.

Combining Morphological Characters and Molecular Characters in a Reduced Data Set

The morphological matrix used by Gerlach (2001) (Figure 2.4), in a cladistic analysis of cranial characters in the Testudinidae, was combined with the mitochondrial genes investigated in this study. The final matrix contained 21 individuals, with several species of the Testudinidae unavailable for analysis (Matrix 4.1). As discussed in Chapter 3 it is recommended that data partitions, should in so far as possible, contain equal numbers of taxa. For this reason, the original combined data set produced in this study and containing 55 individuals was pruned to contain only those taxa that were represented in the Gerlach (2001) matrix. This reduced data set consisted of 1167 bp of mitochondrial DNA and was

combined with the morphological matrix containing 66 morphological characters. This produced a new data set of 1233 characters for 21 taxa and the three outgroups included in the initial analysis. Morphological characters were not available for outgroups i.e. *L. kempii, T. ornata* and *T. coahuila* and the cranial characters were coded as missing in each of these cases. The combined data partition was analysed using differentially weighted parsimony (Ti/Tv of 9:1) for the molecular data and implemented in PAUP* v4.0b2 (Swofford 1999) with TBR branch swapping in effect, 10 random addition replicates and 1000 bootstrap replicates.

The incongruence length difference (ILD) test was used to determine the amount of heterogeneity displayed between the two data partitions, represented by the molecular and morphological characters. The test failed to converge on a probability estimate and was terminated after extensive computation time (100 hours). The data was combined irrespective of this negative result as the ILD test has been shown to be too conservative when estimating levels of heterogeneity between data partitions (Sullivan 1996).

Matrix 4.1: Morphological matrix modified from Gerlach 2001 (see Figure 2.4)

H.areolatus19C	00000120110000000110100011100000000000000010000010000001111001010 0
K.erosa31A	00000120110000000110111011100000000000010010000000000000010100101 01
G.gigantea1A	11011110000000110010001100100000000000000111010110000001000001000 0
G.carbonaria3A	00000020120000000010001100000000000000010001000001000100001110100 00
G.pardalis9B	11011110010000110010001001110000000001100111010000010101001001000 0
G.elegans7A	00000020010000011110000110010000000000000001001000001000000111010001
G.radiata11D	01011110000001111010001100100000000110201011010011101101001001000 0
G.sulcata12B	01010110010000011000001000100000000000010111010010010001011000000 0
G.yniphora13A	01011110000101111010001100100000010100001111010001001101001001000 0
C.angulata2A	00000120110000000110111011110000000100010010001000000000010100101 01
I.elongata14A	00000120110000000110101000100000000100000010011000000000000100100 00
G.agassizii15A	00100021121010001110001100100011111001010001000000211001001100000 00
G.polyphemus18A	00100021121010001110001100100011111001010001000000211001001100000 00
M.tornieri34A	00000120110000000100101101110000000100100100100000000000101100101 00
M.impressa60A	00000010010000010010001000101000010001000110010000000100101010101 1
P.geometricus37D	00000120110000000110100001100000000000000001000001000000101100101 00
P.arachnoides42A	01011110010000111111110110010000000100000001000000100100010100110 00
P.planicauda43C	01011110010000111111110110010000000100000001000000100100010100110 00
T.g.terrestris46A	00000120010000000110101111100000000100100000100013000000011001010 0
T.horsefieldi48A	00000120010000000110001111100000000100000000100013000001010001010 0
T.kleinmanni49A	00000120010000000110101111100000000100100000100013000000011001010 0

Constraint Analysis and Hypothesis Testing

Several tests are available for testing the significance of alternative tree topologies within a phylogenetic context. These include the Shimodaira-Hasegawa (S-H) test (Shimodaira and Hasegawa 1999), the Kishino-Hasegawa (K-H) test (Kishino and Hasegawa 1989) and the Templeton-Wilcoxon signed ranks test (Templeton 1983). Tree topologies, representing alternate phylogenetic hypotheses based on previously published phylogenies for the Family Testudinidae, were constructed in MacClade v4.0 (Maddison and Maddison 2000). The significance associated with these potential alternative topologies, when compared with the "best" estimate inference obtained from the analysis of the combined data set, were tested within a maximum likelihood framework using the S-H test. This test allows for the comparison of *a priori* and *a posteriori* hypotheses (see Chapter 3). The S-H test simultaneously compares multiple topologies and corrects the corresponding P-values to accommodate for multiple testing. All reasonable alternative hypotheses were provided for the calculation of confidence limits. It is important to note, however, that these pre-specified topologies should be carefully selected as the inclusion of too many is thought to increase the conservativeness of the test, thereby reducing the power of the test (Buckley et *al.* 2002). The "best" estimate topology i.e. the *a posteriori* hypothesis is always available for comparison against the alternative maximum likelihood topologies i.e. the *a priori* hypothesis (Goldman et *al.* 2000). The S-H test accounts for this potential bias by readjusting the expectation of the null hypothesis i.e. that the two trees are not different, a process known as "centering" (Buckley et *al.* 2001a). The test was implemented in PAUP* v4.0b2 (Swofford 1999) with full optimisation and 1000 bootstrap replicates. In each case the test was performed with the maximum likelihood parameters re-optimised for the particular data set from ModelTest v3.06 to minimise any selection bias.

The Wilcoxon signed ranks test (Templeton 1983) examines the statistical significance of the overall shortest tree relative to an alternative hypothesis. The test determines whether the most parsimonious tree is significantly shorter than an alternative tree, or if the observed length difference is due to chance alone (Larson 1998). This test, however, was devised to test the significance of the observed fit of the data to *a priori* hypotheses. In this study all comparisons of tree topologies involved the inclusion of at least one *a posteriori* hypothesis. There are recommended methods whereby the statistical significance of the values obtained by using the Wilcoxon or K-H tests, can be manipulated to account for the inclusion of *a posteriori* hypotheses (Goldman et *al.* 2000). However, the affects of these violations are not

fully understood. For this reason, only the S-H test was used within the framework of this thesis to test the significance of alternative tree topologies and for constraint analysis.

Molecular Clock Hypothesis

The relative rate test compares the amount of discrete nucleotide character changes between species and selected outgroups (see Chapter 3). It is assumed the ingroup taxa are of similar evolutionary divergence relative to the outgroup taxa and that their respective rates may be directly compared (Mindell and Honeycutt 1990). Pairwise Relative Rate tests for all species (Tajima 1993) were conducted using the Relative Rate test option in MEGA v2.1 (Kumar et *al.* 2001). This test was used to determine which (if any) of the species included in the study deviated from a null hypothesis of equal rates of evolution i.e. did not conform to a molecular clock.

Likelihood ratio tests were also used to determine whether the taxa conformed to a molecular clock hypothesis. Maximum likelihood analysis was carried out with a molecular clock enforced using parameters estimated from ModelTest v3.06. The clock model is considered a special case of the more generalised non-clock model, with rates of evolution or substitution constrained to being equal, rather than free to vary (see Chapter 3). The significance of the difference in the resulting likelihood scores of the null i.e. with no clock imposed (L_0) and alternative hypothesis i.e. with a clock imposed (L_1), was evaluated using the LRT statistic. The LRT is defined as $\delta = 2(\text{Log}L_1 - \text{Log}L_0)$ with $n - 2$ degrees of freedom, where n is the number of taxa, and the LRT statistic approximates a chi-squared distribution.

CHAPTER 5

RESULTS OF THE PHYLOGENETIC INFERENCE FOR THE FAMILY TESTUDINIDAE Batsch, 1788

Base Composition

The aligned sequence for the Cyt *b* sequence consisted of a total length of 429 bp with no gaps present. Over the entire length of the gene fragment, 206 sites (48.0%) were variable and 172 (40.1%) were parsimony informative (Table 5.1). The majority of the parsimony informative sites occurred at third codon positions (66%), followed by first codon positions (23.5%) with only a few at second codon positions (10.5%). The third codon positions showed a significant bias against guanine (G = 3.1%, Cb = 0.39) (Table 5.1). Gamma shape parameters (a) were calculated for each codon position using PAUP* v4.0b2 (Swofford 1999) and, as expected for protein coding genes, displayed substantial rate heterogeneity (Voelker and Edwards 1998). The low values of a for 1st and 2nd codon positions (a = 0.441 and 0.125 respectively) suggests that most sites have very low substitution rates or are invariable consistent with some degree of functional constraint while other positions are mutating very rapidly. The high values of the gamma shape parameter displayed at 3rd codon positions (a = 2.43) suggests that most of these sites have intermediate rates of change with a few sites evolving either very fast or very slow (Voelker and Edwards 1998) (Table 5.1).

The 738 bp fragment of the ND4 sequence displayed a similar pattern of nucleotide composition to the Cyt *b* gene (Table 5.1). There were 390 variable sites (53%) and 340 parsimony informative sites (46.1%) across the aligned sequences. The number of parsimony informative sites was marginally higher than the Cyt *b* gene at both the 1st and 2nd codon positions (26.5% and 12.5%). Most of the variability occurred at 3rd codon positions, which were represented by a high percentage of parsimony informative sites (62%) and a high inferred value for the gamma shape parameter (a = 2.167). Furthermore, 3rd codon positions were guanine deficient (G = 3.5%, Cb = 0.38).

The combined data set showed a homogenised pattern between the Cyt *b* gene and the ND4 fragment (Table 5.1). There were 1167 bp, of which 596 (51.1%) were variable and 512 (44%) were parsimony informative. Base frequencies were similar to each of the genes

individually with A = 34.4%, G = 12.5%, C = 27.1% and T = 26.0%. The low guanine content at 3rd codon positions implies that the majority of these sites will contain the other three nucleotides and, as a result, are more prone to the effects of saturation with increased divergence times (Irwin et *al.* 1991).

Base frequency statistics for both genes separately showed a strong bias against guanine (Zhang and Hewitt 1996) and high transition rates (Moritz et *al.* 1987), which was consistent with genes being mitochondrial as opposed to nuclear. Furthermore, the fact that there were no nonsense codons or stop codons when the genes were translated with the appropriate codon table, provided additional evidence that both fragments were functional proteins (Waters and Burridge 1999).

Table 5.1: Table showing base frequencies and gene description statistics for the mitochondrial DNA data sets of the Cyt *b* and ND4 genes and the combined data set. All analysis was carried using the programme MEGA 2.1 (Kumar et *al.* 2001) and PAUP* v4.0b2 (Swofford 1999).

DATA SET	Total sites	Variable sites (%)	Parsimony informative sites (%)	*NUCLEOTIDE FREQUENCY (%)*				%AT	Nucleotide Bias*	Gamma Shape Parameter(a)
				T	C	A	G			
CytB										
1st position	143	54 (26.0)	40 (23.5)	26.4	21.9	32.3	19.5	58.7	0.123	0.441
2nd position	143	26 (12.6)	18 (10.5)	37.9	23.0	21.8	17.4	59.7	0.172	0.125
3rd position	143	126 (61.1)	114 (66.0)	17.2	37.0	42.6	3.1	59.8	0.39	2.43
All sites	429	206 (48.0)	172 (40.1)	27.2	27.3	32.2	13.3		-	1.13
ND4										
1st position	246	104 (26.6)	89 (26.5)	19.3	26.8	35.7	18.2	55.0	0.17	0.591
2nd position	246	57 (714.6)	41 (12.5)	39.2	29.5	17.1	14.3	56.3	0.24	0.241
3rd position	246	229 (58.7)	210 (62.0)	17.8	24.9	53.9	3.5	71.7	0.38	2.167
All sites	738	390 (53.0)	340 (46.1)	25.4	27.0	35.6	12.0	61.0	-	1.12
COMBINED										
1st position	389	158 (26.5)	129 (25.2)	21.8	25.0	34.5	18.7	56.3	0.21	0.509
2nd position	389	83 (13.9)	59 (11.5)	38.7	27.1	18.8	15.4	57.5	0.25	0.163
3rd position	389	355 (59.5)	324 (63.0)	17.6	29.2	49.8	3.4	67.4	0.38	2.32
All sites	1167	596 (51.1)	512 (44.0)	26.0	27.1	34.4	12.5	60.4	-	1.11

*Nucleotide bias is calculated as Cb = (2/3) ? I Ci – 0.25 I where Cb = compositional bias and Ci = frequency of the ith base (Irwin et *al.* 1991)

Phylogenetic Signal

Both the Cyt *b* and the ND4 gene fragment displayed base composition homogeneity across all lineages sequenced in the study (Table 5.2). The base frequencies were similar across all sampled taxa and the results followed the same trends with the inclusion of outgroup taxa. The chi-squared test, although violating the assumption of non-independence amongst taxa, was not significant in all comparisons, which initially included all sites and subsequently excluded 1st, 2nd and 3rd codon positions respectively. In addition, LogDet/paralinear transformed distances and maximum likelihood distance estimates resulted in identical topologies, confirming base frequency stationarity across lineages.

Both the Cyt *b* and ND4 gene contained significant non-random structure and the presence of strong phylogenetic signal with correlation among characters was confirmed (Table 5.2). This was assessed using the g1 test statistic and the associated significance tables (Hillis and Huelsenbeck 1992). The g1 test displayed significantly left-skewed tree distributions represented by negative g1 values as calculated in PAUP* v4.0b2 (Swofford 1999).

The Partition Homogeneity Test (ILD test) confirmed that the two gene fragments (Cyt *b* and ND4) did not contain significantly different phylogenetic signal ($p = 0.24$) and so could be combined in the subsequent analysis (Table 5.2). Potential heterogeneity between the three codon positions within each gene was also assessed. The results were non-significant for the ND4 fragment but in the case of the Cyt *b* fragment could not converge on an answer and the run was terminated after 42 hours of computation time.

Table 5.2: Table showing the results of tests to determine the phylogenetic utility of the Cyt *b* and ND4 genes and the combined data set. These tests included the g1 skew test for phylogenetic signal, the chi-squared test for base frequency stationarity and the Partition Homogeneity Test all implemented in PAUP* v4.0b2 (Swofford 1999).

DATA SET	g1 Skew Test	Chi-Squared Test			Partition Homogeneity Test
	g1	χ2	d.f.	P	
CytB					
Excluding 1st position	-0.29*	45.1	147	1	-
Excluding 2nd position	-0.46*	60.1	147	1	-
Excluding 3rd position	-0.264*	12.39	147	1	-
All sites	-	37.4	147	1	-
Excluding constant sites	-0.34*	81.18	147	0.99	Not calculated[1]
ND4					
Excluding 1st position	-0.46*	71.03	162	1	-
Excluding 2nd position	-0.518*	119.99	162	0.99	-
Excluding 3rd position	-0.614*	27.82	162	1	-
All sites	-	80.80	162	0.99	-
Excluding constant sites	-0.56*	175.06	162	0.22	0.73
Combined					
Excluding 1st position	-0.60*	96.46	162	0.99	-
Excluding 2nd position	-0.60*	157.98	162	0.57	-
Excluding 3rd position	-0.40*	28.47	162	1	-
All sites	-	101.36	162	0.99	-
Excluding constant sites	-0.48*	216.15	162	0.2	0.24

d.f. = degrees of freedom
*significant at $p < 0.01$ (Hillis and Heulsenbeck 1992)
[1] test was unable to converge on an answer

Saturation Curves

Uncorrected sequence divergence estimates varied between the two gene fragments sequenced and ranged from 0% - 18.5% (excluding outgroups) for the Cyt *b* sequence and from 0% - 24% (excluding outgroups) for the ND4 gene fragment. The evolutionary relationships between lineages may become obscured if sites become saturated by unobserved multiple substitutions (Swofford et *al.* 1996), which is particularly problematic when analysing ancient radiations (see Chapter 3). To test for this potential saturation of sites and subsequent homoplasy within the sequence data, the Cyt *b* and the ND4 gene fragments were independently tested for saturation by plotting the observed transitions and transversions observed for each codon position against a corrected F84 sequence divergence estimate.

For the Cyt *b* gene fragment the 1st and 2nd codon positions appeared relatively uninformative as there was no linear increase in the number of transitions and transversions with increasing genetic distance (Figure 5.1). The third codon transitions and transversions appeared to be informative and the plot was linear up to approximately 10% sequence divergence of the Cyt *b* gene. The majority of the pairwise divergence estimates seemed to fall within the 11% - 18% sequence divergence range.

In the ND4 sequence the first codon position showed a linear increase in the number of transitions and transversions until approximately 13% sequence divergence (Figure 5.2). At estimates larger than 13% there appeared to be a clustering of the estimates between 14% and 25% sequence divergence. The number of transitions was higher than transversions for all positions. The second codon positions were relatively conserved, whilst third codon positions showed a positive linear relationship between the number of transitions and transversions and increasing genetic distance, with transitions appearing to saturate at approximately 12% sequence divergence.

It is important to bear in mind, however, that the comparisons among taxa are not independent and the saturation curves can not be interpreted in a strictly linear fashion. The absence of a linear plot for the codon positions does not necessarily equate to an absence of phylogenetic signal. This is particularly true for 1st and 2nd codon positions in the Cyt *b* gene. These sites are the most conservative given the deep level divergences and as a result possibly the most informative, despite the absence of a linear increase in transitions and transversions.

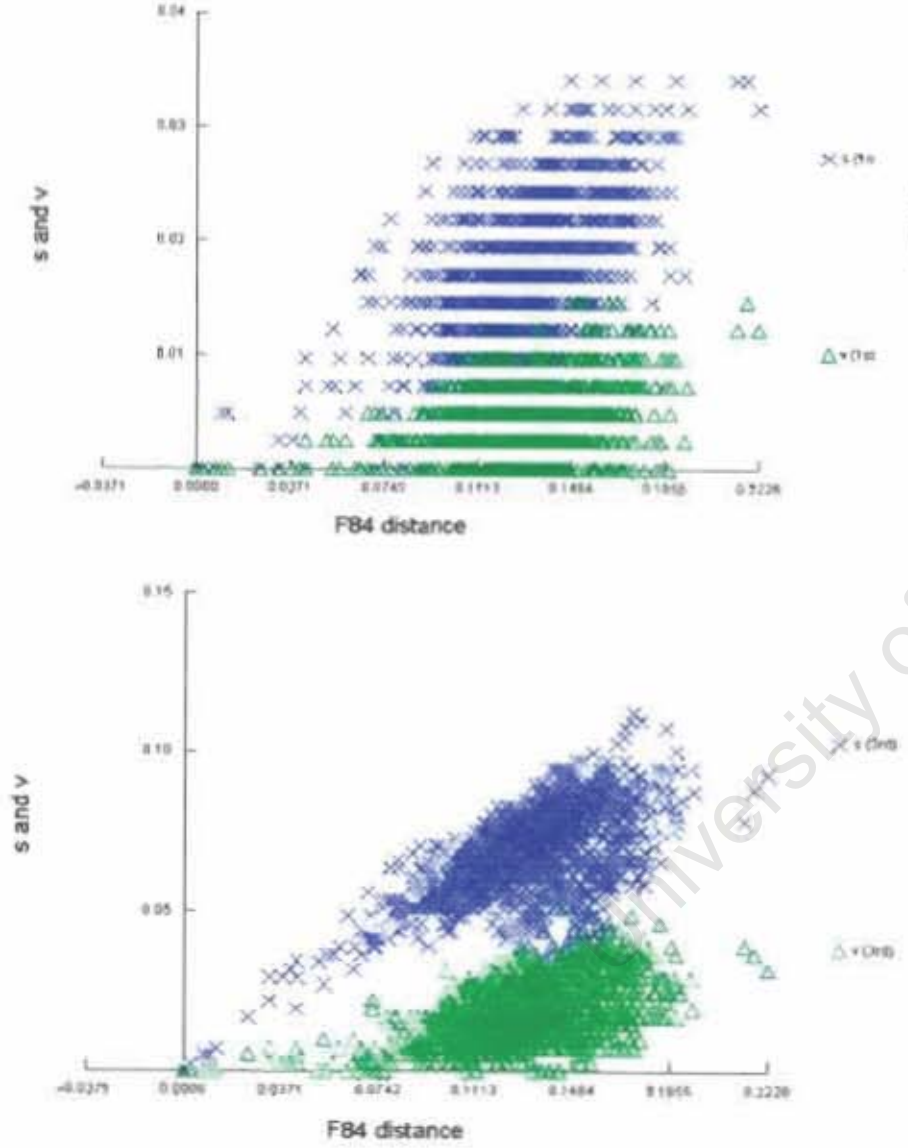
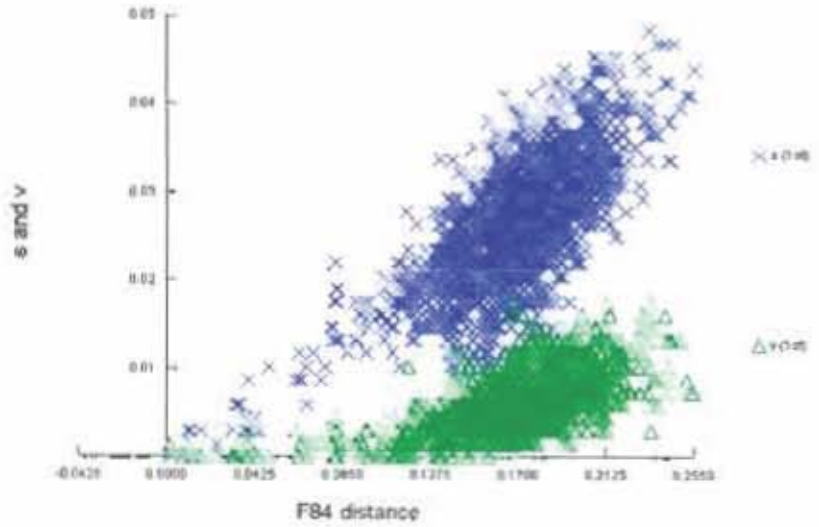
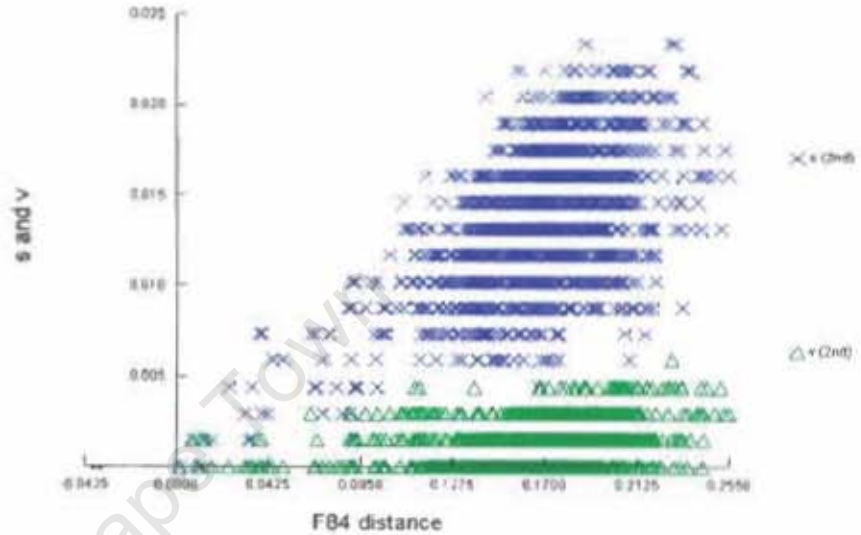


Figure 5.1: Saturation plots for the cytochrome *b* gene fragment. Observed number of transitions and transversions (s and v) are plotted against a corrected sequence divergence estimate (F84) for the 1st, 2nd and 3rd codon positions respectively. Triangles represent transversions whilst transitions are represented by blue crosses. Each data point represents a pairwise comparison between individuals in the data set, including outgroups.





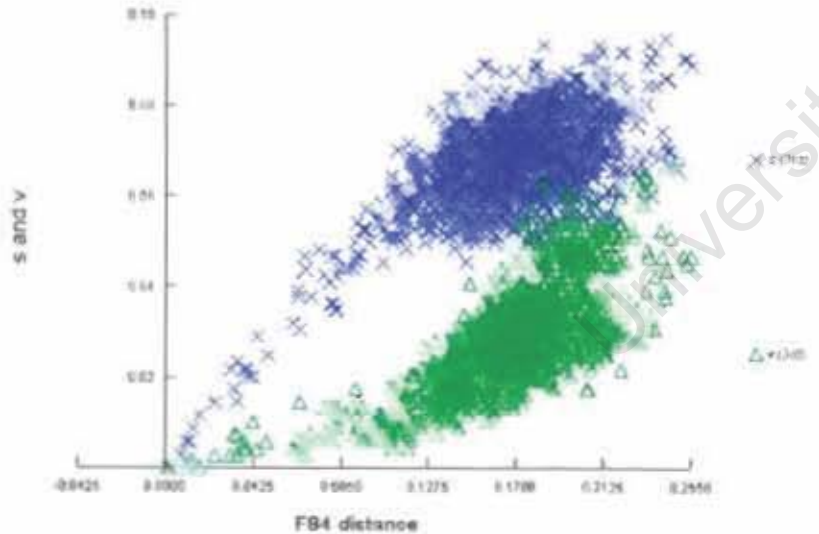
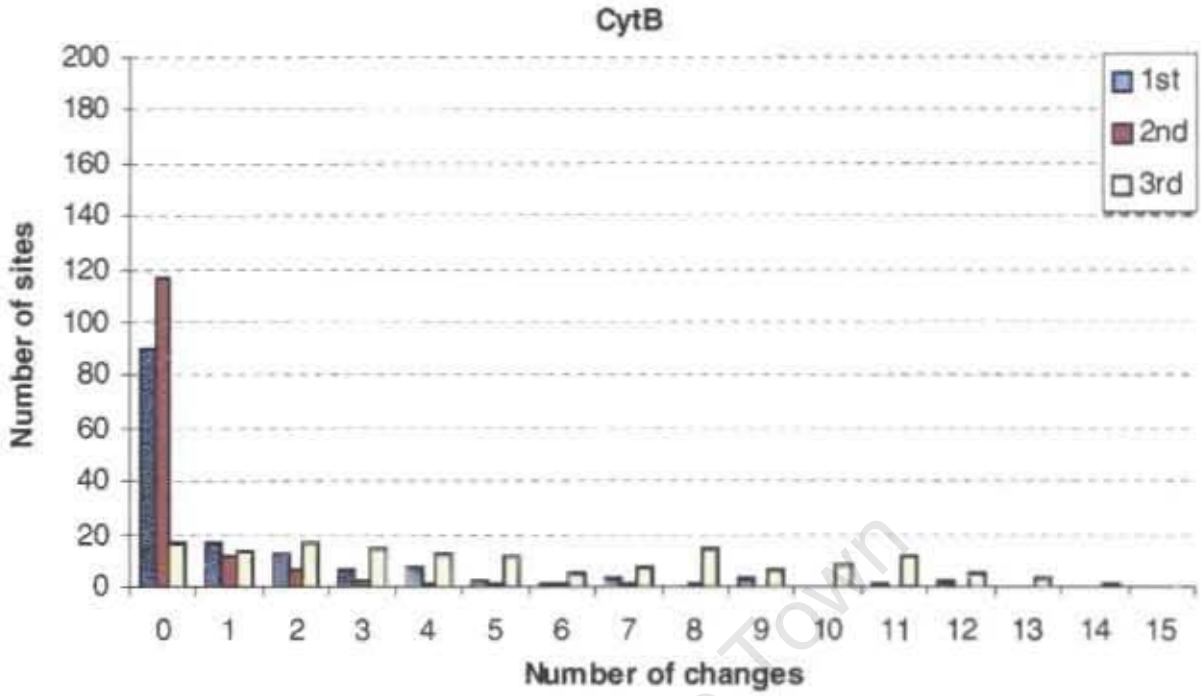


Figure 5.2: Saturation plots for the ND4 gene fragment. Observed number of transitions and transversions (s and v) are plotted against a corrected sequence divergence estimate (F84) for the 1st, 2nd and 3rd codon positions respectively. Triangles represent transversions whilst transitions are represented by blue crosses. Each data point represents a pairwise comparison between individuals in the data set, including outgroups.

An additional method to determine potential homoplasy is to plot the number of changes associated with each site or character in the sequence data set. These estimates are based on the mapping of the number of changes associated with each site, for each codon position, on the most parsimonious tree (Voelker and Edwards 1998) (see Materials and Methods) (Figure 5.3 and 5.4). Second codon positions for both gene fragments appeared relatively invariant with only a few characters requiring more than three steps on the most parsimonious tree. First codon positions showed limited saturation with some characters requiring up to six changes (ND4). The third codon positions for both genes showed an increase in the number of changes associated with these sites (see gamma shape parameters in Table 5.1), and there were as many as thirteen changes for third codon position sites, indicating potential homoplasy in the data sets. The overall levels, however, were higher for the Cyt *b* sequence. Nucleotides that exhibit these multiple changes are more likely to be saturated and thus potentially uninformative phylogenetically (Mardulyn and Whitfield 1999).



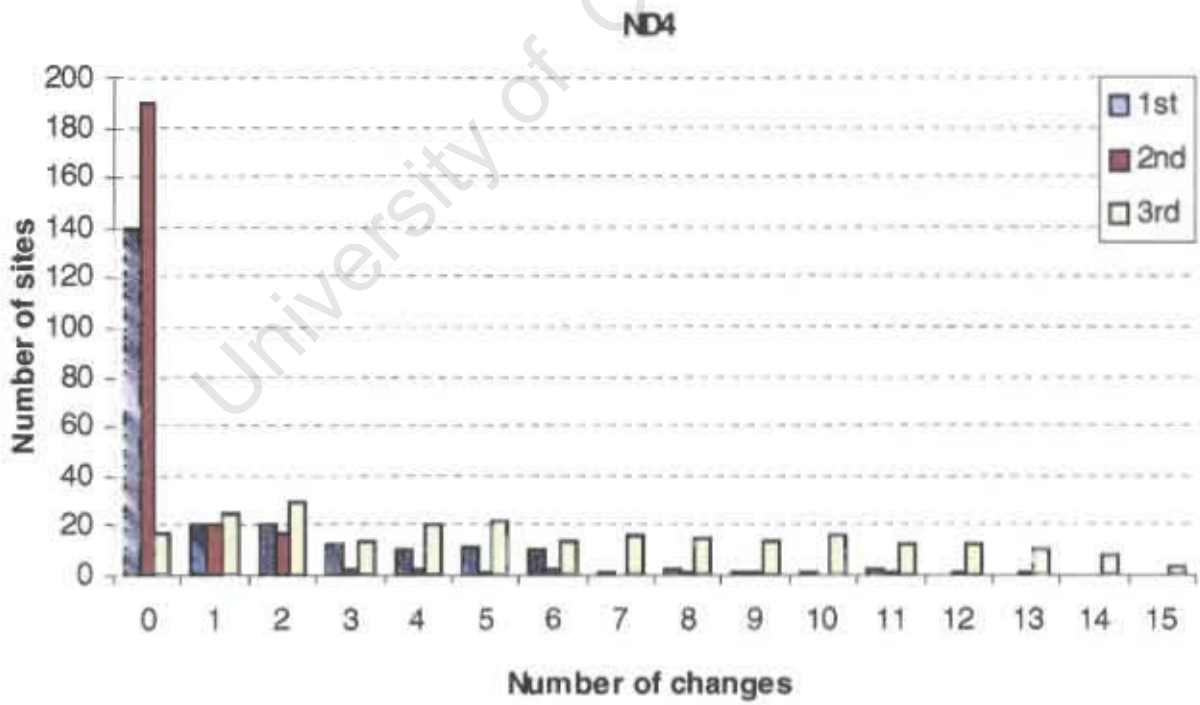


Figure 5.3 and 5.4: Bar charts showing the number of steps or changes associated with the characters at each of the 1st, 2nd and 3rd codon positions in both the Cyt *b* (top) and ND4 (below) gene fragment. The plot gives an indication of codon-specific rate variation in the sequence data.

Corrected Sequence Divergence Estimates

The analysis of saturation plots and rate heterogeneity among codon positions for both the Cyt *b* and the ND4 sequence fragments indicated that both genes could be susceptible to the effects of multiple hits that could obscure the phylogenetic signal in the data sets. To account for this, corrected sequence divergence estimates using the Kimura-2-parameter model, that allows for differences in the transition and transversion ratio, as well as the more complex maximum likelihood model were calculated. These estimates were calculated for intra-specific and intra-generic comparisons for the combined data set only. The majority of the intra-specific pairwise comparisons were found to correspond to less than 0.5% sequence divergence. However, a few appeared to be higher for example, within *Psammobates tentorius* spp. the upper range of the pairwise divergence estimates was 5.2% (K-2P), for *Homopus areolatus* spp. it was 3% (K-2P) and 2.6% (K-2P) for *Manouria emys* spp. In the intra-specific comparisons, the maximum likelihood and K-2P estimates were within 0.1% sequence divergence of each other. Sister species sequence divergence estimates were less than 10% using both the maximum likelihood and K-2P estimates (and the tree topology in Figure 5.14). Exceptions included *the M. tornieri + T. horsfieldii* pair (15%), the *P. geometricus + P. oculifer pair* (16%) and *the G. nigra + G. chilensis* combination (12%).

For the intra-generic comparisons, however, the differences between the two corrected estimates was substantially higher, particularly at the upper range of each generic comparison, whilst the lower ranges showed similar values (Figure 5.5). For both the maximum likelihood and the K-2P measures, the average divergence estimates were similar within each genus. At the upper range, the maximum likelihood estimates were always higher suggesting that the K-2P model has not adequately accounted for potential multiple hits, which are expected given the ancient divergence dates for some of the taxa (estimated at more than 30 million years). The genera *"Geochelone", Homopus, Psammobates* and *Testudo* displayed particularly high levels of intra-generic variability and substantial differences in the values of their upper range estimates as calculated by K-2P and maximum likelihood. The maximum likelihood divergence estimates for between species comparisons within a genus ranged from 0.65% (between *H. bergeri* and *H. boulengeri*) to 28.5% (between *G. platynota* and *G. pardalis*). Using the K-2P estimates, the upper range of sequence divergence in the *Homopus* genus was only 16% whilst the maximum likelihood estimate was 26% between *H. femoralis* and *H. signatus*. *Chersina* and *Malacochersus* were the least variable genera within the family with low average divergence estimates (2.4% and 0.1% respectively) and limited ranges with both the K-2P and maximum likelihood estimates.

This is not unexpected as both are defined as monotypic genera. Below approximately 12% sequence divergence, both the maximum likelihood and K-2P estimates showed similar patterns with regard to the upper and lower range estimates, for example with *Indotestudo* and *Gopherus*. Above this 12% estimate, however, the K-2P model consistently underestimated the upper range values. The majority of the species within a genus did not show more than 12% pairwise sequence divergence at the intra-generic level, with the exception of the *"Geochelone"* (maximum 28.5%), *Psammobates* (maximum 24%), *Homopus* (maximum 26%) and the *Testudo* (maximum 16%) (Figure 5.5).

This trend was also evident in the inter-generic comparisons. For the K-2P divergence estimates, excluding outgroups, the highest pairwise difference was 21.7% between *G. berlandieri* and *P. oculifer*. Maximum likelihood estimates were higher and showed a highest divergence estimate of 50.1% between *G. agassizii* and *P. oculifer* when outgroups were excluded and 67% between *L. kempii* and *P. oculifer* when outgroup taxa were included. All maximum likelihood pairwise estimates involving outgroup taxa were greater than 28%, whilst the highest estimate for the K-2P model was 23.2% sequence divergence between *L. kempii* and *P. tentorius*.

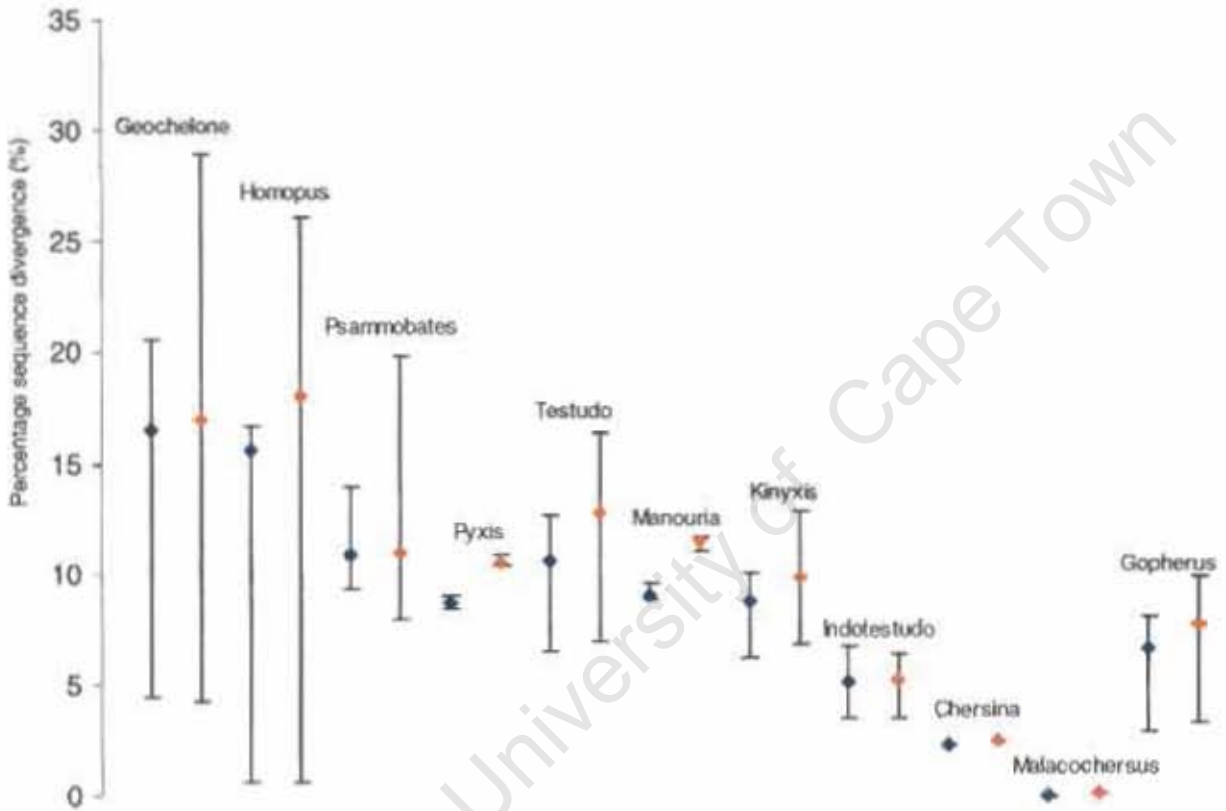


Figure 5.5: Graph showing mean intra-generic sequence divergence estimates based on the combined molecular data set. Red data points represent maximum likelihood distance estimates, whilst blue data points represent K-2P distance estimates. Bars represent the upper and lower range of the divergence estimate between species within the genus.

Phylogenetic Inference Methodology

Parsimony[5]

The Cyt *b*, ND4 and combined data sets were initially analysed using unweighted parsimony. In each case, the resulting inferences were highly unresolved, although each gene offered resolution at particular levels of phylogenetic divergence.

The Cyt *b* gene tree showed low CI and RI scores (0.277 and 0.520 respectively) and was unable to resolve any of the deep level relationships (Figure 5.6). There was strong support for species level relationships at the terminal nodes of the tree, for example the African *Kinixys* clade (bootstrap = 95) and the North American *Gopherus* clade consisting of *G. agassizii*, *G. berlandieri*, *G. flavomarginatus* and *G. polyphemus* (bootstrap = 96). The *Indotestudo* clade was also strongly supported (bootstrap = 98), as was the sister relationship between the Indian and Southeast Asian representatives of *Geochelone*, *G. elegans* and *G. platynota* (bootstrap = 99). An initial unweighted parsimony analysis of the Cyt *b* data set could not place *L. kempii* as an outgroup taxon and it was removed from subsequent analysis (see Materials and Methods) leaving *T. coahuila* and *T. ornata* as outgroup taxa for the Cyt *b* data set. The Cyt *b* gene provided strong support for the monophyly of the Testudinidae using *Terrapene* as outgroup taxa (bootstrap = 100).

[5] Parsimony estimates have not been displayed as phylograms due to the known tendency of this inference method to underestimate branch lengths (Swofford et *al.* 1996).

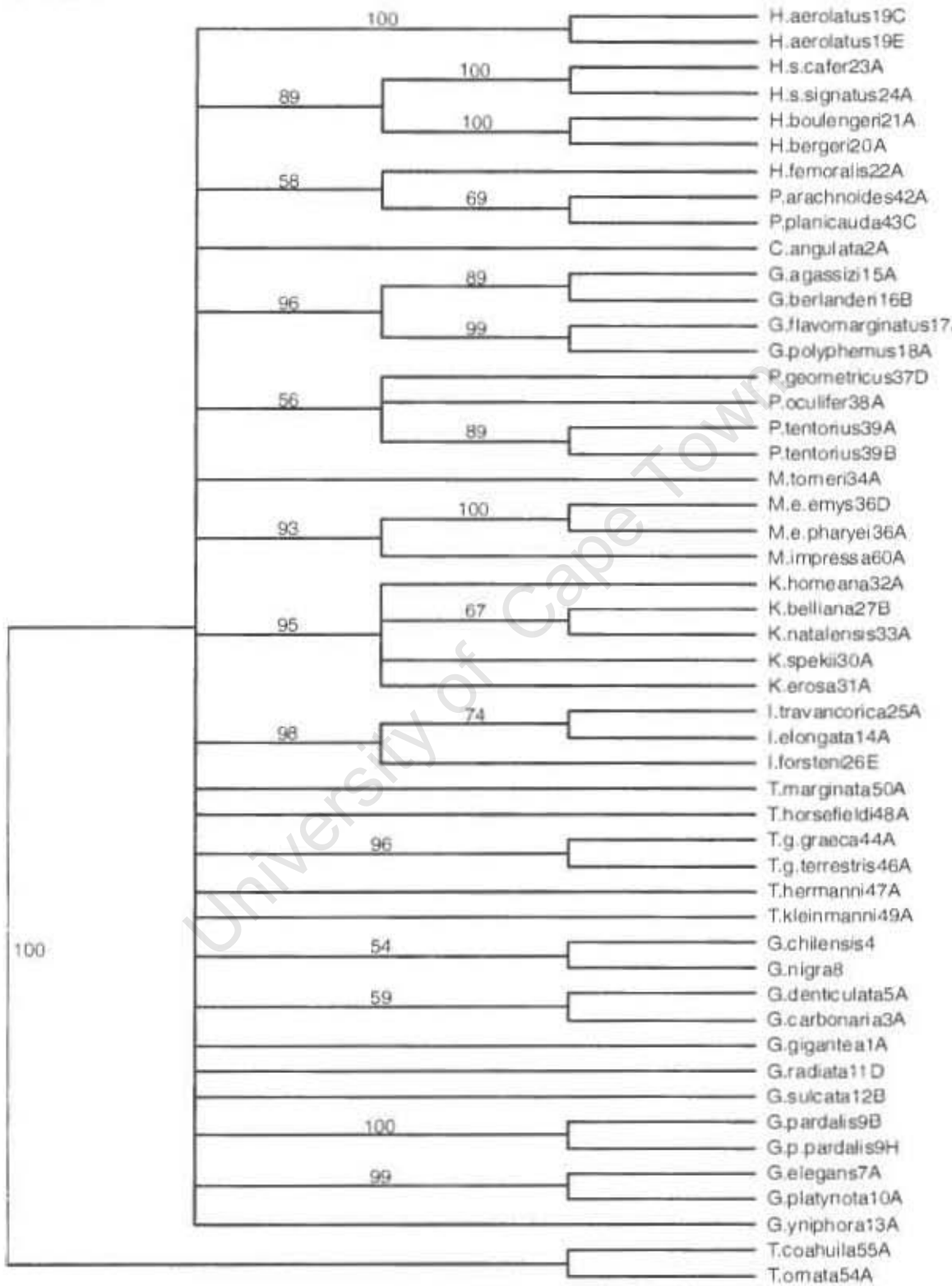


Figure 5.6: An unweighted parsimony estimate (50% majority rule) based on the Cytochrome *b* gene for the Family Testudinidae. Values on the branches represent bootstrap proportions, tree length=969, CI=0.247 and RI=0.520.

The ND4 gene and the combined data set provided more resolved trees than the Cyt *b* inference in the unweighted parsimony analysis, particularly for the deeper divergence levels. Both data sets produced similar topologies (Figure 5.7 and Figure 5.8) and provided strong support for the monophyly of the Testudinidae (bootstrap = 91 for the ND4 gene and 99 for the combined data set). The combined inference analysis showed support for a monophyletic assemblage, which included a monophyletic *Manouria* and a monophyletic *Gopherus* clade (bootstrap = 73). The ND4 gene tree resolved the genera as two highly supported monophyletic groups, *Manouria* with bootstrap support of 85 and *Gopherus* with bootstrap support of 100, although the placement of the *Gopherus* clade was not resolved.

Within the *Gopherus* clade, both trees resolved two assemblages. The first of these contained *G. flavomarginatus* and *G. polyphemus*, and the second contained *G. agassizii* and *G. berlandieri*. The *Testudo* spp., *Indotestudo* spp. and *Malacochersus* genus formed a well-supported clade (bootstrap = 98). *Malacocherus tornieri* and *T. horsfieldii* were resolved as well-supported sister taxa in both inferences. Furthermore, both trees displayed a large derived clade that contained the remaining species in the family, including the genera *Geochelone*, *Psammobates*, *Homopus*, *Pyxis*, *Chersina* and *Kinixys*. The above mentioned clade was not particularly well supported in either the combined data set (bootstrap = 54) or the ND4 tree (bootstrap = 60). However, it was found to contain some well resolved groups, including a clade consisting of *H. s. signatus*, *H. s. cafer*, *H. boulengeri* and *H. bergeri* (bootstrap = 100). The *Homopus* clade was shown to be affiliated with *Chersina angulata* (combined bootstrap = 69, ND4 bootstrap = 68). In all the analyses, the monophyly of the genus *Homopus* was not supported and the relationship between *H. areolatus* and *H. femoralis* remained unresolved.

Geochelone elegans and *G. platynota* were shown to be sister taxa (bootstrap = 100) in the combined topology and occurred within a well-supported trichotomy, which included the African *G. sulcata* (combined bootstrap = 78, ND4 bootstrap = 60). The monophyly of the *Kinixys* clade was well supported in both trees. Within the *Kinixys* clade, the sister relationship between *K. natalensis* and *K. belliana* was strongly supported (combined bootstrap = 95, ND4 bootstrap = 94).

Geochelone pardalis and the *Psammobates* clade were found to group together in both the ND4 topology and in the combined tree (bootstrap = 73 and 71 respectively).

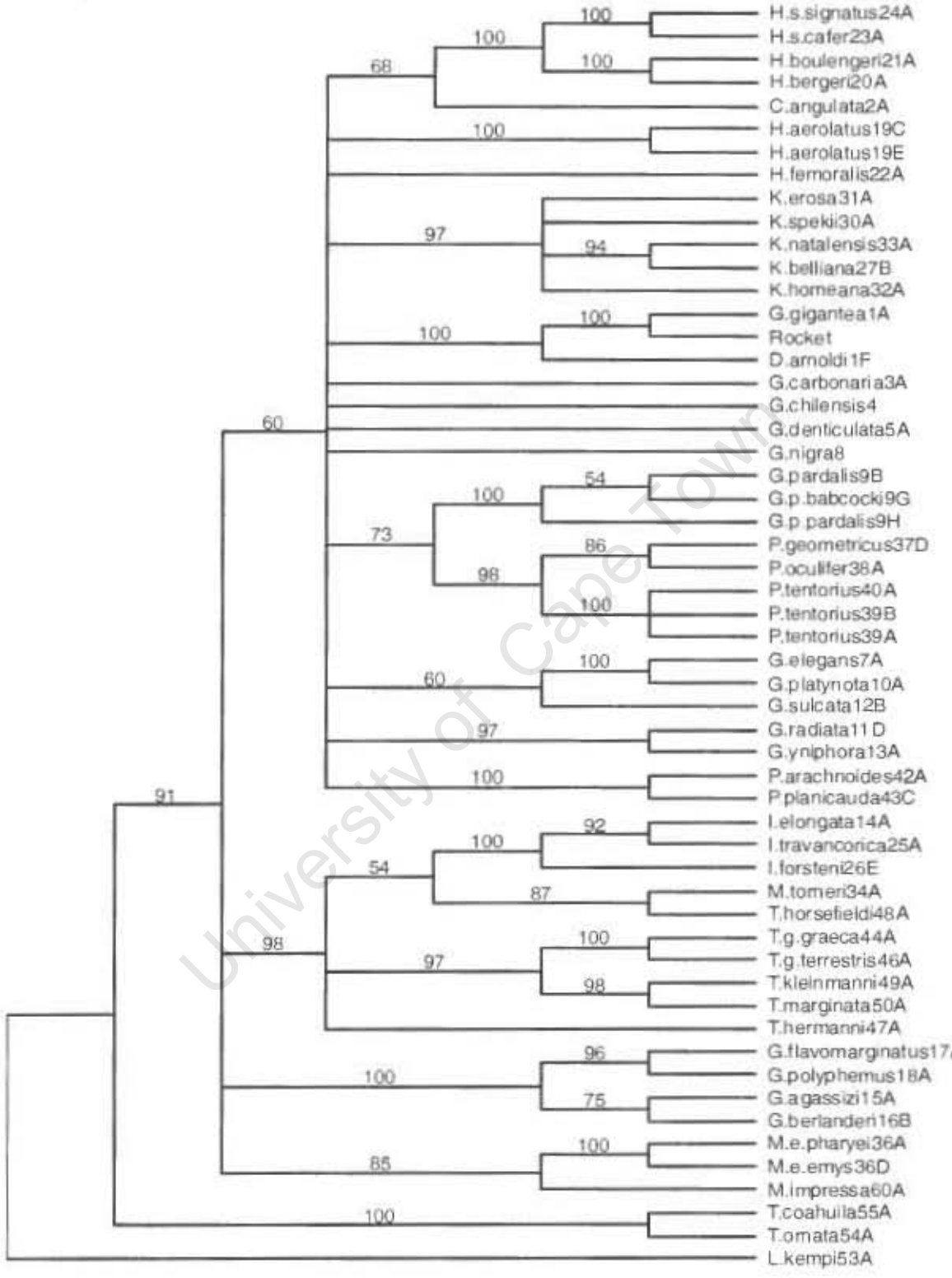


Figure 5.7: An unweighted parsimony estimate (50% majority rule) based on the ND4 gene for the Family Testudinidae. Values on the branches represent bootstrap proportions, tree length=2066, CI=0.398 and RI=0.678.

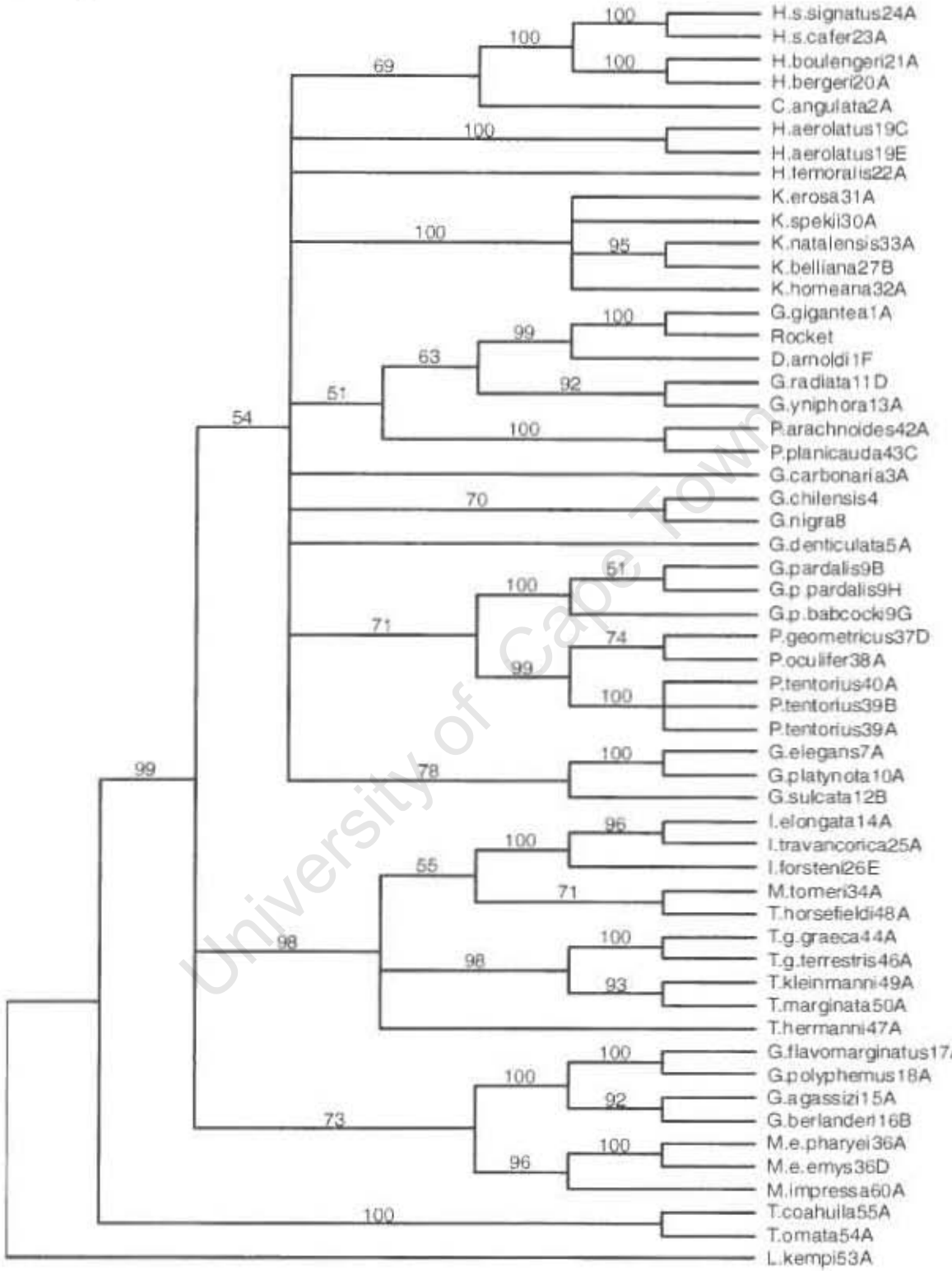


Figure 5.8: An unweighted parsimony estimate (50% majority rule) based on the combined data set (Cyt *b* + ND4) for the Family Testudinidae. Values on the branches represent bootstrap proportions, tree length=3091, CI=0.279 and RI=0.551.

Unweighted parsimony was also carried out for the reduced data sets, including the African clade, the *Manouria-Gopherus* clade and the *Psammobates* clade (see Materials and Methods). These trees (not reported) showed a similar trend to that reported for the complete data sets above. The Cyt *b* gene was unable to resolve deep level divergences for the African and *Manouria-Gopherus* data sets, but produced a fully resolved and well-supported tree for the *Psammobates* clade. The ND4 gene and the combined gene trees showed more resolution at the deeper nodes in all sub-trees analysed, but remained insufficiently resolved for detailed analysis and interpretation of the relationships between the genera.

All unweighted parsimony inferences were characterised by low CI and RI scores (Table 5.3). This, in combination with the poor resolution of nodes, justified the differential weighting of the data sets. In all cases the application of weighting schemes improved both the tree scores (Table 5.3) and the resolution of the nodes in the trees, particularly for the ND4 and combined data sets. In addition to aiding in the resolution of previously unidentified nodes, there was also an improvement in the bootstrap proportions associated with the branches. This aspect of tree robustness and the effect of differential weighting schemes on clade support will be discussed in more detail further in the Chapter. Optimal Ti/ Tv ratios were determined empirically and were calculated as 9:1 for the complete data sets for the Cyt *b*, ND4 and combined genes, 5:1 for the African data set (for all genes) 3:1 for the *Manouria-Gopherus* data set (for all genes) and 1:1 and 3:1 for the *Psammobates* group.

Table 5.3: Parsimony tree scores and tree statistics for the differential weighting schemes (Ti/ Tv) applied to the total data set (55 individuals), the *Manouria-Gopherus* clade (20 individuals) and the African clade (25 individuals), for the Cyt *b*, ND4 and combined genes respectively.

DATA SET	Tv:Ti = 1:1			Tv:Ti = 3:1			Tv:Ti = 5:1			Tv:Ti = 7:1			Tv:Ti = 9:1		
	TL	*CI*	*RI*	TL	*CI*	*RI*	TL	*CI*	*RI*	TL	*CI*	*RI*	TL	*CI*	*RI*
TOTAL SET															
CytB	**969**	*0.277*	*0.520*	-	-	-	-	-	-	**1711**	*0.423*	*0.631*	**1812**	*0.434*	*0.650*
ND4	**2066**	*0.398*	*0.678*	-	-	-	-	-	-	**4129**	*0.385*	*0.670*	**4815**	*0.401*	*0.683*
Combined	**3091**	*0.279*	*0.551*	-	-	-	-	-	-	**6015**	*0.393*	*0.651*	**6229**	*0.410*	*0.667*
Manouria-Gopherus															
CytB	**352**	*0.491*	*0.576*	**425**	*0.520*	*0.590*	**577**	*0.611*	*0.685*	-	-	-	-	-	-
ND4	**772**	*0.513*	*0.607*	**1058**	*0.567*	*0.675*	**1344**	*0.598*	*0.710*	-	-	-	-	-	-
Combined	**1161**	*0.504*	*0.591*	**1591**	*0.563*	*0.658*	**2021**	*0.598*	*0.694*	-	-	-	-	-	-
African Clade															
CytB	**768**	*0.539*	*0.620*	-	-	-	-	-	-	-	-	-	-	-	-
ND4	-	-	-	**1330**	*0.507*	*0.629*	**1694**	*0.539*	*0.651*	-	-	-	-	-	-
Combined	**2699**	*0.538*	*0.639*	**1933**	*0.496*	*0.610*	-	-	-	**2885**	*0.557*	*0.656*	**3361**	*0.574*	*0.669*

CI - Consistency index
RI - Retention index
TL - Tree Length (number of steps)
- not calculated

Maximum Likelihood Analysis

Optimal models of evolution and rate parameters for each of the data sets were selected using ModelTest. In the initial discussion of the results for maximum likelihood analysis the effect of factors, such as the inclusion of outgroup taxa, on parameter estimates and on the selection of models of evolution was assessed. In addition, the effect of alternate model selection on tree topology and subsequent likelihood scores was assessed. These comparisons of tree topology were based on the "best-fit" likelihood estimate obtained after a heuristic search of the available tree space using the optimal models designated by the ModelTest programme. The specific topologies and the relationships within the Testudinidae that were obtained from weighted parsimony analysis, maximum likelihood inference and bayesian analysis, for each data set and including the sub-clade analysis, will be discussed under the framework of "Topology Estimation And Clade Support". All topologies were similar within each data set analysed, irrespective of the inference method and model of evolution used.

i. "Best-fit" Model Selection

Parameter estimates and model selection were found to be robust to differences in the starting tree topology. Identical models were selected when both a neighbour joining tree and a "best" estimate parsimony tree were used as starting topologies for estimation in ModelTest v3.06. Using the hLRT, the most appropriate model of evolution to best explain the Cyt *b*, ND4 and combined data sets was the GTR+Γ+P*inv* model. The AIC index, however, selected a different model for the combined data set, the HKY85+Γ+P*inv* model. The latter model differs from the former in that the GTR has six substitution types, whereas the HKY85 model only has two, in the form of a Ti/ Tv ratio. The log likelihood scores for these two models were almost identical (GTR+Γ+P*inv* = 14479.95 and HKY85+Γ+P*inv* = 14483.04) and the addition of parameters required for the more complex GTR model did not significantly improve the likelihood score. For this reason, the simpler HKY85+Γ+P*inv* model was selected for the analysis of the combined data set.

Natural log likelihood scores (-lnL) were plotted against all the available models tested (x-axis) and the trend of the improvement of likelihood scores with increasing model complexity was shown to be similar for all three data sets (Figures 5.9 and 5.10). The only difference being the actual values of the scores, which were higher for the combined and ND4 data sets as these both have more characters and more taxa than the Cyt *b* data set.

In each of the data sets the model that displayed the poorest fit to the data was found to be the JC model, the simplest of all the models tested. For the Cyt *b* gene, the -lnL score for the JC topology was 5675.8115, for the ND4 gene was 11335.8 and for the combined data set was 17113.8700. The log likelihood scores were **significantly** improved with increasing model complexity and particularly with the addition of the gamma distribution (Γ) (Figures 5.9 and 5.10). The addition of the P*inv* sites parameter did not allow for any significant improvement in any of the log likelihood scores (data not included). The addition of the P*inv*+Γ parameter combination, however, provided the "best fit" to the data in all cases i.e. the lowest log likelihood score.

The Cyt *b* log likelihood scores ranged from 5675.8115 (JC model of evolution) to the "best-fit" GTR+Γ+P*inv* of 4779.3564. In the ND4 gene the scores ranged from 11335.8 (JC model) to 9624.366 (GTR+Γ+P*inv*) and for the combined data set the range was from 17113.87 (JC model) to 14479.95 (GTR+Γ+P*inv*) (Figures 5.9 and 5.10).

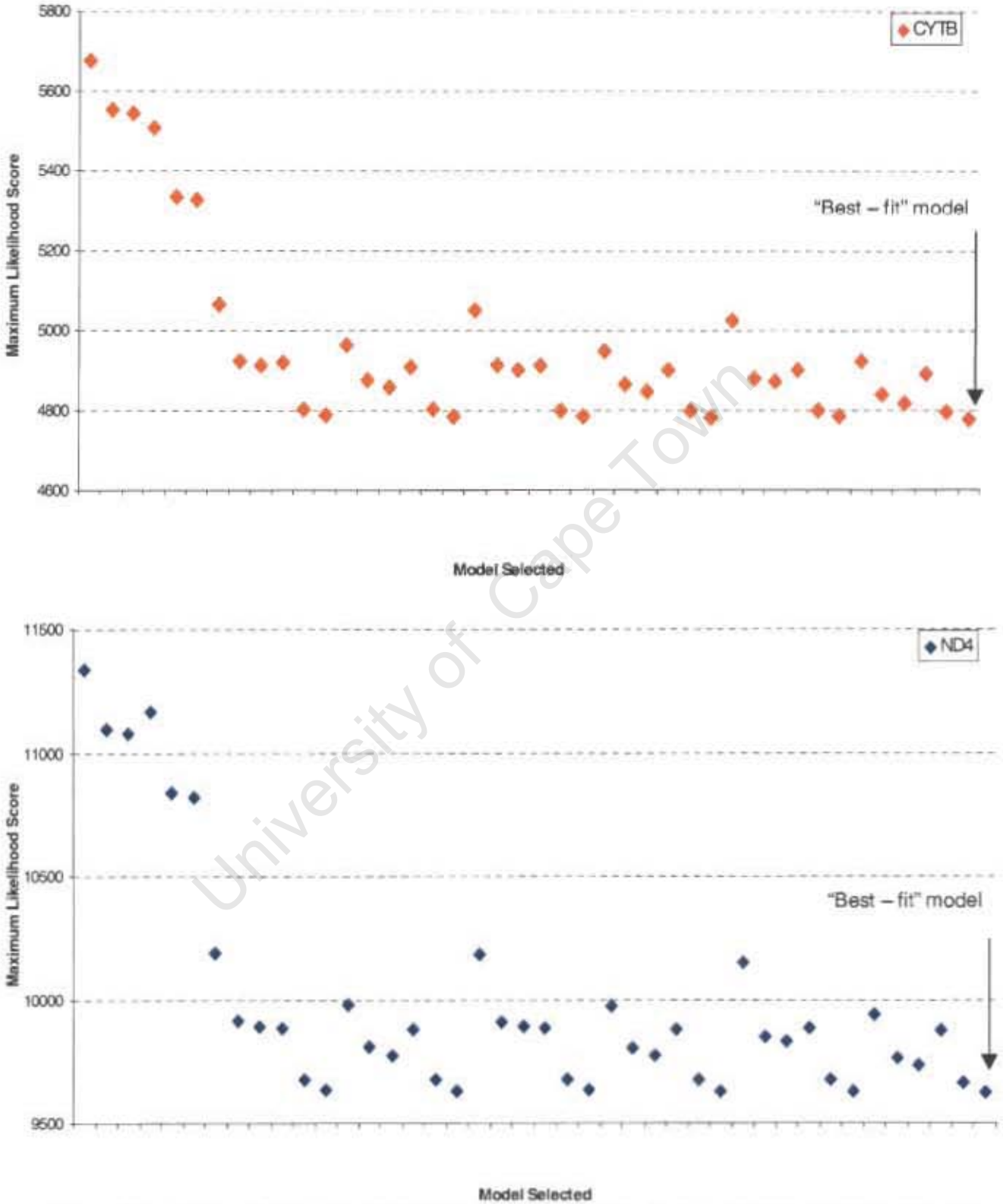


Figure 5.9: Graph showing the relationship between nested models of DNA evolution and log likelihood scores as calculated by ModelTest v3.06b (Posada and Crandall 1998) using the hierarchical LRT for the Cyt *b* and ND4 sequences. Starting trees were generated using a neighbour-joining algorithm. "Best-fit" models were selected based on the -lnL scores and used in a maximum likelihood inference.

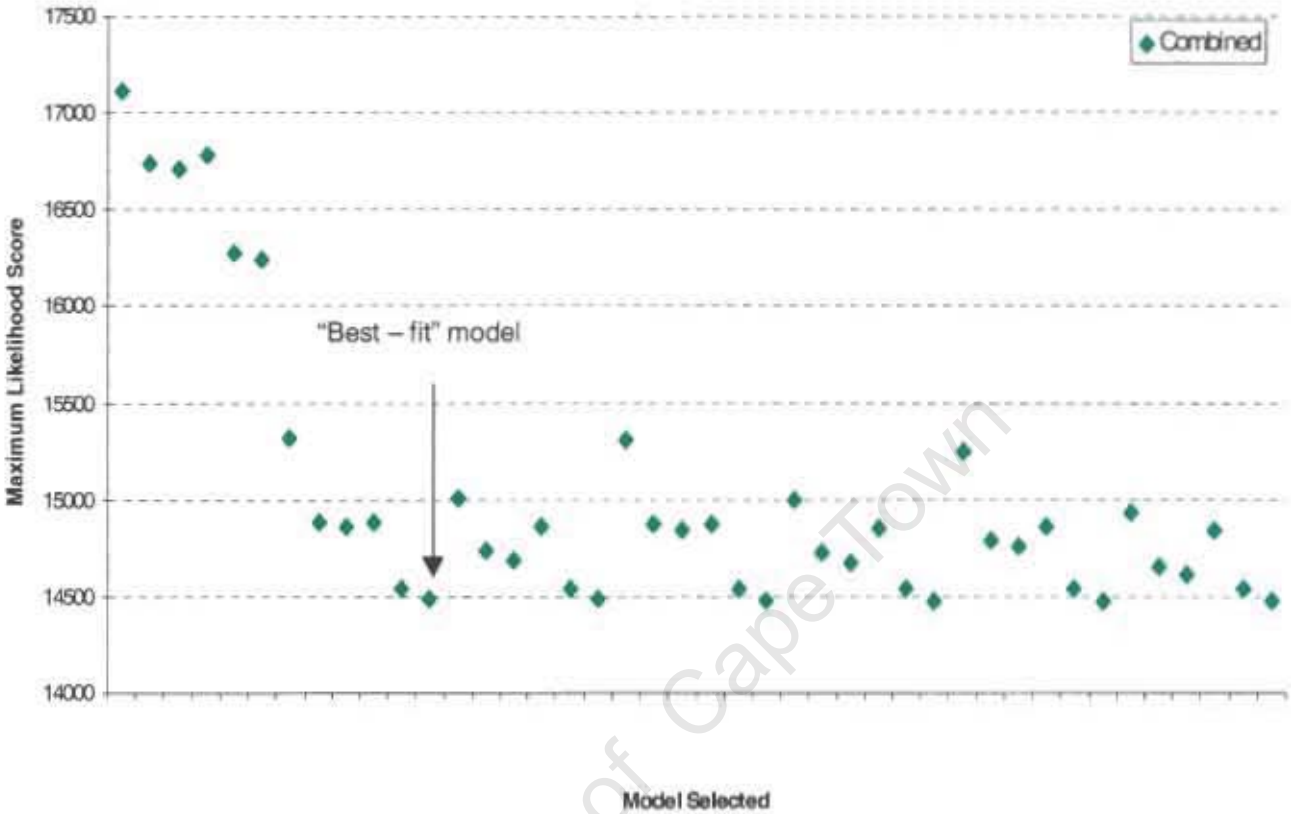


Figure 5.10: Graph showing the relationship between nested models of DNA evolution and log likelihood scores as calculated by ModelTest v3.06b (Posada and Crandall 1998) using the hierarchical LRT for the combined data set. Starting trees were generated using a neighbour-joining algorithm. "Best-fit" models were selected based on the -lnL scores and used in a maximum likelihood inference.

ii. Parameter Estimation

Parameters for each of the complete Cyt *b*, ND4 and combined data sets were estimated with and without the outgroup taxa to determine what effect, if any, these more divergent taxa would have on these parameters (Table 5.4). The "best-fit" models of evolution and parameter estimates were also determined for the sub-clade data sets for the Cyt *b*, ND4 and combined gene partitions, with outgroup taxa included (Table 5.4).

For the large Cyt *b* data set the GTR+Γ+P*inv* model was selected as the model of evolution that best described the sequence data (Table 5.4). The estimated frequency of each of the four nucleotides was found to correspond closely to those found in the analysis of the gene fragment using MEGA v2.1, A = 35%, C = 30%, G = 11% and T = 24%. The six rate parameters showed a strong bias toward transitions (r[A – G] = 22.43 and r[C – T] = 42.33) with a concurrent low rate for the transversions. The rate heterogeneity among sites was a = 1.13 and 45% of the sites in the fragment were invariant. The exclusion of outgroup taxa did not have a significant effect on these parameter estimates with only a small increase in the gamma value (a = 1.16) and a marginal decrease in the proportion of invariable sites (44%). For this reason, the parameter estimates including outgroup taxa were used in the subsequent maximum likelihood inference. An alternate model, the HKY85+Γ+P*inv*, was also used in a likelihood inference to determine the effect of model selection on tree topology. Apart from a non-significant (using the LRT statistic) increase in the log likelihood score, from 4601.494 to 4655.435, there was no change in the tree topology when incorporating this simpler model (tree not shown).

Both the ND4 gene and the combined data set showed a similar pattern when excluding the outgroup taxa, with only marginal changes in each of the estimated parameters that did not have an effect on the subsequent tree topology (Table 5.4, trees not shown). The model of evolution selected for the ND4 gene was the GTR+Γ+P*inv* model although the HKY85+Γ+P*inv* model was selected as an alternate model, again to test the effect of model selection on topology and tree score. As with the Cyt *b* gene, there was no effect on the subsequent maximum likelihood topologies but an increase in likelihood scores was observed (Table 5.4, tree not shown). The ND4 sequence showed higher estimates for the frequency of adenine (40%) and lower estimates for the frequency of guanine (6%) than the Cyt *b* fragment. The rate parameter estimates were lower than those found in the Cyt *b* gene, particularly in the number of transitions (r[A – G] = 7.91 and r[C – T] =11.60).

The combined data set (including outgroups) did not show a completely homogenised pattern between the Cyt *b* and the ND4 data sets. Base frequency estimates (A = 38%, C = 29%, G = 6.5% and T = 25%), rate parameters for the GTR model, particularly with respect to transitions (r[A – G = 12.43 and r[C – T = 14.15), as well as the gamma estimate (a = 1.11) and the proportion of invariant sites (43%) showed closer affinities with the ND4 data set than with the Cyt *b* data set (Table 5.4). The HKY85+Γ+P*inv* model of evolution showed an improved likelihood score (14445.481) when compared to that obtained for the GTR+Γ+P*inv* (14469.229), but there was no difference in the subsequent topologies (tree not shown).

Table 5.4: Table showing parameter estimates from the ModelTest v3.06b (Posada and Crandall 1998) for the complete data sets and with (+ O) and without outgroups (- O) included. If a different model was selected under the AIC both trees were constructed to check for potential differences in the topologies.

Parameters	**Cyt *b* + O**	**Cyt *b* - O**	**ND4 + O**	**ND4 - O**	**Combined + O**	**Combined - O**
Model Selected (hLRT)	GTR+Γ+P*inv*	GTR+Γ+P*inv*	GTR+Γ+P*inv*	GTR+Γ+P*inv*	GTR+Γ+P*inv*	GTR+Γ+P*inv*
-lnL	4601.494	4424.348	9599.444	9368.856	14469.229	13499.995
FreqA	0.35	0.34	0.40	0.40	0.38	0.38
FreqC	0.30	0.30	0.30	0.30	0.29	0.28
FreqG	0.11	0.11	0.06	0.06	0.065	0.07
FreqT	0.24	0.25	0.24	0.24	0.25	0.27
Rate [A – C]	2.41	2.30	0.34	0.33	0.62	0.63
Rate [A – G] - Ti	**22.43**	**21.64**	**7.91**	**7.70**	**12.43**	**12.64**
Rate [A – T]	1.37	1.35	0.61	0.63	0.63	0.54
Rate [C – G]	0.72	0.61	0.34	0.34	0.50	0.48
Rate [C – T] - Ti	**42.33**	**43.18**	**11.60**	**11.82**	**14.15**	**13.5**
Rate [G – T]	1.00	1.00	1.00	1.00	1.00	1.00
Gamma value (Γ)	1.13	1.16	1.12	1.11	1.11	1.16
P*inv*	0.45	0.44	0.42	0.43	0.43	0.44
Model Selected (AIC)	GTR+Γ+P*inv*	-	GTR+Γ+P*inv*	-	HKY85+Γ+P*inv*	-
Alternative models	HKY85+Γ+P*inv*	-	HKY85+Γ+P*inv*	-	-	-
-lnL	4655.435	-	96664.256	-	14445.481	-
Topology	No difference	-	No difference	-	No difference	-

Ti = transitions
P*inv* = proportion of invariant sites

iii. Sub-Clade Model Selection and Parameter Estimation

The reduced data sets for the *Manouria-Gopherus* assemblage and the African clade showed similar estimations to the larger data sets for base frequency estimations. Nucleotide frequency estimates ranged from 32% - 39% for A, 28% - 30% for C, 9% - 13% for G and 22% to 25% for T, across the Cyt *b*, ND4 and combined data partitions (Table 5.5). Gamma parameter estimates were higher than for the larger sets (up to a = 1.87 for the ND4 gene for the *Manouria-Gopherus* clade), as well as displaying an increased proportion of sites that were invariable (55% for the Cyt *b* gene for the *Manouria-Gopherus* data set). Transition rates were higher than transversion rates, particularly for the Cyt *b* gene. Similar "best-fit" models of evolution were selected using both hLRT's and the AIC index (Table 5.5).

In both the *Manouria-Gopherus* and the African clade, the ND4 and the combined data sets showed similar estimated parameter values and model selection. For the *Manouria-Gopherus* clade, the K8luf+Γ+P*inv* model was selected for both ND4 and the combined genes. This model differs form the more complex GTR model in that only three rate parameters, one for transitions and two for transversions are calculated. The combined data set displayed higher rate values for both transitions and transversions than the ND4 gene, probably as a result of the effects of the Cyt *b* rate parameter estimates (Table 5.5). "Best" estimate log likelihood tree scores ranged from 4366.2556 for the ND4 gene to 6660.7005 for the combined gene. The GTR+Γ+P*inv* model of evolution was selected for the African clade for both ND4 and the combined data sets. Parameter estimates were again similar although the combined data set displayed higher transition rate estimates than the ND4 gene (r[C – T] = 28.79 and r[C – T] = 17.74] respectively).

The Cyt *b* gene showed differences in both parameter estimation and model selection for both clades. Furthermore, transition rates were substantially higher for this gene, particularly in the *Manouria-Gopherus* clade than for the other two data partitions (r[C – T = 75.70). In the *Manouria-Gopherus* data set, the GTR+Γ+P*inv* (-lnL score = 2091.8558) was selected. For the African clade, however, the TRN+Γ+P*inv* model of evolution was selected and when implemented in a maximum likelihood inference, there were over 200 "best" trees saved and the PAUP* run was terminated after approximately 400 hours of computation time. The implementation of alternate models of evolution did not rectify the lack of convergence onto a "best" estimate topology for the African clade with the Cyt *b* gene.

Table 5.5: Table showing parameter estimates from the ModelTest v3.06b (Posada and Crandall 1998) for the sub-tree data sets for the *Manouria-Gopherus* clade (M&G) and the African clade. If a different model was selected under the AIC, both trees were constructed to check for potential differences in the resulting topologies.

Parameters	Cyt *b* (M&G)	ND4(M&G)	Combined(M&G)	Cyt *b* (African)	ND4(African)	Combined(African)
Model Selected (hLRT)	GTR+Γ+P*inv*	K8luf+Γ+P*inv*	K8luf+Γ+P*inv*	TRN+Γ+P*inv*	GTR+Γ+P*inv*	GTR+Γ+P*inv*
-lnL	2091.855	4366.255	6660.700	> 200 trees	5106.772	7695.980
FreqA	0.32	0.39	0.37	0.36	0.38	0.35
FreqC	0.30	0.29	0.30	0.28	0.28	0.28
FreqG	0.13	0.09	0.09	0.10	0.092	0.11
FreqT	0.24	0.22	0.24	0.226	0.25	0.25
Rate [A – C]	6.203	1	1	1	0.88	1.57
Rate [A – G] - Ti	**28.69**	**15.07**	**13.11**	**12.6**	**15.69**	**18.06**
Rate [A – T]	2.31	0.51	0.45	1	1.28	1.58
Rate [C – G]	0	0.51	0.45	1	1.29	1.09
Rate [C – T] - Ti	**75.70**	**15.07**	**13.11**	**18.38**	**17.74**	**28.79**
Rate [G – T]	1	1	1	1	1	1
Gamma value (+Γ)	1.47	1.87	1.74	1.62	1.69	1.67
P*inv*	0.55	0.52	0.54	0.48	0.48	0.48
Model Selected (AIC)	GTR+Γ+P*inv*	K8luf+Γ+P*inv*	K8luf+Γ+P*inv*	TRN+Γ+P*inv*	GTR+Γ+P*inv*	GTR+Γ+P*inv*

Ti = transitions
P*inv* = proportion of invariant sites

iv. The Effect of Default PAUP* Model Parameters on Tree Topology

To determine the extent to which the different tree topologies, produced from the large data sets and the sub-clade data sets, were sensitive to parameter estimation, a number of default parameters were used within a maximum likelihood inference to test the effect on subsequent tree topology. The significance of the difference in tree topologies was evaluated using the Shimodaira-Hasegawa test (1999). The ND4 and the combined data sets for both the larger analysis sets and the sub-clade data sets were more robust to changes in the parameter estimates than the Cyt *b* gene sets (Table 5.6).

In the ND4 data sets and the combined data sets, the log likelihood scores for the default topologies were found to be significantly worse than the "best estimate" topology (using the LRT test) with particularly low levels of resolution when the gamma parameter was set to zero. With gamma set to a = 0.5, however, differences in the tree topologies were only in branch length estimation, whilst the arrangement of taxa remained consistent with "best" estimate topologies, apart from limited branch swapping evident in the terminal taxa. The terminal taxa that were prone to this branch swapping effect occurred in unresolved clades in the final tree topologies (see "Topology Estimation and Clade Support"). All trees were significantly different from the "best" estimate using the S-H test.

The Cyt *b* gene, however, was very sensitive to changes in the parameter estimation (Table 5.6). There were significant differences in both tree score (using the LRT test) and tree topology (using the S-H test). The differences were in branch length estimation and taxon placement at both the terminal nodes and the deeper nodes in the trees. The Cyt *b* estimation for the African clade was unable to converge on a "best" estimate topology for any of the default parameters, even under the "best-fit" model of evolution.

Table 5.6: Table showing the log likelihood scores of the trees calculated using the default model parameters: gamma = 0 or 0.5, P*inv* = 0 and base frequencies = 0.25, for the total data set (55 individuals), the *Manouria-Gopherus* clade (20 individuals) and the African clade (25 individuals).

Data set	Max -lnL score from best tree	Max -lnL score Gamma = 0	Max -lnL score Gamma = 0.5	Topology different to best estimate [1]
Whole data set:				
Cyt *b*	4601.494	5909.083**	5363.696**	Yes
ND4	9599.444	12815.121**	11093.100**	Yes
COMBINED	14445.487	17124.456**	16254.263**	Yes
***Manouria-Gopherus* clade:**				
Cyt *b*	2091.856	2521.214**	2224.538**	Yes
ND4	6660.701	7919.377**	7003.262**	Yes
COMBINED	4366.256	5176.924**	4934.709**	Yes
African clade:				
Cyt *b*	Saved> 200 trees	Saved > 200 trees	Saved > 200 trees	-
ND4	5106.773	6124.470**	5764.886**	Yes
COMBINED	7695.980	9337.633**	8769.741**	Yes

[1] topologies that were different from the best estimate were significantly different using the S-H test (Shimodaira and Hasegawa 1999)
**$p \leq 0.05$ indicates that the log likelihood score is significantly worse under the default parameters as calculated by the LRT (where the degrees of freedom is equal to the difference in the number of parameters between the best estimate and the simpler model of evolution i.e. the default parameters)

Bayesian Inference

The GTR+Γ+P*inv* model was used for all bayesian analyses. The parameter estimates for the final trees were similar to those calculated for the maximum likelihood inferences (Table 5.7). Log likelihood scores were calculated for each of the final tree topologies calculated from MrBAYES and were not different from those under the maximum likelihood models.

Table 5.7: Table showing the bayesian parameter estimates for each data set averaged over the selected trees (36000) and the resulting maximum likelihood scores under the defined models. Parameter estimates included outgroup taxa.

Parameters	**Cyt *b***	**ND4**	**Combined**
Bayesian Model	GTR+Γ+P*inv*	GTR+Γ+P*inv*	GTR+Γ+P*inv*
-lnL	4596.68796	9600.39304	14472.89146
FreqA	0.32	0.41	0.35
FreqC	0.31	0.30	0.31
FreqG	0.11	0.05	0.07
FreqT	0.26	0.24	0.24
Rate [A – C]	1.62	0.47	1.05
Rate [A – G]	15.14	11.57	21.17
Rate [A – T]	1.33	0.48	0.91
Rate [C – G]	0.82	0.87	1.22
Rate [C – T]	35.14	16.8	22.15
Rate [G – T]	1	1	1
Gamma value (+Γ)	1.16	1.03	1.1
Pinv	0.41	0.42	0.4

P*inv* = proportion of Invariant sites

In all analyses for all data sets, the bayesian estimates showed identical topologies to maximum likelihood estimates. For all discussions on tree topologies the bayesian inferences will be represented diagrammatically, whilst maximum likelihood bootstraps (where applicable) and weighted parsimony bootstraps proportions for the corresponding relationships will be reported. The bayesian probabilities have been reported as percentages for ease of comparison with the non-parametric bootstrap proportions.

Topology Estimates and Clade Support

Cyt *b*

As with the parsimony analysis, the Cyt *b* gene trees were not well resolved and showed little structure for both the bayesian analyses and maximum likelihood analyses, particularly at the deeper nodes in the tree (Figure 5.11). There were, however, several strongly supported relationships represented in the terminal nodes. Strongly supported relationships are represented by Pr > 94. In the analysis of the large Cyt *b* data set the monophyly of the Testudinidae was fully supported (Pr = 100). The Indian representatives of the *Geochelone, G. platynota* and *G. elegans*, represented a monophyletic assemblage (Pr = 100), which was sister clade to the rest of the Testudinidae. *Geochelone sulcata* was associated with the two Indian *Geochelone*, although the relationship was not resolved. *Geochelone denticulata* and *G. carbonaria* were supported as sister taxa (Pr = 87), with no relationship evident for *G. nigra* and *G. chilensis*. The monophyly of the *Kinixys* clade was well supported (Pr = 100) although the placement of the clade was not resolved with any degree of confidence. Within this clade there was evidence for a sister relationship with *K. belliana* and *K. natalensis* (Pr = 100), which were placed in a trichotomy with *K. b. spekii* (Pr = 82).

There was limited support (Pr = 51) for a large paraphyletic clade containing two assemblages. One of these was composed of African representatives, excluding *Kinixys, G. sulcata* and *M. tornieri* (Pr = 70). Within the African assemblage, there was strong support for a *H. femoralis-Pyxis* clade (Pr = 94) and a relationship between *Chersina* and several of the small *Homopus* species (Pr = 100). The Indian Ocean Island endemics (*G. gigantea, G. yniphora, G. radiata* and the *Pyxis* species) did not cluster together. The *Psammobates* species formed a monophyletic clade (Pr = 100), which was characterised by longer branch lengths, particularly for *P. oculifer* and *P. tentorius*. *Geochelone pardalis* grouped within the African assemblage and not with other members of the genus *Geochelone.*

The second assemblage was well supported (Pr = 99) and contained two clades, one of which resolved *Manouria* and *Gopherus* as a monophyletic group (Pr = 99) and the other which contained *Testudo, Indotestudo* and *Malacochersus* (Pr = 60). Although the placement of these clades is questionable due to the limited support associated with the large clade, the terminal relationships within them were well resolved.

The Asian Indotestudo species were resolved as monophyletic (Pr = 100) with a sister relationship between *I. travancorica* and *I. elongata* (Pr = 90). Within *Testudo, T. horsfieldii,*

M. tornieri and *T. hermanni* were characterised by longer branch lengths but their position within the *Testudo* group remained undefined. There was strong support for the monophyly of both the *Manouria* and *Gopherus* species groups (Pr = 88 and Pr = 100 respectively). Within each of these clades, well resolved relationships between *G. flavomarginatus* and *G. polyphemus* (Pr = 100), *G. berlandieri* and *G. agassizii* (Pr = 100), and the two sub-species of *Manouria emys* (Pr =99) were evident.

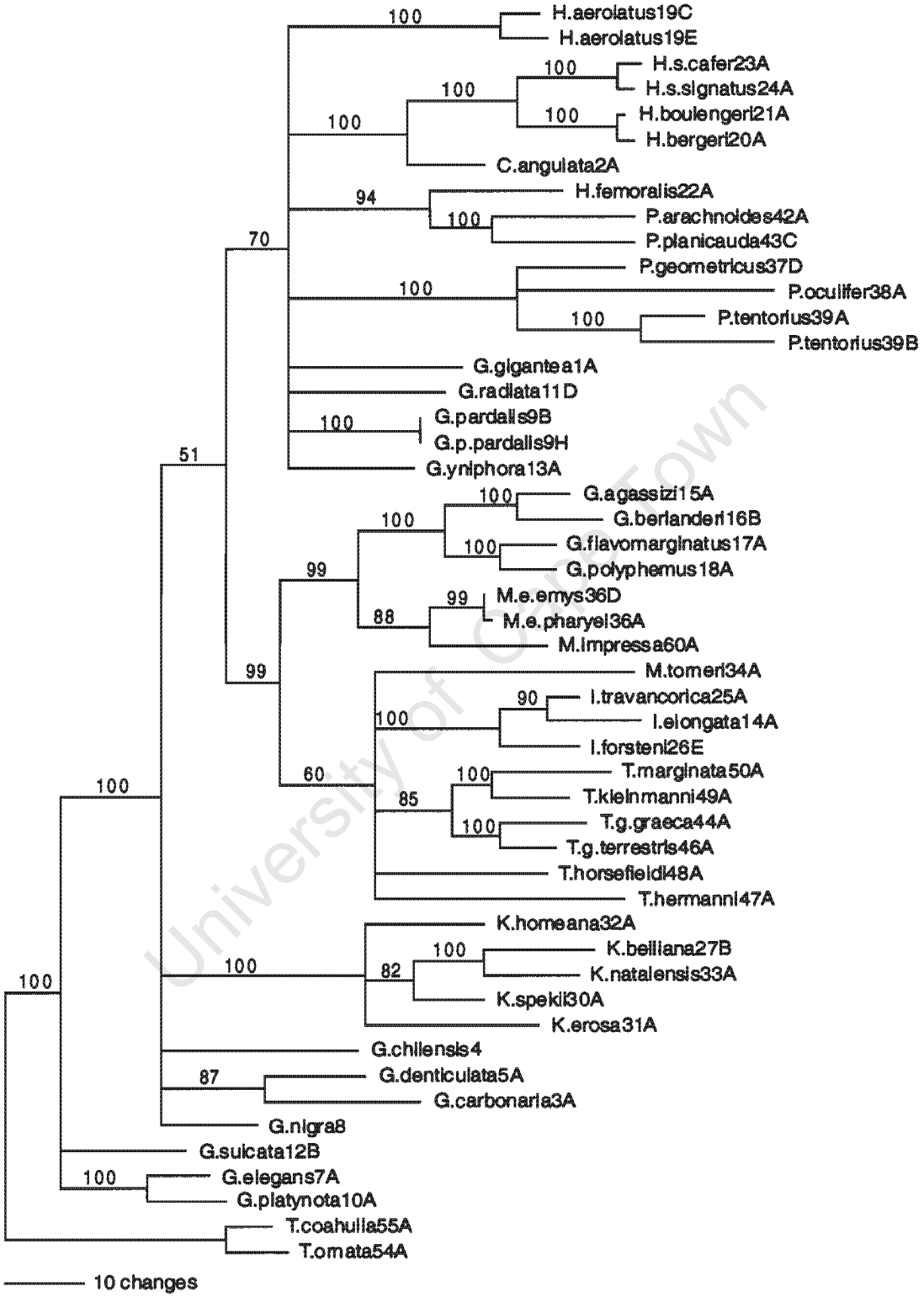


Figure 5.11: A bayesian estimate (GTR+Γ+P*inv*) tree topology for the Cyt *b* gene for the Family Testudinidae (50 individuals). Values above the branches represent clade Probability estimates. The topology was the same as that obtained using maximum likelihood inference (GTR+Γ+P*inv*, with P*inv* = 0.45, Γ = 1.13 and -lnL = 4601.494)

In an attempt to improve the resolution obtained with the Cyt *b* gene, the smaller data sets containing the African taxa and the data set containing *Manouria, Gopherus, Testudo, Indotestudo* and *Malacochersus* were analysed using bayesian inference, maximum likelihood inference and weighted parsimony.

Maximum likelihood failed to resolve a single best estimate tree topology for the African sub-set and the run was terminated after extensive computation time failed to converge on a solution (more than 200 trees saved). The tree topologies obtained for the bayesian analysis and weighted parsimony (Ti: Tv = 5:1) were similar. The relationships within the African taxa remained poorly resolved and there was a decrease in the probability values and bootstrap proportions associated with the terminal relationships within the clade. For example, the *Kinixys* clade, although still monophyletic was only weakly supported (Pr = 71, bootstrap = 69), as was the monophyly of the *Psammobates* group (Pr = 65, bootstrap = 68). *Psammobates oculifer* and *P. tentorius* were characterised by long branches. The African genus *Malacochersus* was resolved as a monophyletic sister group with respect to the remaining African genera (Figure 5.12).

Analysis of the *Manouria-Gopherus* sub-clade showed some differences to the larger set with *Gopherus* and *Manouria* no longer supported as a monophyletic assemblage (Figure 5.13). The Asian members of the genus *Manouria* were placed as a sister group to the European *Testudo* (containing *Malacochersus*) (Pr = 88 and bootstrap = 76). The monophyletic North American *Gopherus* clade (Pr = 88 and bootstrap = 72) was placed as a sister group to this larger assemblage. Terminal relationships were characterised by lower support values than seen in the larger data set.

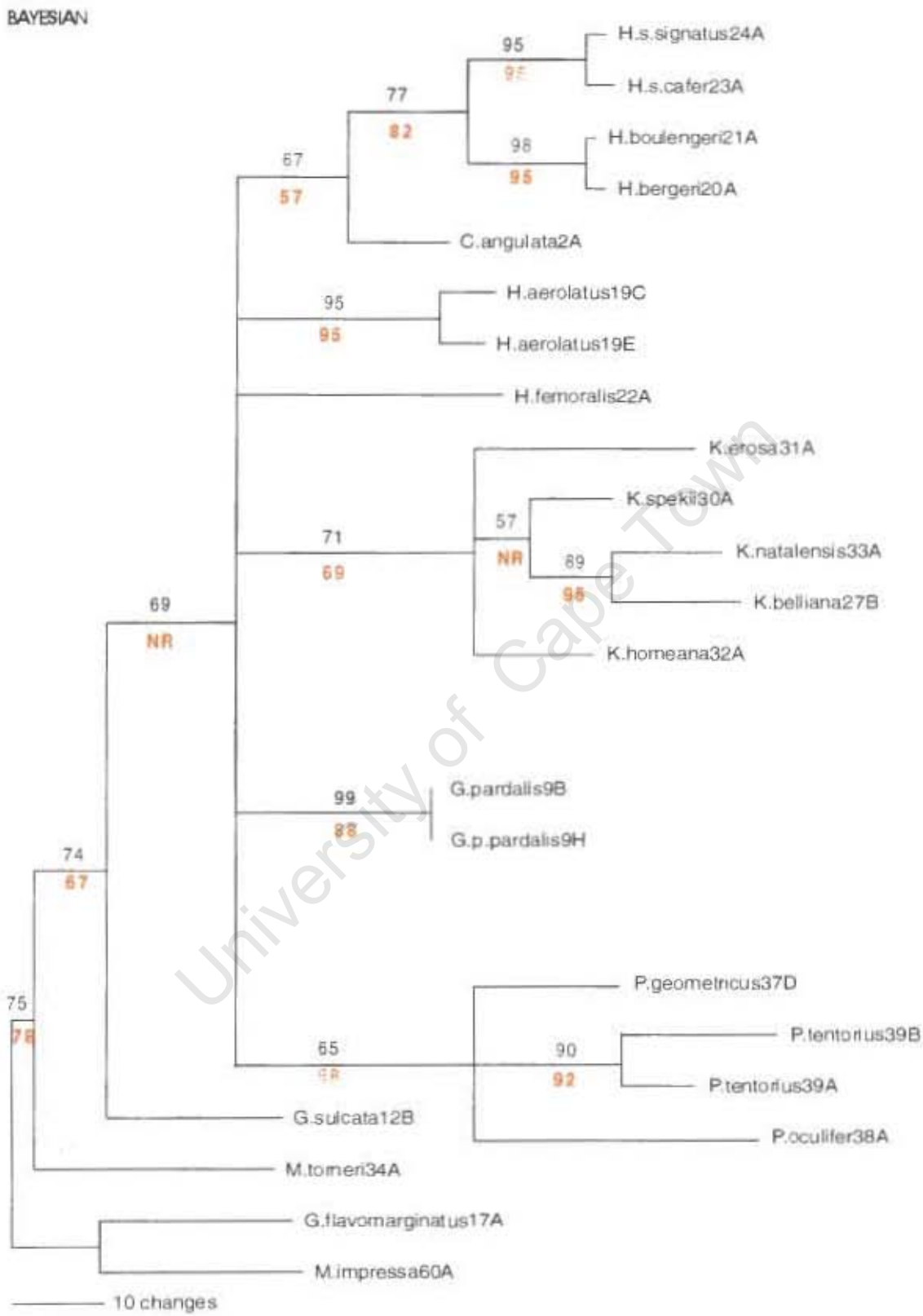


Figure 5.12: A bayesian estimate (GTR+Γ+P*inv*) tree topology for the Cyt *b* gene for the African clade (23 individuals). Values above the branches represent clade Probability estimates. Values in red are bootstrap proportions for a differentially weighted maximum parsimony estimate (Ti/ Tv = 7:1). NR = not resolved.

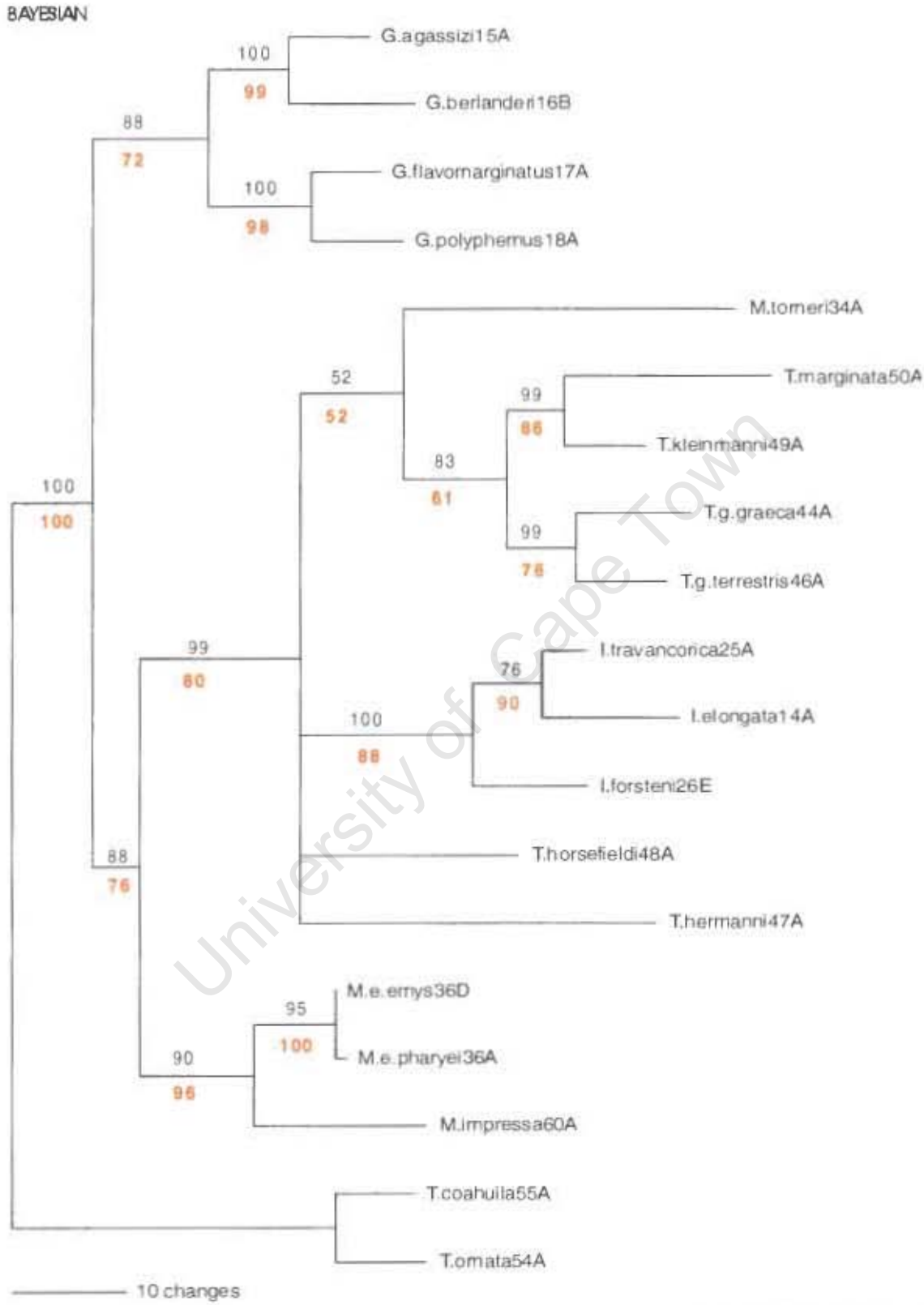


Figure 5.13: A bayesian estimate (GTR+Γ+P*inv*) tree topology for the Cyt *b* gene for the *Manouria-Gopherus* clade (19 individuals). Values above the branches represent clade Probability estimates. The topology was the same as that obtained using maximum likelihood inference (GTR+Γ+P*inv*, with P*inv* = 0.55, Γ = 1.47 and -lnL = 2091.855). Red values below the branches represent bootstrap proportions obtained from a differentially weighted maximum parsimony analysis (Ti/ Tv = 5:1).

ND4 Gene and the Combined Data Set

The topologies obtained for the large ND4 and combined data sets for all inference methods were similar. Both data sets were weighted with a Ti/ Tv of 9:1. The trees were characterised by high bootstrap support and probability values at the deep levels of divergence, as well as the terminal nodes (Figure 5.14, Table 5.8). In all cases, the combined gene tree was better resolved than the ND4 tree, particularly with maximum likelihood and bayesian analysis. Sub-clade analysis for the African and *Manouria-Gopherus* data sets showed similar topologies with respect to the larger data sets. Furthermore, all topologies within these smaller data sets were similar irrespective of the inference method used (differentially weighted parsimony, maximum likelihood and bayesian analysis), for the ND4 gene and the combined data set (Figures 5.15 and 5.16, Tables 5.9 and 5.10)

The monophyly of the Testudinidae was well-supported (node 2 in Figure 5.14 and Table 5.8, node CC in Figure 5.15 and Table 5.9). The Family consisted of two well-defined assemblages corresponding to the sub-family Gopherinae (*Manouria* + *Gopherus*) (node 3 in Figure 5.14 and Table 5.8). Sub-family Gopherinae was a well-supported sister clade to the sub-family Geocheloninae, containing all the remaining genera.

i. Sub-family Gopherinae

Within the Gopherinae, both *Manouria* and *Gopherus* were monophyletic (nodes 4, 6 respectively in Figure 5.14 and Table 5.8, nodes HH, EE in Figure 5.15 and Table 5.9). In the ND4 gene tree the sub-family Gopherinae was not monophyletic, and although both *Manouria* and *Gopherus* remained sister groups to the rest of the genera in the Testudinidae, the position of the genus *Gopherus* was not resolved (tree not shown). Removing *L. kempii* as an outgroup taxon and re-analysing the ND4 gene with only the *Terrapene* taxa as outgroups, resolved the monophyletic *Manouria* as a sister group to the remaining genera, which included *Gopherus* (Pr = 70). Within the larger clade a monophyletic *Gopherus* was sister to the rest of the Testudinidae (Pr = 100). Altering outgroups did not change the topology in the combined data set, although the monophyly of the Gopherinae was more strongly supported when *L. kempii* was used as the only outgroup in the analysis (node 3, Pr = 90). Furthermore, support for the *M. tornieri* + *T. horsfieldii* relationship (node 15) increased to Pr=90 when *L. kempii* was removed from the analysis and using the ND4 sequence data only.

In both the ND4 tree and the combined tree, the *Gopherus* clade consisted of two well defined monophyletic groups, *G. agassizii* + *G. berlandieri* (node 7 in Figure 5.14 and Table 5.8, node GG in Figure 5.15 and Table 5.9) and *G. flavomarginatus* + *G. polyphemus* (node 8 in Figure 5.14 and Table 5.8, node FF in Figure 5.15 and Table 5.9).

ii. Sub-family Geocheloninae

The Geocheloninae assemblage was monophyletic (node 18 in Figure 5.14 and Table 5.8) and contained two robust sister clades. The first contained the *Testudo, Malacochersus* and the *Indotestudo* genera (node 9 in Figure 5.14 and Table 5.8, node JJ in Figure 5.15 and Table 5.9). The genus *Testudo* was paraphyletic. Within the *Testudo, T. graeca, T. kleinmanni* and *T. marginata* occurred as a monophyletic group (node 10 in Figure 5.14 and Table 5.8, node NN in Figure 5.15 and Table 5.9). This group was sister to a poorly supported clade containing *T. hermanni,T. horsfieldii, Malacochersus* and the *Indotestudo* (node 13 in Figure 5.14 and Table 5.8). The ND4 gene tree failed to resolve *T. hermanni* (tree not shown), which was also poorly resolved in the combined data set and in the sub-clade analyses (Figure 5.14 and Figure 5.15). The monotypic genus *Malacochersus tornieri* was closely related to *Testudo horsfieldii* in all trees analysed (node 15 in Figure 5.14 and Table 5.8, node MM in Figure 5.15 and Table 5.9). These two sister species were resolved as a poorly supported sister clade to the *Indotestudo* spp. (node 14 in Figure 5.14 and Table 5.8). The Indian/ Southeast Asian *Indotestudo* species were monophyletic (node 16 in Figure 5.14 and Table 5.8, node KK in Figure 5.15 and Table 5.9).

The second and larger clade within the Geocheloninae contained all the remaining species in the Family Testudinidae. It was a paraphyletic assemblage containing two sister clades.

The first clade, although not well supported (node 20 in Figure 5.14 and Table 5.8), contained members of the genus *Kinixys*, the South American *Geochelone*, the Asian/ Indian *Geochelone*, the African *G. sulcata* and all the Indian Ocean Island endemics. The clade contained two well-resolved assemblages. The one was a monophyletic group containing all the Indian Ocean Island endemics (node 21 in Figure 5.14 and Table 5.8). Within this clade, the two *Pyxis* species formed a monophyletic pair (node 22 in Figure 5.14 and Table 5.8). *Pyxis* was sister to a clade containing *G. radiata* + *G. yniphora* (node 24 in Figure 5.14 and Table 5.8) and the Island giants, *D. arnoldi (G. gigantea* and "Rocket") (node 25 in Figure 5.14 and Table 5.8). In the ND4 parsimony tree, the relationship *between G. radiata* and *G. yniphora* was not resolved.

The second assemblage was also well-supported (node 26 in Figure 5.14 and Table 5.8). Within this clade, the African genus *Kinixys* was monophyletic (node 32 in Figure 5.14 and Table 5.8, node E in Figure 5.16 and Table 5.10). Within the *Kinixys, K. natalensis* and *K. belliana* were sister species (node 34 in Figure 5.14 and Table 5.8, node G in Figure 5.16 and Table 5.10) and closely related to *K. b. spekii* (node 33 in Figure 5.14 and Table 5.8, node F in Figure 5.16 and Table 5.10).

The genus *Kinixys* was sister clade to a larger diphyletic group containing the South American endemics (node 64 in Figure 5.14 and Table 5.8), and a well resolved trichotomy (node 28 in Figure 5.14 and Table 5.8) containing the African *Geochelone sulcata* and the closely related sister species pair, *G. elegans and G. platynota* (node 29 in Figure 5.14 and Table 5.8). Within the South American assemblage there was a close relationship between the Galapagos giant tortoise, *G. nigra*, and the smaller Argentinean endemic, *G. chilensis* (node 31 in Figure 5.14 and Table 5.8).

The next strongly supported clade within the Geocheloninae contained most of the southern African endemics except *K. natalensis and K. b. spekii* (node 35 in Figure 5.14 and Table 5.8, node D in Figure 5.16 and Table 5.10). The genus *Homopus* was not monophyletic although there were several well-supported relationships within the genus. These included *H. s. signatus* + *H. s. cafer* (node 47 in Figure 5.14 and Table 5.8, node R in Figure 5.16 and Table 5.10) *and H. bergeri* + *H. boulengeri* (node 48 in Figure 5.14 and Table 5.8, node S in Figure 5.16 and Table 5.10). These two sister species pairs formed a monophyletic group (node 46 in Figure 5.14 and Table 5.8, node Q in Figure 5.16 and Table 5.10) that was closely affiliated with the monotypic genus *Chersina* (node 45 in Figure 5.14 and Table 5.8, node H in Figure 5.16 and Table 5.10). This assemblage was a sister clade to the remaining southern African taxa, which included *H. areolatus and H. femoralis.* In the larger combined tree, *H areolatus* and *H. femoralis* formed a weakly supported monophyletic group (node 43 in Figure 5.14 and Table 5.8). However, in the ND4 bayesian analysis and the sub-clade analysis for the combined data set and the ND4 data set, *H. femoralis* was a monotypic lineage, sister to a larger clade containing the *G. pardalis* species complex and the genus *Psammobates* (node K in Figure 5.16 and Table 5.10). In all trees analysed, with all data sets, the southern African representative of the *Geochelone, G. pardalis,* was closely associated with the *Psammobates* clade (node 37 in Figure 5.14 and Table 5.8, node L in Figure 5.16 and Table 5.10). Within the *Psammobates* clade, *P. geometricus* and *P. oculifer*

were sister taxa (node 41 in Figure 5.18 and Table 5.8, node O in Figure 5.16 and Table 5.10). They were closely related to a well-supported group containing the representatives of the *P. tentorius* species complex (node 40 in Figure 5.14 and Table 5.8, node P in Figure 5.16 and Table 5.10). The genus *Psammobates* was monophyletic and long branches were associated with *Psammobates oculifer* (Figure 5.16).

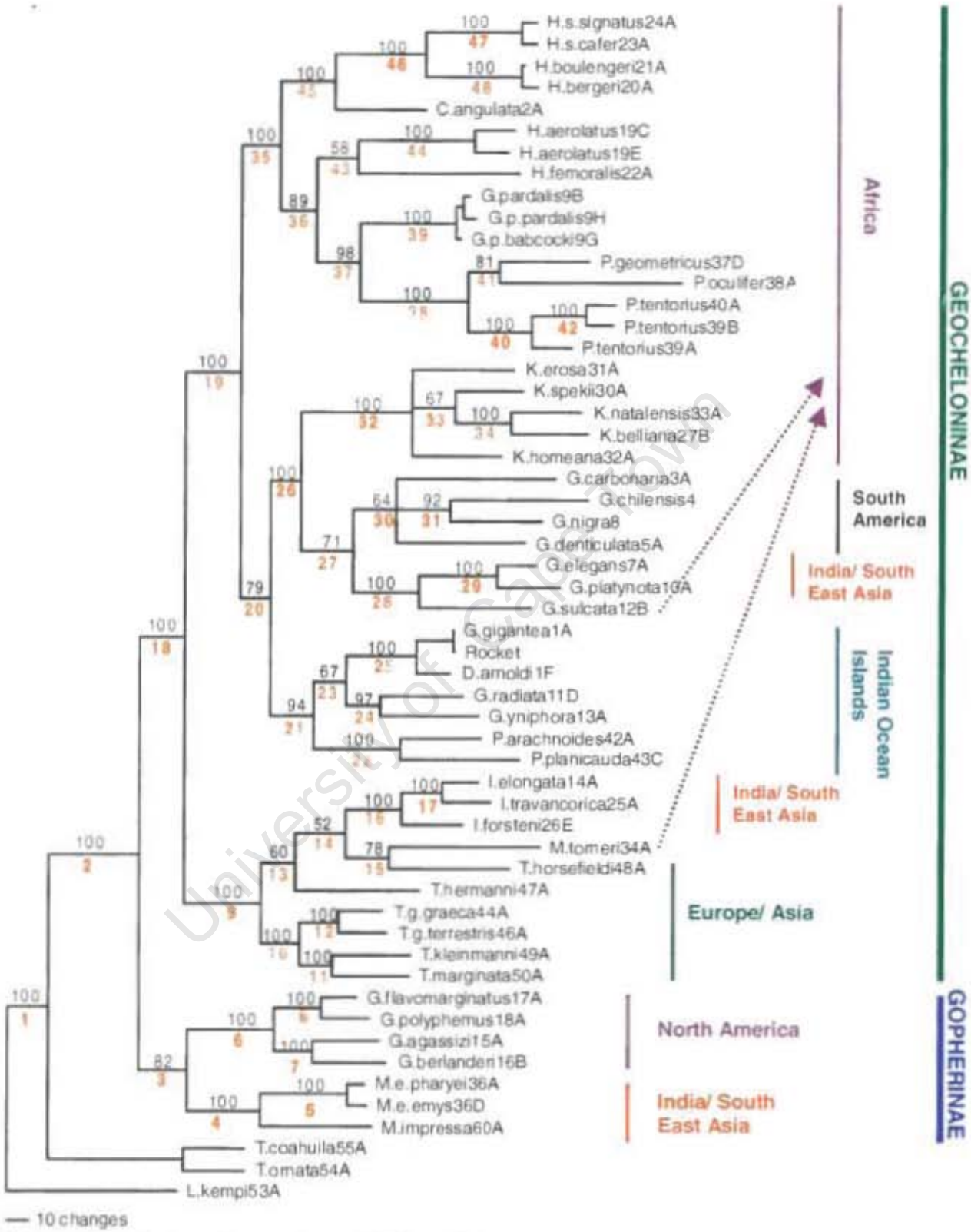


Figure 5.14: A bayesian estimate (GTR+Γ+P*inv*) tree topology for the combined data set (55 individuals and 1167bp). Values above the branches represent clade Probability estimates. The topology was the same as that obtained using maximum likelihood inference (HKY85+Γ+P*inv*, with P*inv* = 0.43, Γ = 1.11 and -lnL = 14445.481). Red values below the branches correspond to node labels (refer to text and Table 5.8).

Table 5.8: Non-parametric bootstrap support values for parsimony (differential weighting and 100 replicates), maximum likelihood (60 replicates) and probability values from bayesian analysis for the complete data set (ND4 gene and the combined data set respectively) (refer to Figure 5.14).

NODE	ND4		COMBINED		
	MP	Pr	MP	ML	Pr
1	100	100	100	100	100
2	100	100	100	100	100
3	51	NR	78	86	82
4	80	100	96	100	100
5	100	100	100	100	100
6	100	100	100	100	100
7	61	100	98	100	100
8	79	100	100	100	100
9	98	100	99	100	100
10	96	100	100	100	100
11	93	100	95	100	100
12	100	100	99	100	100
13	NR	72	NR	NR	60
14	NR	NR	NR	NR	52
15	71	92	75	70	78
16	88	100	98	100	100
17	89	69	99	98	100
18	87	100	95	93	100
19	89	100	90	100	100
20	NR	79	NR	77	79
21	NR	79	NR	92	94
22	88	100	100	100	100
23	NR	NR	NR	68	67
24	NR	100	53	82	97
25	94	100	100	100	100
26	79	100	90	89	100
27	NR	67	NR	70	71
28	95	100	99	100	100
29	100	100	100	100	100
30	62	59	64	74	64
31	62	86	72	72	92
32	95	100	100	100	100
33	NR	61	67	NR	67
34	100	100	100	100	100
35	50	100	NR	100	100
36	NR	58	NR	77	89
37	61	98	65	90	98
38	99	100	100	100	100
39	100	100	100	100	100
40	96	100	100	100	100
41	85	65	76	72	81
42	67	100	77	100	100
43	62	NR	71	NR	58
44	100	100	100	100	100
45	100	100	100	100	100
46	100	100	100	100	100
47	100	100	100	100	100
48	100	100	100	100	100

NR - not resolved

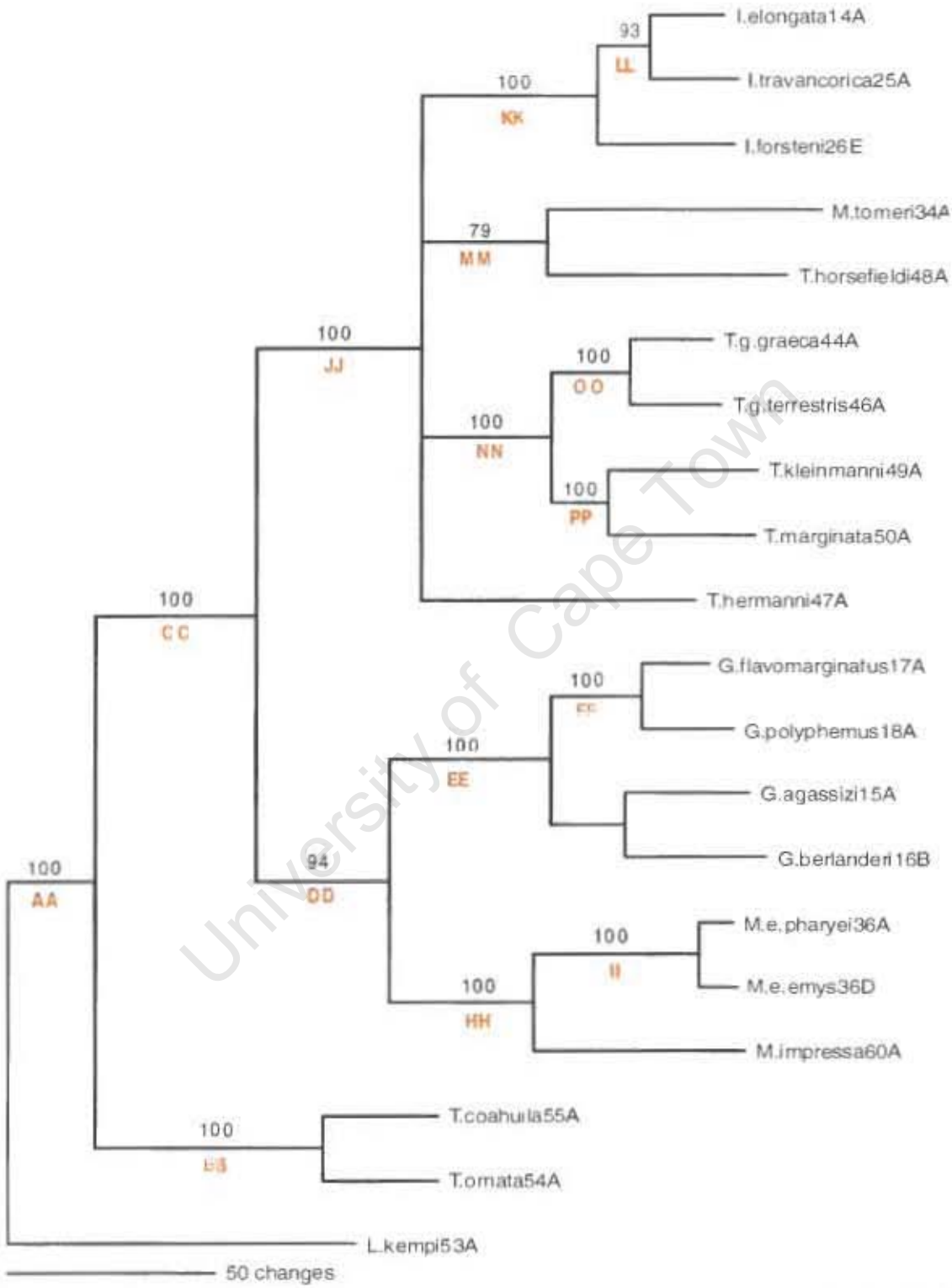


Figure 5.15: A bayesian estimate (GTR+Γ+P*inv*) tree topology for the combined data set for the *Manouria-Gopherus* clade (20 individuals). Values above the branches represent clade Probability estimates. The topology was the same as that obtained using maximum likelihood inference for both the combined and ND4 data sets (K8Luf+Γ+P*inv*, with P*inv* = 0.54, Γ = 1.74 and -lnL = 4366.255 for the combined set). Red values below the branches correspond to node labels (refer to text and Table 5.9).

Table 5.9: Non-parametric bootstrap support values for parsimony (differential weighting and 100 replicates), maximum likelihood (100 replicates) and probability values from bayesian analysis (Pr) for the *Manouria-Gopherus* clade (ND4 gene and the combined data set respectively) (refer to Figure 5.15).

NODE	ND4			COMBINED			
	MP 3:1	MP 5:1	Pr	MP 3:1	MP 5:1	ML	Pr
AA	100	100	100	100	100	100	100
BB	100	100	100	100	100	100	100
CC	100	100	100	100	100	100	100
DD	70	66	69	90	84	68	94
EE	100	100	100	100	100	100	100
FF	96	92	100	100	100	100	100
GG	81	75	81	100	100	100	100
HH	94	90	100	98	96	97	100
II	100	100	100	100	100	99	100
JJ	100	100	100	100	100	99	100
KK	98	95	100	100	100	100	100
LL	88	90	69	100	97	89	93
MM	89	88	100	86	84	58	79
NN	98	95	100	100	100	100	100
OO	99	96	100	100	100	100	100
PP	96	96	100	100	100	100	100

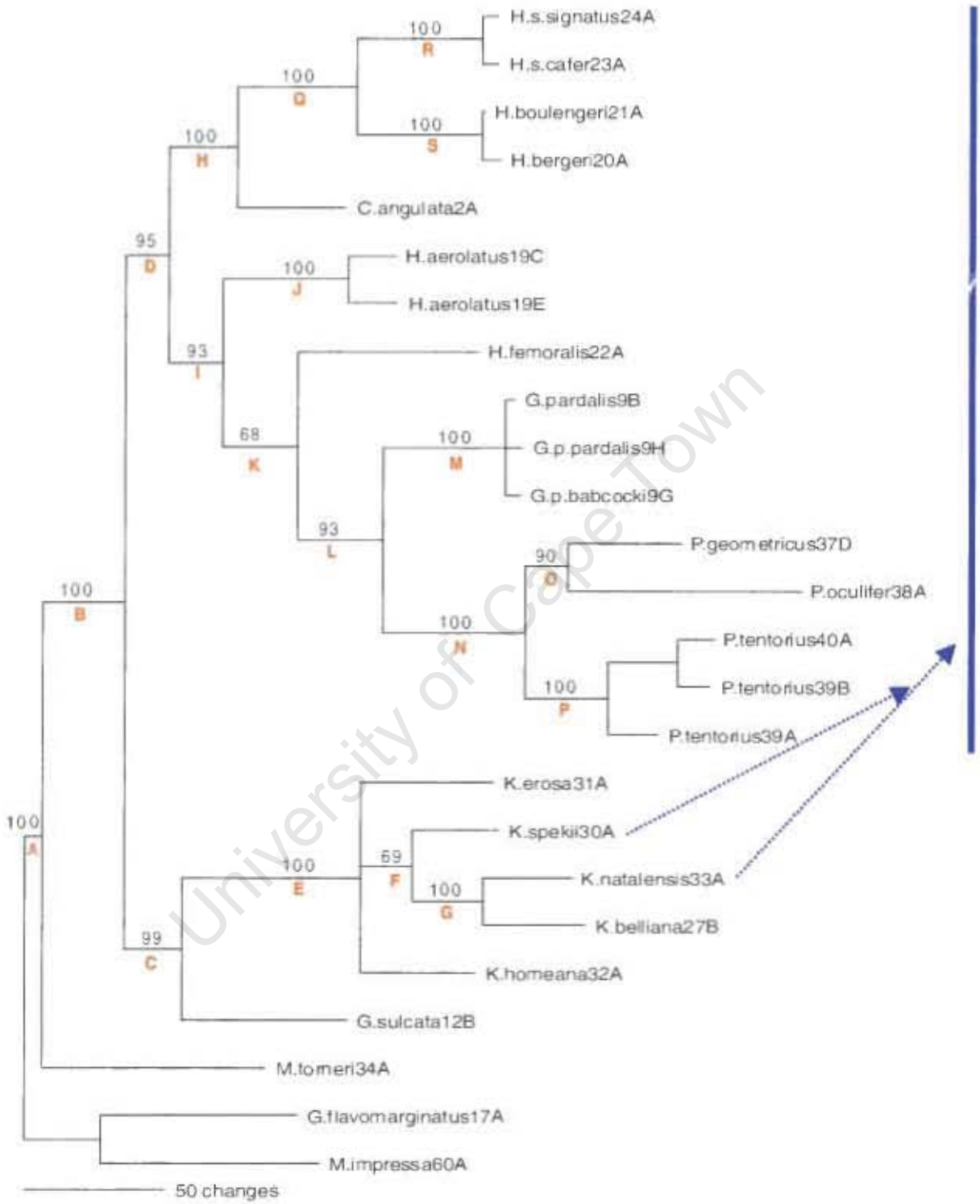


Figure 5.16: A bayesian estimate (GTR+Γ+P*inv*) tree topology for the combined data set for the African clade (25 individuals). Values above the branches represent clade Probability estimates. The topology was the same as that obtained using maximum likelihood inference (GTR+Γ+P*inv*, with P*inv* = 0.48, Γ = 1.67 and -lnL = 7695.980). Red values below the branches represent node labels (refer to text and Table 5.10).

Table 5.10: Non-parametric bootstrap values for parsimony (differential weighting and 100 replicates), maximum likelihood (100 replicates) and probability values from bayesian analysis for the African clade (ND4 gene and the combined data set respectively) (refer to Figure 5.16).

	ND4			COMBINED			
NODE	MP 3:1	MP 5:1	Pr	MP 3:1	MP 5:1	ML	Pr
A	95	95	100	96	95	89	100
B	51	88	100	95	95	89	100
C	82	91	99	81	88	76	99
D	87	85	100	89	89	95	100
E	100	97	100	100	100	100	100
F	70	70	76	50	58	52	69
G	99	98	100	100	100	100	100
H	76	86	100	89	89	95	100
I	67	72	75	NR	NR	76	93
J	100	100	100	100	100	100	100
K	68	68	88	NR	NR	62	68
L	87	84	91	88	88	83	93
M	100	100	100	100	100	100	100
N	100	100	100	100	100	100	100
O	83	85	78	77	79	62	90
P	97	100	100	100	100	100	100
Q	100	100	100	100	100	100	100
R	100	100	100	100	100	100	100
S	100	100	100	100	100	100	100

NR – not resolved

Combined Morphological and Molecular Analysis

The combined morphological and molecular data set contained 21 individual taxa and three outgroups for which there were no morphological characters available. Outgroup taxa did not have a significant impact on the topology whether they were included or excluded. There was 1167 bp of mitochondrial DNA and 66 morphological characters represented. The parsimony (Ti/ Tv = 9:1) estimate showed a similar overall topology to the larger data sets analysed, although it was not as well resolved (Figure 5.17). Support for the nodes on this reduced phylogenetic estimate were lower than for the previous analyses, which contained all the available members of the Family Testudinidae.

To elucidate the effect that the morphological characters had on the phylogenetic estimate, the analysis was repeated with morphological characters excluded. For the majority of the nodes there was an improvement in the branch support with the inclusion of the morphological data set, particularly at the base of the tree. The Family Testudinidae was shown to be a monophyletic group, both with and without the morphological characters (bootstrap = 100). *Manouria* and *Gopherus* were resolved as a monophyletic sister clade to the remaining genera (Figure 5.17). This relationship was well supported by the molecular data (bootstrap = 83) and was improved with the addition of the morphological characters (bootstrap = 91). *Testudo, Indotestudo* and *Malacochersus* spp. were retained as a monophyletic group (bootstrap = 96) that was sister to a larger clade containing the remaining genera. The sister relationship between *M. tornieri* and *T. horsfieldii* was resolved although not strongly supported, both with and without the morphological data (bootstrap = 54 and 56 respectively).

The larger assemblage containing all the remaining members of the Testudinidae was well supported as a monophyletic group (bootstrap = 94) with morphological characters included. In the absence of the morphological data, the relationships within this clade, particularly with the Indian Ocean island endemic, were not well resolved. The addition of the morphological matrix did not significantly improve the nodal support except for the relationship between *G. radiata* and *G. ynlphora* (bootstrap = 72 with morphological data included).

There was strong support for the sister relationship between *G. elegans* and *G. sulcata,* both with and without morphological characters (bootstrap = 99 and 98 respectively), as well as between the genus *Kinixys* and the South American *G. carbonaria.* As in the case of the large molecular data set, there was evidence for the monophyly of this group containing

representatives of the African species (*Kinixys* and *G. sulcata*), the Indian species (*G. elegans*) and South American species (*G. carbonaria*).

The monophyly of the southern African endemics, *C. angulata, H. areolatus, P. geometricus* and *G. pardalis* (excluding *K. natalensis* and *K. b. spekii*), was not strongly supported either with or without the addition of the morphological characters (bootstrap = 67 and 59 respectively). Furthermore, the addition of the morphological characters resulted in lower bootstrap support in all cases (Figure 5.17). *Psammobates* and *Geochelone pardalis* were again shown closely associated, although this relationship was not well corroborated by bootstrap support values.

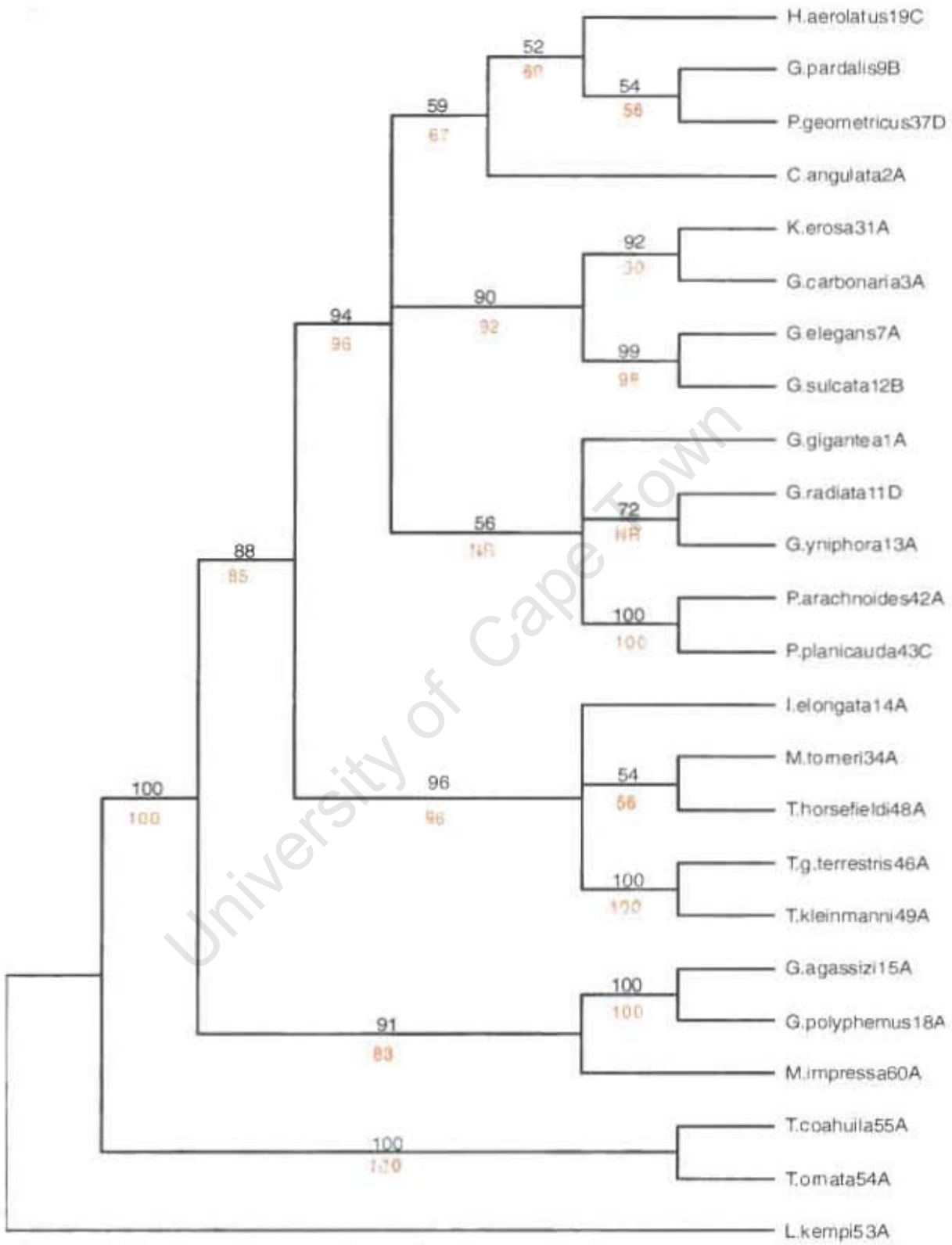


Figure 5.17: A parsimony estimate for the combined data set containing 1167bp of mitochondrial DNA sequence + 66 morphological characters (from Gerlach 2001). Molecular characters were weighted Ti/ Tv = 9:1. Values above the branches represent bootstrap proportions for the morphological+molecular characters whilst values below the branches represent the bootstrap proportions for the molecular characters only. NR = not resolved.

The *Psammobates* Clade

In all initial maximum likelihood and bayesian tree estimates, the genus *Psammobates* had long branches associated with *P. oculifer*. A detailed analysis was conducted on this genus, both to elucidate the potential causes of these long branches and to determine the extent of the proposed species complex within *P. tentorius* (Boycott and Bourquin 1989). Furthermore, several representatives of the species *P. geometricus* were included in this analysis to determine the effects of habitat fragmentation on the population genetic structure in this endangered South African endemic (see Chapter 7).

All phylogenetic topologies with all methods of inference were identical. The only differences were in the level of nodal support achieved, particularly between the model-based approaches as compared to the parsimony inferences (Figure 5.18). The "best-fit" model of evolution under the maximum likelihood optimality criterion was the TRN+Γ model with no invariant sites (P*inv*) assumed. The value of the gamma shape parameter was low (a = 0.359) indicating that most sites were invariable with a limited number of sites evolving very fast. Rate parameter estimates were high for transitions (r[C – T] = 17.97 and r[A – G] = 11.2) whilst all transversion rates were set to one. Parsimony analysis was implemented with two weighting schemes. The first was an unweighted analysis and the second was a differential weighting of Ti/ Tv = 3:1. For most of the nodes resolved within the genus *Psammobates*, parsimony analyses gave the highest support as measured by non-parametric bootstrap proportions, whilst the maximum likelihood and bayesian estimates of support were more conservative (Figure 5.18). This suggests that there are a sufficient number of synapomorphies to elucidate the inter-relationships amongst the species within this complex.

The monophyletic status of the genus *Psammobates* was robustly supported (node A). *Psammobates oculifer* and *P. geometricus* are sister species (node C). There was little to no differentiation discernible in the *P. geometricus* grouping (< 0.1% sequence divergence). The *P. oculifer* + *P. geometricus* clade was resolved as a sister group to a strongly supported clade containing all the *P. tentorius* representatives (node *B*). *Psammobates oculifer* was characterised by particularly long branch lengths (Figure 5.18).

Within the monophyletic *Psammobates tentorius* complex, two assemblages were evident both of which were well supported with the parsimony analysis (nodes G and F). The first clade (supported by node F) consisted of three individuals of *P. tentorius* (*P. tentorius*39A,

39D and 39E) that were obtained from Chafee Zoo Gardens and were not identified to sub-species level. This clade formed a sister group to a diphyletic clade containing the remaining representatives from the sample set (node G). This diphyletic clade consisted of two assemblages, both of which were well-resolved (nodes I and J). The first group (node I) contained *P. tentorius*40C which belongs to the *P. t. trimenii* sub-species. *Psammobates tentorius*39B and *P. tentorius*39C are individuals obtained from the Chafee Zoo Gardens and were not identified to sub-species level. The remaining two individuals (supported by node J) belong to the *P. t. verroxii* sub-species (*P. t. verroxii*40A and *P. t. verroxii*40B).

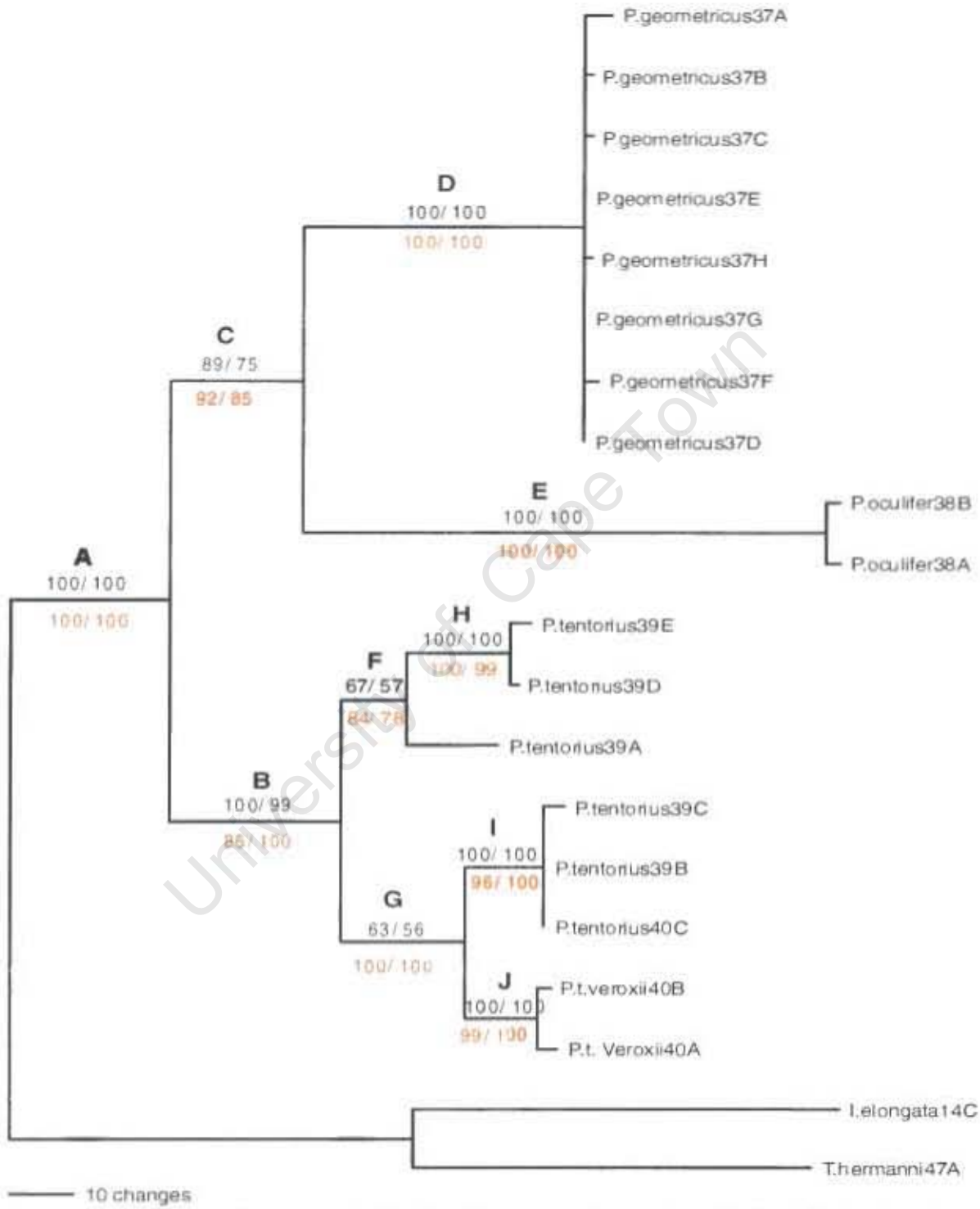


Figure 5.18: A bayesian estimate for the *Psammobates* clade (GTR+Γ+P*inv*). Topologies were similar irrespective of the inference method used (maximum likelihood model = TrN+Γ where Γ = 0.359 and -lnL = 3775.868). Values above the branches are clade Probability estimates/ and maximum likelihood bootstrap proportions whilst values below the branches represent bootstrap proportions from weighted maximum parsimony analysis with Ti/ Tv = 1:1 and 3:1.

The Relationship between Probability Values and Non-Parametric Bootstrap Support Values

Hillis and Bull (1993) conducted a study using known phylogenies of bacteriophages and found that approximately 70% bootstrap support corresponded to a 95% confidence interval. To compare the two measures of clade robustness within this study i.e. non-parametric bootstrapping and bayesian branch support, the two measures were plotted against one another using data from Table 5.8, Table 5.9 and Table 5.10 (Figure 5.19). The points corresponded to each of the nodes represented in the respective tables. A Pearson's product moment correlation (two tailed with n = 76) was conducted using SPSS v9.0 (SPSS Inc. 1998) to determine whether a linear relationship exists between the two variables. This test measures the degree to which the two variables are proportional to one another. The relationship was found to be significantly positive at the $\alpha = 0.01$ level, $r = 0.92$, indicating a strong correlation between the two estimates.

All bootstrap proportions of 100% corresponded to 100% bayesian support. Bayesian estimates were generally less conservative and ≥70% bootstrap corresponded to 90% - 100% bayesian support, with few exceptions. As a result of this and in keeping with current literature, branches that displayed probability estimates of more than 94% were taken as an approximation of robust branch support. Similar trends were displayed for the parsimony non-parametric bootstrap results (Tables 5.8, 5.9 and 5.10).

There was some variation around these points, however, which made a general relationship between the two difficult to interpret. For both the maximum likelihood and parsimony analyses, nodes supported by bootstrap proportions of less than 70% showed bayesian estimates lower than 85%, whilst bootstrap proportions of less than 55% generally resulted in probabilities lower than 60%. This could be because of the more conservative nature of the non-parametric bootstrap (Felsenstein 1985). The opposite relationship was observed with the analysis of the *Psammobates* clade (Figure 5.18) where bayesian support for the nodes was more conservative than the bootstrap values obtained.

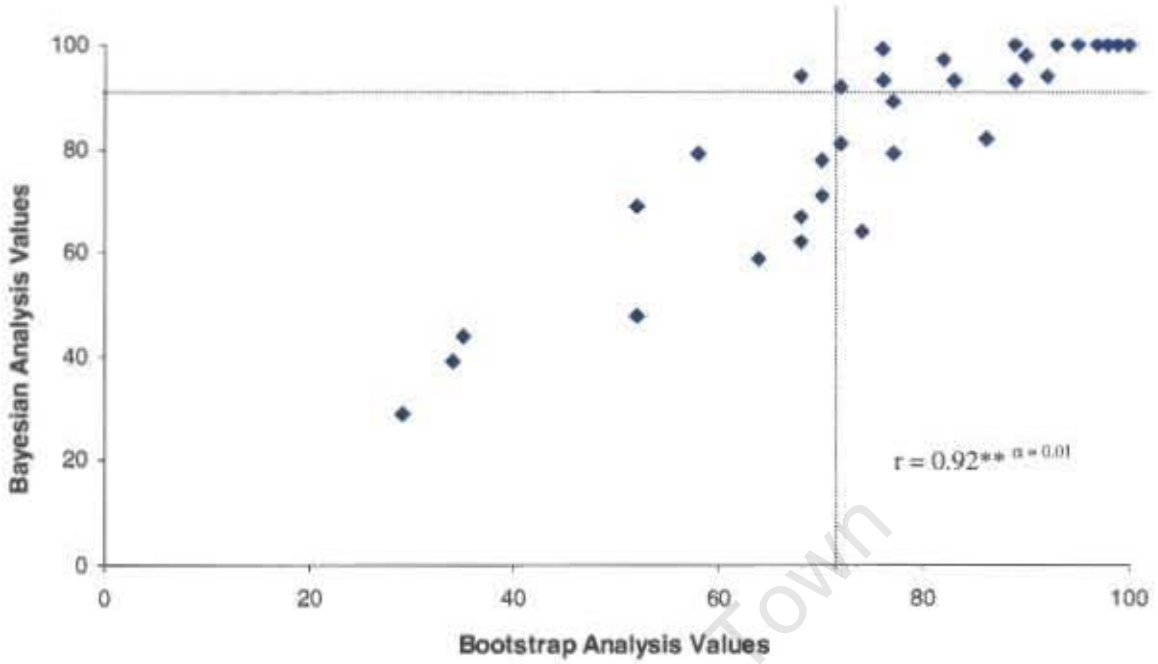


Figure 5.19: Comparison of bayesian and Bootstrap support. Each dot represents pairwise values as shown in Table 5.8, 5.9 and 5.10. The vertical line represents non-parametric bootstrap estimates over 70% and the vertical line represents bayesian probabilities of 90%.

Constraint Analysis and Hypothesis Testing

The Shimodaira-Hasegawa test (S-H) (1999) was used to test competing hypotheses of evolution in the Testudinidae. These alternative hypotheses are based on currently accepted phylogenies from both the morphological literature and the more recent molecular literature (Table 5.11, Crumly 1982, 1984, Gerlach 2001, Palkovacs et *al.* 2002, van der Kuyl et *al.* 2002). Furthermore, the monophyly of several of the currently accepted genera and biogeographic assemblages was tested. All alternative topologies were tested against the "best" estimate phylogeny obtained in this study (Figure 5.14). The same model of evolution and parameter estimates as calculated for the combined data set were used in the testing procedure i.e. HKY85+Γ+P*inv*, with P*inv* = 0.43, Γ = 1.11 and -lnL = 14445.481 (Table 5.4, Figure 5.14).

The southern African Clade was a well-supported group (including *Homopus, Chersina, G. pardalis* and *Psammobates*) that excluded the African species *Kinixys* (including the southern African endemics, *K. natalensis and K. b. spekii*), *Geochelone sulcata* and the monotypic *Malacochersus tornieri* (Figure 5.14). The clade did however support a strong relationship for *Geochelone pardalis* as a sister group to the genus *Psammobates*. In this analysis *Geochelone pardalis* was not placed with the other members of the genus *"Geochelone"* (see Auffenberg 1974, Crumly 1984 and Gerlach 2001). To test this novel "southern African" association, *G. pardalis* was constrained as a sister group to *G. sulcata* and subsequently as a sister lineage to the Indian Ocean Island clade whilst leaving the rest of topology unchanged. Both these trees were found to be significantly worse when compared to the "best" estimate topology obtained for the combined data set (Table 5.11). The evolution of *Geochelone pardalis* within the other southern African genera was therefore corroborated. To test the paraphyletic status of the African genera, the *Kinixys* clade, *Geochelone sulcata* and *Malacochersus tornieri* were each constrained as taxa embedded within the southern African clade. These trees were significantly worse than the trees that supported i) *Geochelone sulcata* as associated with the South American and Indian representatives of the *Geochelone* ii) *Malacochersus tornieri* related to the *Testudo* and iii) the *Kinixys* clade resolved with the "*Geochelone*". Consequently, the monophyly of the African genera could not be supported.

Palkovacs et *al.* (2002) hypothesised that the Indian Ocean Island endemics were closely affiliated with the African genera, particularly *G. pardalis*. The placement of the Indian Ocean clade as a monophyletic sister group (not including the sister clade relationship with *Kinixys*)

to the southern African endemics (including *G. pardalis*) was assessed. The resulting tree score was not significantly different from the "best" estimate log likelihood score (Table 5.11). This suggests that the Indian Ocean Island endemics could be affiliated with either of the two African assemblages within the Geochelonini i.e. to the smaller southern African genera or the larger African genera, including *Kinixys* and *G. sulcata* (as inferred from the "best" estimate topology).

In all phylogenetic estimates, the genus *Homopus* was not monophyletic and consisted of the *H. signatus* + *H. bergeri* + *H. boulengeri* assemblage, including the closely related *Chersina angulata* and an inconsistent *H. areolatus* + *H. femoralis* relationship (Figure 5.14, 5.16). The paraphyletic status of the genus *Homopus* was confirmed because the tree constraining the genus to a monophyletic grouping (excluding *Chersina*) had a natural log likelihood score that was significantly worse than that obtained for a paraphyletic *Homopus* as per the original estimate (Table 5.11). The relationship between *H. areolatus* and *H. femoralis* could not be clarified, as there was no significant difference between the scores of the "best" estimate tree and the topology that placed *H. femoralis* as a monophyletic sister group to *G. pardalis* (Table 5.11, Figure 5.16).

There has been considerable debate surrounding the monophyletic status of the genus *"Geochelone"*. All currently accepted members of the genus *Geochelone* were constrained to form a monophyletic group. This tree was found significantly worse than the "best" estimate topology that resolved the *Geochelone* as a paraphyletic assemblage (Table 5.11).

Although the genus *Testudo* was identified as a strongly supported group that included the monotypic genus *Malacochersus tornieri*, the relationships within this assemblage were not well resolved (Figure 5.14 and 5.15). This was particularly true for the placement of *T. hermanni, T. horsfieldii* and *M. tornieri*. *Testudo hermanni* and *M. tornieri* were each constrained to be monophyletic sister taxa to the remainder of the members in the clade. Neither of these two constrained topologies was significantly different from the "best" estimate topology (Table 5.11). No further comment could be made regarding their position within the clade although constraining them to any other assemblage in the tree produced a significantly worse fit to the data (results not shown).

A constraint tree was created that followed the phylogeny produced by Gerlach (2001) (Figure 2.4). This topology was compared with the "best" estimate topology obtained in this

study (Figure 5.14) and was found to be a significantly worse estimate as calculated by the S-H test, given the available molecular data and the specified model of evolution (Table 5.11).

Table 5.11: Table showing results of constraint analyses using the S-H test (Shimodaira and Hasegawa 1999) implemented in PAUP* v4.0b2 (Swofford 1999). All constraint analysis and topology tests were implemented with the combined data set only.

Constraint tree	- ln L	?Ln
"Best" combined ML inference	14445.481	best
Indian Ocean Clade with African Clade	14446.653	1.171
***G (S.). pardalis* with Indian Ocean Clade**	14496.922	51.441**
G (S.). pardalis* with *G (C.). sulcata	14493.201	47.720**
H. femoralis* monophyletic with *H. areolatus	14446.321	0.838^
***Homopus* Clade monophyletic**	14522.773	77.292**
***G (C.). sulcata* with African Clade**	14512.130	66.650**
***"Geochelone"* monophyletic**	14496.370	50.889**
***Testudo* Clade with African Clade**	14490.418	44.937**
***M. tornieri* monophyletic**	14448.717	3.235^
***M. tornieri* basal to *Testudo* Clade**	14450.967	5.486^
***M. tornieri* with African Clade**	14512.292	66.811**
***T. hermanni* basal to *Testudo* Clade**	14448.130	2.635^
Gopherus* Clade monophyletic with a basal *Manouria	14448.152	2.671^
"Gerlach" phylogeny	14675.321	229.84**

^ The S-H test was repeated on the sub-trees, where applicable, with the same significance result
**p < 0.005
S-H tests were implemented with full optimisation and 1000 bootstrap replicates

The Molecular Clock Hypothesis

The assumption of rate constancy amongst lineages was tested using information obtained from the initial estimates of tree topology. Branch length estimates indicated the species in the *Psammobates* clade, the two *Pyxis* species, *Testudo hermanni, Testudo horsfieldii* and *Malacochersus tornieri* were prone to longer branch lengths, particularly in the Cyt *b* gene tree (Figure 5.11, 5.12, 5.13, 5.14, 5.15, 5.16, 5.18). The Relative Rates Test (Tajima 1993) was used to determine any difference in the rate of evolution for these taxa. Pairwise comparisons revealed that *Psammobates oculifer* violated the assumption of rate constancy in almost all the pairwise tests conducted (85% of comparisons). Furthermore, *P. geometricus* and *P. tentorius* were found to differ significantly from the null hypothesis of equal rates of evolution in over 50% of the pairwise comparisons conducted. The pairwise rates within the *Psammobates* genus, however, were not significant indicating that members of this group are evolving at similar rates. The remaining taxa only produced significant results in less than 10% of the pairwise comparisons.

In addition to the Tajima Relative Rates Test, the hypothesis that lineages were evolving at similar rates was tested using the likelihood ratio test (Table 5.12). The LRT statistic was calculated for the large data set (using the "best-fit" model selected with ModelTest) containing all the taxa (including and excluding outgroups) and then excluding the *Psammobates* clade. The test was repeated for the *Manouria-Gopherus* clade (with and without outgroups) and for the African sub-set (with and without the outgroup taxa) and for the smaller *Psammobates* subset, including the outgroup taxa. The results of the test were the same regardless of the inclusion or exclusion of the outgroup taxa. The combined data set, with all taxa included, was found not to conform to rate homogeneity amongst lineages (Table 5.12). When the *Psammobates* clade was excluded, however, the LRT test showed a non-significant result indicating conformity to the molecular clock hypothesis or rate constancy amongst lineages. The analysis of the smaller data sets showed a similar pattern. The African clade did not conform to the molecular clock with the *Psammobates* genus included, whilst the *Psammobates* clade, when analysed in isolation (with outgroups included), was found to be evolving at a constant rate. This corroborated the initial observation as indicated by the longer branch lengths, that members of the genus *Psammobates* were evolving at a different rate to the remainder of the taxa in the Family Testudinidae (Table 5.12).

Table 5.12: Table showing the significance values for the molecular clock tests using the Likelihood Ratio Test and the combined data for the total sample set (55 individuals), for the *Manouria-Gopherus* clade (20 individuals), the African clade (25 individuals) and the *Psammobates* clade (20 individuals). Each test was repeated excluding outgroup taxa.

Data set	$-\ln L_1$	$-\ln L_0$	χ^2	d.f.	Significance including outgroups	Significance excluding outgroups
Total data set	14547.491	14445.481	204	53	0.00**	0.00**
Excluding *Psammobates*	13362.737	13307.531	110.4	48	> 0.1	> 0.1
African Clade	7732.783	7695.986	73.6	23	< 0.05**	< 0.05**
***Psammobates* clade**	3788.868	3775.868	13	18	> 0.1	-
***Manouria* Clade**	6670.079	6660.700	18.76	18	> 0.1	> 0.1

**$p \leq 0.05$
d.f. - n-2 where n is the number of taxa
$-\ln L_0$ – without molecular clock imposed
$-\ln L_1$ – with molecular clock imposed
$2^*(-\ln L_1 - -\ln L_0)$ approximates a chi-squared distribution

Divergence Estimates

The error or variance associated with the calculation of divergence estimates using misleading molecular clock assumptions can be large (Hillis et *al.* 1996). This is of particular concern when incorrect rates for the clocks are assumed or calibration rates from one part of the tree are used to date other nodes in the tree that may not necessarily exhibit equal rates of evolution (Rambaut and Bromham 1998). Furthermore, incorrect models of evolution can severely affect the estimation of branch lengths in the phylogenetic inferences, which will have a direct affect on the subsequent divergence estimates (Gaut and Lewis 1995, Yang 1996). Despite these associated limitations, divergence estimates for the Testudinidae were calculated using pairwise sequence divergence values from the maximum likelihood inference and two rate calibrations of the molecular clock to get some indication of the evolutionary time span that defines the Family. The standard or "universal" vertebrate rate of 2% per million years was used as well as a reduced rate of between 0.25% (Avise et *al.* 1992) to 0.6% (Caccone et *al.* 1999a, Palkovacs et *al.* 2002) per million years, which has been calibrated for the Order Testudines. To avoid violating the molecular clock assumption of rate homogeneity amongst lineages, the genus *Psammobates* was not included in the calculations.

Assuming a molecular clock rate of 0.6% per million years, the sequence divergence estimates suggest that the evolution of the Family Testudinidae began in the late Cretaceous

at least, approximately 65 million years ago (MYA) (node 2, Table 5.13). The "universal" rate of 2% per million years places this split from the Emydidae at only 18 MYA, which corresponds to a period well into the Miocene. Considering that fossil evidence for the Testudinidae is available from the Early Eocene (Auffenberg 1974), this estimate appears too conservative. The Kimura-2-parameter estimates were also found to be too conservative and using the 0.6% per million years calibration, placed the initial split between the Emydidae and the Testudinidae at approximately 30 MYA (mid Oligocene).

The extant members of the Gopherinae (*Manouria* and *Gopherus)* are the oldest members of the Testudinidae, a placement supported by plesiomorphic morphological characters these genera share with the Bataguridae and Emydidae turtles (see Chapter 2). This is corroborated by large sequence divergence estimates between *Manouria* and *Gopherus* spp. and the remaining representatives of the terrestrial tortoise family. Within the genus *Gopherus*, the two assemblages corresponding to the *G. agassizii* + *G. berlandieri* clade and the *G. flavomarginatus* + *G. polyphemus* clade, diverged some 16 MYA (node 6, Table 5.13). This estimate agrees closely with that obtained by Lydeard and Lamb (1994) of between 17 - 18 MYA.

Diversification in Europe and Asia had occurred by the early Eocene (node 18, Table 5.13) with the establishment of new species lineages still occurring into the Pliocene in both the *Testudo* (node 12, Table 5.13) and *Indotestudo* (node 17, Table 5.13) genera. The split between the African genus, *Malacochersus tornieri* and the Asian *Testudo horsfieldii* is estimated at 25.8 MYA (node 15, Table 5.13).

The remaining species of terrestrial tortoises diversified within a short period, beginning approximately 34.1 MYA (node19, Table 5.13), which corresponds broadly to the Eocene-Oligocene transition boundary.

The colonisation of Madagascar was estimated at approximately 24.7 MYA (node 21, Table 5.13). This estimate is older than the estimates from Caccone et *al.* (1999b) and Palkovacs et *al.* (2002) that date the colonisation of Madagascar at between (17.5MYA – 22 MYA). The split between the closely related *G. radiata* and *G. yniphora* species (node 24, Table 5.13) is estimated as having occurred 15 MYA, which is a close approximation to that obtained by Caccone et *al.* (1999b) of 14.5 MYA. The separation of the two *Pyxis* species appears to have occurred within the same period, 16.6 MYA (node 22, Table 5.13).

The divergence of the *Kinixys* clade and the clade containing the South American *Geochelone*, the Indian *Geochelone* and the African *G. sulcata* appears to have occurred before the colonisation of Madagascar, approximately 31.4 MYA (node 26, Table 5.13). Within this clade, the South American endemics separated from the African-Indian Geochelone at the interface of the Oligocene-Miocene transition some 24.3 MYA (node 27, Table 5.13). The Galapagos Island endemic, *Geochelone nigra*, separated from the South American mainland endemic, *G. chilensis,* approximately 20 MYA (node 31, Table 5.13). This divergence estimate is older than that estimated by Caccone et *al.* (1999b) who place the split at approximately 12 MYA. The diversification of the African *Kinixys* began in the mid Oligocene, approximately 28.1 MYA (node 32, Table 5.13) and appears to correspond closely to the diversification of the other southern African tortoise assemblage some 25 MYA (node 35, Table 5.13). Diversification within the southern African clade continued into the late Pleistocene/ early Holocene (nodes 39, 44, 47 and 48, Table 5.13).

Table 5.13: Table showing divergence times (in million years) based on two molecular clock estimates for the Testudinidae of 0.6%/ MY (Caccone et *al.* 1999) and 2.0%/ MY (van der Kuyl et *al.* 2002). The estimates are based on the sum of pairwise maximum likelihood distances for the combined data set excluding the *Psammobates* clade (refer to Figure 5.14).

NODE	0.6%/ MY	2.0%/ MY
1	69.5	20.7
2	65	18
3	25.3	7.59
4	18.3	5.49
5	2.3	0.69
6	16.6	4.98
7	10	3
8	5.3	1.59
9	-	-
10	16	4.8
11	11.6	3.48
12	6.2	1.86
13	-	-
14	-	-
15	25.8	7.74
16	8.3	2.49
17	5.8	1.74
18	48.3	14.59
19	34.1	10.2
20	-	-
21	24.7	7.41
22	16.6	4.98
23	-	-
24	15	4.5
25	-	-
26	31.4	9.42
27	24.3	7.29
28	14.3	4.29
29	4.1	1.23
30	-	-
31	20	6
32	28.1	8.43
33	14.5	4.35
34	11.6	3.41
35	23	6.9
36	-	-
37	-	-
38	-	-
39	2	0.6
40	-	-
41	-	-
42	-	-
43	23	6.9
44	5	1.5
45	18.3	5.49
46	12.4	3.72
47	1.3	0.39
48	1	0.3

- Not calculated

CHAPTER 6

DISCUSSION
PART I: PHYLOGENETIC INFERENCE

i. Phylogenetic Utility of the Cytochrome *b* gene fragment

The Cyt *b* gene has been used extensively in the elucidation of the phylogenetic relationships in a variety of taxa. This gene is generally considered most appropriate for recent divergence estimates of less than 50 million years (Wilson et *al.* 1985) although it has been used for the inference of phylogenies dating more than 345 MYA (Meyer and Wilson 1990). This is because variation in the evolutionary rate for this mitochondrial protein occurs at several levels. These include i) faster evolving changes at 3rd codon position (generally transitions), which are useful for recent divergence estimates and ii) the slowly evolving changes at the 1st and 2nd codon positions (generally transversions), which are able to resolve more ancient relationships within groups. This gene should therefore be appropriately matched for the evolutionary divergence levels of interest in Testudinidae although it is difficult to determine this suitability *a priori*, as this appears to be a taxon specific property (Graybeal 1993).

Limitations exist in the utilisation of this gene over deeper divergences, for example in the Bufonidae, which is a large family that occurs on all continents except Australia, Madagascar and the Polar Regions. The Bufonidae are an ancient lineage (Savage 1973) and have a poorly resolved taxonomy based on Cyt *b* sequence data. This is due largely to the saturation or near saturation of variable positions of the gene amongst the more deeply diverged Bufonoid species (Graybeal 1993). This trend is also apparent in phylogenies for the Xantusiid lizards (Hedges et *al.* 1991), where Cyt *b* has been more appropriate for the elucidation of recent relationships within the group. Similar results to these were apparent within this study of the Family Testudinidae. The Cyt *b* gene fragment provided high levels of phylogenetic signal in the terminal branches of the trees using parsimony, bayesian and maximum likelihood estimates, for example as seen within the genus *Gopherus, Manouria* and for the sister relationships between species in the genus *Psammobates* and *Geochelone platynota* and *G. elegans*.

In most cases, the deeper branches of the phylogenies for the Testudinidae were poorly resolved or not resolved at all, with little structure available to infer inter-generic relationships among species that could be supported with any degree of confidence. This could be due to the conserved nature of the Cyt *b* gene fragment, particularly in reptiles (Kocher et *al.* 1989). It has been suggested that there are differential selection pressures operating on this gene due to ectothermic physiology, which appears to place a greater constraint on the amino acid composition of the resulting protein (Kocher et *al.* 1989). This allows for variation at 3rd codon positions, which is sufficient for the resolution of recent relationships, but becomes saturated at synonymous sites at deeper divergence levels. This is due to functional constraints and results in the phylogenetic signal becoming swamped by random noise. The number of transitions calculated using model-based approaches was almost triple that of the number of transversions observed in this data set. This resulted in low levels of observed amino acid pairwise distances, even between ingroup and outgroup taxa. Complete exclusion of the potentially homoplastic 3rd codon positions did not improve resolution and instead reduced the bootstrap proportions supporting the terminal relationships. This suggests that although there is saturation at some 3rd position sites, there is also site specific rate heterogeneity (as supported by reported α values) implying several of these sites do in fact contain informative phylogenetic signal.

Despite the inefficiency of this gene in providing resolution and structure for deeper levels of divergence within the trees, the monophyly of the Testudinidae was fully supported. This was achieved after the exclusion of *L. kempii* as an outgroup taxon in the unweighted parsimony analysis. *L. kempii* could not be placed as an outgroup which, could be a result of the randomisation of the phylogenetic signal over deep divergence levels i.e. no synapomorphies to support the relationship.

The Cyt *b* gene was particularly well suited for the analysis of the *Psammobates* species complex, and particularly so using parsimony methods, suggesting confidence for utilisation at the intra-generic and conspecific level due to signal provided by the rapidly evolving codon position transitions.

Phylogenetic Utility of the ND4 gene fragment

The Nicotinamide Adenine Dinucleotide Dehydrogenase subunit 4 gene has been used to elucidate the inter-generic relationships in reptiles and amphibians for example, in Iguanid lizards (Sites et *al.* 1996, Wiens and Hollingsworth 2000), the Cyclura lizard family (Malone et *al.* 2000) and in the sub-family Lygosominae (Honda et *al.* 2000). All studies to date, including the Testudinidae, have displayed significant adenine-cytosine (AC) bias across all sites, and particularly at 1st and 3rd codon positions, and this appears to be a common pattern in animal mitochondrial DNA genes (Naylor et *al.* 1995). Furthermore, guanine (G) deficiency at 3rd codon positions has also been observed. Within the Family Testudinidae, the ND4 gene fragment offered greater resolution, particularly at the deeper levels of divergence than was afforded by the Cyt *b* gene fragment. This was particularly evident in unweighted parsimony analyses. All phylogenetic estimates based on the ND4 gene fragment were similar, irrespective of the methods of inference utilised, suggesting strong underlying phylogenetic signal. This was corroborated by saturation plots in which 1st and 3rd position transitions and transversions were shown to saturate at higher sequence divergence estimates than the Cyt *b* sequences and displayed higher overall amino acid sequence estimates suggesting fewer functional constraints operating on the ND4 protein. Despite the high levels of resolution offered by this gene in the Testudinidae, there has been some debate as to its utility resolving of deep level divergences in other groups (Wiens and Hollingsworth 2000).

The variability in the suitability of the two genes utilised in this study highlights the importance of correct selection of mitochondrial markers that should be appropriately matched and optimised for the divergence levels of interest. The suitability of genes used in molecular phylogenetic studies should not be assumed *a priori* from parallel studies.

ii. Combined Analysis

Due to the increasing realisation that no particular data set or type of data can be assumed *a priori* to be a better indicator of phylogenetic or evolutionary relationships, the Cyt *b* gene fragment and the ND4 gene fragment were combined using a Conditional Combination Approach as advocated by Sullivan (1996) and Cunningham (1997). Independent analysis of both gene fragments showed several similar highly supported nodes. The ND4 gene topologies, however, offered more structure particularly at deeper divergence levels. The combination of the data sets was supported by homogeneity in the signal from the data sets as indicated by the ILD test (Farris et *al.* 1994). The resulting combined phylogenetic estimate was well resolved at both deep and terminal levels within the tree, as indicated by higher bootstrap proportions, higher probability values and similar topologies irrespective of the inference method utilised, than was achieved by either of the genes in isolation. This suggests that the signal from the two genes was additive and complementary in the combined approach resulting in a partially resolved phylogenetic estimate for the Family Testudinidae. This has also been shown in the Xantusiidae lizards (Hedges et *al.* 1991) where estimates based on the Cyt *b* gene fragment alone displayed high levels of homoplasy and low bootstrap support, but when combined with the more slowly evolving 12s RNA, resolved a well-supported phylogeny.

Molecules or Morphology?

Different rates of evolution are known to underlie different data partitions (Sullivan 1996). To further investigate the effects that these differences have on estimates of phylogeny, a morphological data matrix by Gerlach (2001) was used in combination with the molecular data partition in the phylogenetic inference.

The initial morphological phylogeny reported by Gerlach (2001) was not congruent with the best estimate phylogeny obtained using the combined molecular data set as reported in this study. As described in Chapter Two, high levels of parallelism and convergent evolution appear to characterise the Family Testudinidae and have plagued morphological estimates for the group. Despite the fact that morphological characters were unavailable for analysis for the outgroup taxa used in this molecular study, characters from the original study (Gerlach 2001) were polarised using members of the closely related Bataguridae. The Bataguridae are considered a sister group to the Testudinidae and closely associated with the *Terrapene,* which were used as outgroup taxa in the molecular analysis. The exclusion of outgroup taxa did not, however, have a detrimental impact on the resulting inference (data not shown).

Although no result was obtainable for the ILD test, the morphological data sets and molecular data set (Cyt *b* + ND4) were combined (see Sullivan 1996). The resulting phylogenetic estimate was congruent with that obtained for the molecular data set. These results further corroborate the novel molecular phylogeny obtained for the Family Testudinidae as described within the framework of this study, and suggest that high levels of homoplasy within the terrestrial tortoises may limit current estimates based on morphological characters alone. This is particularly evident in the derived clade containing the African endemics, which displayed lower bootstrap support when morphological characters were included in the analysis. Gaffney and Meylan (1988) and Meylan and Sterrer (2000) have reported that these more derived members of the family show higher levels of character reversal than is present in other clades (and Gerlach *pers. comm.*) and this could explain their poor resolution.

The result of the combined morphological and molecular analysis also indicates decoupled rates of morphological and molecular evolution within the Family Testudinidae and suggests particular caution when using morphological characters in isolation to infer deep level divergences.

iii. More Taxa or more Characters and the effects of Long-branch Attraction

There has been some debate as to whether it is more effective to increase the number of characters (Mitchell et *al.* 2000) or to increase the number of taxa (Johnson 2001) to improve the accuracy and consistency of phylogenetic estimates, particularly in data sets that display high levels of homoplasy (Mitchell et *al.* 2000). Furthermore, increased taxon sampling has been shown to increase the accuracy of the resulting inference even at the expense of a decrease in the number of characters sampled per taxon (Hillis 1996, 1998, Poe 1998, Johnson 2001 but see Kim 1998, Mitchell et *al.* 2000).

Support and resolution for inter-generic relationships in the Family Testudinidae were improved with increased taxon sampling, as indicated by increased bootstrap proportions and probability values associated with the analysis of the larger data sets. The only exception to this trend was found in the analysis of the *Psammobates* clade, where the intra-generic analysis of the smaller data set was dramatically improved when analysed in isolation (Figure 5.18). This was particularly true for the sister relationship between *P.*

geometricus and *P. oculifer*. The genus *Psammobates* was characterised by increased rates of evolution, which could be a factor in the poor resolution for this in the larger data set.

Insufficient or incorrect taxon sampling can manifest as long-branch attraction at deep and low levels of phylogenetic hierarchies (Stiller and Hall 1999, Wiens and Hollingsworth 2000), resulting in misleading inferences (Sites et *al*. 1996, Wiens and Hollingsworth 2000). Within the Family Testudinidae, van der Kuyl et *al*. (2002) report a sister relationship between the *Testudo* and *Indotestudo* genera. This relationship was attributed to insufficient taxon sampling within the group and long-branch attraction, leading to an incorrect association between these two lineages. However, given the results reported in the present study, which had all genera and all species for the Family represented, the sister relationship between these two genera was supported and well-resolved and was not due to the effects of long-branch attraction as has been previously suggested. Furthermore, the novel relationship reported between the African endemic *M. tornieri* and the Eurasian *T. horsfieldii*, both of which were defined by longer branches, was confirmed using all methods of phylogenetic inference. Model-based methods are known to be less susceptible to the effects of long-branch attraction unless an incorrect model of evolution is assumed (anti-Felsenstein Zone) (Yang 1996, Swofford et *al*. 2001). However, neither bayesian estimates nor maximum likelihood estimates for the ND4 and combined data sets separated these lineages using the selected "best-fit" models of evolution. This relationship is further supported by independent morphological evidence (Crumly 1984). The possibility of long-branch attraction between *M. tornieri* + *T. horsfieldii*, arising as a result of model inadequacy, can be further investigated using parametric bootstrapping. This option, however, was beyond the scope of the study due to the computational time constraints involved in parametric bootstrap analysis, particularly on large data sets (Bolback 2002).

Character sampling may have been an additional problem in the analysis of phylogenetic estimates for the Family Testudinidae. For example, one of the limitations of the Cyt *b* gene may have been due to the small size of the gene fragment analysed. There were 172 parsimony informative sites present within the Cyt *b* sequence, with few synapomorphies available to resolve the deeper divergence levels. Resolution within these estimates was only marginally improved after the application of complex weighting schemes and the paucity of the number of informative characters available for the gene could have been a contributing factor in this poor resolution. Both the bayesian and maximum likelihood estimates were poorly resolved when based on Cyt *b* sequence analysis alone. This suggests that saturation

of the variable sites and constraints on amino acid composition change were additional factors contributing to the poor resolution achieved with Cyt *b* at deeper nodes in the trees.

The ND4 and combined data sets (contain 340 and 512 parsimony informative sites respectively), however, displayed improved resolution at terminal and deep level relationships in the estimates, even in unweighted analysis. It appears that increasing the number of characters in the analysis, particularly from different genes, offers increased resolution, provided all major lineages that define the group have been sampled. This may prevent the potential formation of long-branches and, in addition, increase the number of characters from alternate genes available for enhanced phylogenetic signal, particularly at different levels in the taxonomic hierarchy. The paucity of characters for the Cyt *b* gene fragment, may explain the poor resolution achieved with this marker.

Iv. Parameter Estimation

The effects of parameter estimation on tree topology and tree scores was assessed as the importance of estimates derived empirically from the data set under study is becoming increasingly recognised (Voelker and Edwards 1995). Rate heterogeneity among sites was found to decrease i.e. increasing alpha values, with increasing taxonomic level, whilst the proportion of invariant sites decreased. Estimated parameters were robust to starting tree topology (also in Malone et *al.* 2000) and significant improvement in both tree topology and tree scores was reported when parameters were estimated empirically from the data set as opposed to using default parameter options implemented in PAUP*. The ND4 and combined topologies were found to be more robust to model violations and differences in model selection than Cyt *b* topologies. Branch lengths were consistently underestimated when default parameters were used. Cyt *b* topologies, however, were sensitive to model selection, particularly concerning the placement of taxa, which were significantly different from "best" estimate topologies. For example, using a gamma shape parameter of 0 and frequency of 0.25 for each of the four nucleotides resulted in *Psammobates* spp. being resolved with *Manouria* spp, albeit weakly (bootstrap = 54). This difference could be due to the strength of the underlying signal in the ND4 and combined data sets, evident even in the unweighted parsimony analysis. The poorly resolved Cyt *b* gene tree, in particular, was not sensitive to improvements in resolution with weighting (Voelker and Edwards 1998), which could point to high levels of homoplasy or a rapid radiation in the Family Testudinidae (see PART III: Biogeography). In all cases, there was a significant improvement in the fit of all the data sets with the addition of Γ and *Pinv* rate heterogeneity parameters.

Although site specific rates (SSR) did not alter tree topology, did consistently underestimate branch lengths (data not shown) (Voelker and Edwards 1998, Danforth and Ji 2001). Buckley et *al.* (2001b) have also commented on this phenomenon in a taxonomic study on the *Maoricicada* genus. It has been suggested that this could be due to the fact that SSR models explicitly assume rate homogeneity within each codon class, even when there is extreme rate heterogeneity evident as shown in this study. Within the Testudinidae, in all data sets analysed, 1st and 2nd codon positions showed extreme levels of rate heterogeneity, with low levels of alpha (the gamma shape parameter) (see Voelker and Edwards 1998). This implies that most sites are invariable, with only a few evolving either very fast or very slow. The rates were lowest for 2nd codon positions in each case. This was not unexpected as 2nd codon positions are known to be the most conserved due to functional constraints on subsequent proteins produced from the mitochondrial protein-coding genes. Gamma shape parameters were highest at 3rd codon positions, which implies that most sites are evolving at moderate rates with a few evolving very fast. This is manifested as high levels of variability associated with 3rd codon positions in protein coding genes as a result of the inherent degeneracy of the genetic code.

The effects of parameter estimation and model selection on subsequent topology and likelihood scores suggests that such information should be derived empirically from the data set at hand, and should not be assessed using default parameters, nor assumed on an *ad hoc* basis from studies on closely related groups. The fact that these properties do indeed have a significant impact on the final phylogenetic estimate implies that they should be optimised for each group and gene studied. Furthermore, phylogenetic estimates should be assessed using a range of potential models to assess the effects of alternate assumptions on topology, branch lengths and nodal support. In so doing, this could provide insight into the mode of molecular evolution of DNA and provide a framework within which to refine the application and assumptions pertaining to model-based approaches in particular, which are still subject to debate (see Chapter 3).

Analysis of bootstrap proportions was found to display similar trends to that of posterior probabilities from bayesian estimates, although the bootstrap values were generally more conservative (Rannala and Young 1996). This could be due to either the inherently conservative nature of the bootstrap (Hillis and Bull 1993) or the effects of "burn-in". The process of "burn-in" is specific to bayesian analysis and occurs when initial posterior

probabilities, which have not reached stationarity, are excluded from the selection of probabilities used to generate the final Posterior Probability estimate. Therefore, "burn-in" can potentially bias the estimation of the final probability values. The results presented within the framework of this study suggest a positive correlation between bootstrap and posterior probability values and as such provide a comparative measure of confidence when comparing branches or clade support when using different methods of inference.

v. Methods of Inference

An inherent problem with parsimony is that it is unable to accurately estimate branch lengths. Furthermore, the implication that both the Cyt *b* gene fragment and the ND4 sequence were susceptible to multiple hits indicated the need to address the phylogenies using model-based approaches. Within this study, the Cyt *b* gene was not effective at resolving deeper nodes. ND4 and the combined data partitions, however, provided well-resolved estimates of phylogeny using all methods of inference. For parsimony analysis, there was an adequate distribution of synapomorphic characters in the ND4 gene set to resolve deep level relationships. The combined data set provided additional improvement in the robustness of terminal relationships in the trees using parsimony and model-based approaches (Figure 5.14 and Table 5.8).

Multiple data partitions and the application of a variety of inference methods produced highly congruent estimates of phylogeny for the Family Testudinidae. Bayesian analysis allows for the efficient and rapid analysis of sequences, particularly with large data sets, and gives some measure of the statistical confidence that can be placed in the resulting estimate. One limitation, however, is the requirement for improved computer speed and capacity than is not widely available.

The accuracy of maximum likelihood depends largely on a lack of Type II systematic error i.e. a good fit between the assumptions of the substitution model and the true underlying evolutionary process (Swofford et *al.* 1996, Rogers 1997). This was accomplished by deriving substitution and rate parameters empirically from the data sets. The fit of all models of evolution to the data sets was assessed and the one shown to offer a significantly "best-fit" for the available data was selected. The disadvantage of maximum likelihood and parsimony is that they both only provide point estimates. However, in the analysis of the relationships within the Family Testudinidae, these point estimates agreed closely with the consensus

phylogeny achieved with bayesian analysis, offering some level of confidence in the inferred tree.

This aspect of the study highlights the importance of selection of efficient molecular markers for the elucidation of robust relationships between the organisms under study. Furthermore, parameter estimates for model based approaches should always be derived empirically from the data set at hand and not assumed *a priori* from information obtained from parallel studies and a number of inference methods should be utilised to infer the best estimate topology that can be obtained from the data set under study.

PART II: A REVISED TAXONOMY FOR THE FAMILY TESTUDINIDAE Batsch, 1788

The molecular phylogeny for the Testudinidae is not congruent with currently accepted morphological estimates. This could be due to a number of factors including i) the two data partitions (morphological and molecular) are exposed to different underlying evolutionary pressures ii) the morphological characters may be prone to adaptive or parallel evolution and iii) the addition of taxa and/ or characters may alter tree topologies. This incongruency, however, can be termed "soft incongruence" as the combined molecular + morphological data set is congruent with the best estimate tree topology obtained with molecular characters alone.

This molecular data set represents the most taxonomically complete data set analysed to date for the Testudinidae; which may be one possible reason why several unique relationships within the group were resolved. The novel phylogenetic estimate presented does, however, support several traditionally accepted relationships within the Testudinidae. In all cases, the monophyly of the terrestrial tortoises was fully supported, using a variety of outgroup taxa from the closely related Emydidae and the more distant Lepidochelys. Outgroup taxa were also defined by high levels of sequence divergence estimates[6] in all comparisons with ingroup taxa (all of which were greater than 28%).

The intra-generic sequence divergence estimates calculated for the family Testudinidae were shown to have particularly large ranges within the *"Geochelone", Homopus, Testudo* and *Psammobates* genera (see Chapter 5). This could more than likely be a result of the incorrect classification of species and/ or sub-genera within these groups, which could bias the final values obtained. Sequence divergence estimates used in isolation are by no means an indication of the taxonomic delimitations within groups. However, in this study they have been used together with relationships obtained from molecular and morphological phylogenetic estimates to get some idea of their potential use in defining currently accepted species and generic boundaries within the family Testudinidae (see Hedges et *al.* 1991, Bradley and Baker 2001).

[6] All sequence divergence values reported are based on maximum likelihood estimates calculated in PAUP* and based on the combined molecular data set (Swofford 1999).

Manouria and *Gopherus* were resolved as the most basal or oldest extant members of the Family using the molecular and morphological characters. Several plesiomorphic morphological characters as well as high pairwise genetic distances associated with the remaining species in the family support this hypothesis. *Manouria* has been considered the least derived of the extant tortoises and this association is supported by the retention of several ancestral features, for example a non-enlarged pygal bone and the presence of class II mental glands, which are shared with the Bataguridae turtles (Winokur and Legler 1975). Within the monophyletic genus *Manouria*, the sub-species rank afforded *M. e. emys* and *M. e. pharyei* was supported with a sequence divergence estimate of less than 1.5%.

Manouria and *Gopherus* are a monophyletic assemblage, united by a lack of surungular processes and the presence of mental glands (Crumly 1984) i.e. supporting sub-family Gopherinae (Crumly 1984). The current phylogenetic estimate suggests a single evolutionary history for these two lineages. This could be due to a lack of resolution in the basal part of the tree due to missing fossil taxa that would allow for the elucidation of an alternate relationship between these genera i.e. the separation of these lineages. An alternate relationship would place *Manouria* as the most ancient extant member of the Family as suggested by Crumly (1982, 1984). This hypothesis, however, remains to be tested and can only be achieved by i) the inclusion of the morphological characters which define the associated fossil forms or ii) the sequencing of DNA fragments isolated from ancient DNA, representing such fossil forms.

The North American genus *Gopherus* has been identified as an older genus due to the close affiliation with fossil species such as *Stylemus* and *Hesperotestudo* (Auffenberg 1966, Crumly 1984, Gerlach 2001) and the retention of plesiomorphic features shared with *Manouria*, for example, the presence of mental glands (Crumly 1984). The monophyly of this group was strongly supported, as was the monophyly of two sub-genera represented by *Gopherus polyphemus* + *G. flavomarginatus* (*G. sensu stricto*) and *G. agassizii* + *G. berlandieri (Agassizii)*. Sequence divergence estimates within these species groups agree closely with those reported by Lamb and Lydeard (1994) of 3% and 6% respectively. The two groupings were consistent with sequence divergence estimates for within genus classifications in this study of less than 12%, and agree with previously defined clades identified by Bramble (1971), Auffenberg (1976) and Lamb and Lydeard (1994). The pairwise molecular divergence estimates, however, do not endorse separate generic designations for

each of these species complexes (as suggested by previous authors including Bramble 1982, Bour and Dubois 1984). They should be maintained within a single genus.

Testudo spp. and *Indotestudo* spp. were resolved as sister taxa, basal to the remaining Geochelonini, although several authors have not supported these relationships, for example Crumly (1984) and Gerlach (2001). These two genera are usually associated with the smaller more derived members of the family, namely *Psammobates*, *Chersina*, *Kinixys* and *Homopus* although these relationships are not united by any cranial autapomorphies (Gerlach 2001). These relationships have been questioned due to the high levels of parallelism associated with these derived groups (Meylan and Sterrer 2000).

The genus *Testudo* was not resolved as a monophyletic clade due to the inclusion of the African taxon *M. tornieri* within the assemblage. This relationship was corroborated using constraint analysis and does not support the placement of this monotypic genus with the remaining African taxa. The relationship was initially thought to be a result of long-branch attraction but there is little available evidence to support this reasoning, particularly as the relationship was well supported using all methods of inference.

Testudo horsfieldii, *T. hermanni* and *M. tornieri* were divergent to the remaining *Testudo* species and were found to group with the monophyletic *Indotestudo*. This was corroborated by the high sequence divergence estimates between the three species and the remaining *Testudo*, with all pairwise comparisons greater than 14%. The fact that *Malacochersus tornieri* grouped with the genus *Testudo* is not unexpected. This monotypic genus has been shown to share several morphological characters with *Testudo* spp., including the presence of supramarginal scales (Loveridge and Williams 1957) and three marginals contacting the second pleural (Bour 1982). It has been suggested that the species *Testudo horsfieldii* be elevated to the sub-genus *Agrionemys* (Khozatsky and Mlynarski 1966, van der Kuyl et *al.* 2002). Separation of *Agrionemys* is supported by additional morphological features, notably that *T. horsfieldii* is unique in having four foreclaws whilst the remaining representatives of the *Testudo* have five (Ernst and Barbour 1989). The pairwise genetic distances between *T. horsfieldii*, *T. hermanni* and *M. tornieri* are particularly high at greater than 15%. When compared to other available intra and inter-generic distances within the Testudinidae, these estimates suggest that each species could be regarded separately at the generic level. The little evidence available does not indicate accelerated rates of molecular evolution that might otherwise account for these high levels of variation. However, given the reported sister

relationship between *T. horsfieldii* and *M. tornieri*, combined with reports that *T. horsfieldii* has hybridised with *T. hermanni* (Highfield 1990, Gmira 1993), the re-evaluation of relationships among these three species is advocated. Additional molecular and morphological data should be assessed, combined with behavioral observations before assumptions regarding their taxonomic status at the generic level can be made with any degree of certainty. It does seem plausible that they could represent transition species between the southern *Testudo* (consisting of *T. graeca, T. kleinmanni* and *T. marginata*) and the Asian *Indotestudo* (see PART III: Biogeography).

There is evidence of a relationship between *Indotestudo* and *T. horsfieldii*. Although not a novel result, it has been previously ascribed to long-branch attraction due to inadequate taxon sampling (see van der Kuyl et *al.* 2002). The geographical distributions between *I. elongata* and *T. horsfieldii* are similar, which could predict close phyletic affinities in support of this relationship (Blyth 1853). Furthermore, the affinity between the Eurasian *T. hermanni* and *T. horsfieldii* with the Asian/ Indian *Indotestudo* suggests a "northern" association. The inclusion of *M. tornieri* within this assemblage remains a biogeographic enigma (see PART III: Biogeography).

Within the *Testudo*, there was evidence for a strong association between *T. kleinmanni, T. marginata* and *T. graeca*. *Testudo graeca*, in particular was found to display high levels of intra-specific variation with up to 4% sequence divergence (also reported by Highfield 1990, Gmira 1993, van der Kuyl et *al.* 2002) supporting sup-specific species status reported for this group (Highfield and Martin 1989).

It was interesting to note that *T. marginata*, the largest tortoise in the genus *Testudo*, and *T. kleinmanni*, corresponding to the smallest currently recognised species, clustered together in all analyses. This association was also observed by Gmira (1993), who suggested a new genus *Chersus* to describe the relationship. However, the close sister relationship with *T. graeca* and the low levels of pairwise sequence divergences estimated between *T. marginata* (Mediterranean), *T. kleinmanni* (North Africa) and *T. graeca* spp. (North Africa and Mediterranean) suggests that they should be maintained as members of the genus *Testudo* (less than 8% sequence divergence). Furthermore, this designation would be consistent with the grouping of these individuals in a biogeographic context as a "southern" species group.

The genus *"Geochelone"* is not monophyletic. The phylogenetic estimate obtained in this study supports Gerlach (2001) in elevating several previously designated sub-genera to full generic status. These new designations are supported by high intra-specific sequence divergence estimates within the *"Geochelone"*. Relationships among *"Geochelone"* are typified by high levels of variability with as much as 28% sequence divergence as exemplified by *Geochelone platynota* and *Geochelone pardalis*. It appears that original assignment of individuals to the genus *Geochelone* (sensu Auffenberg 1974) was based for the most part on i) biogeographic affinities and ii) additionally impeded by high levels of parallelism, in particular related to body size (Crumly 1982).

The elevation of the sub-genera *Chelonoidis* (*G. nigra, G. chilensis, G. denticulata* and *G, carbonaria*), *Stigmochelys* (*G. pardalis*), *Asterochelys* (*G. radiata* and *G. yniphora*) and *Dipsochelys* (*G. gigantea*) to generic rank is advocated. Although *Geochelone gigantea, G. radiata* and *G. yniphora* have pairwise sequence divergence values less than 10%, these species are morphologically distinct, with specific adaptations to their ecological niches and respective habitats (Crumly 1982). These three species are representatives of a speciation process unique to the Indian Ocean Islands. This finding endorses their recognition and conservation as separate entities, as indicated by their placement in the genera *Dipsochelys* and *Asterochelys* respectively. The sub-genus *Centrochelys* (represented by the African endemic *Geochelone sulcata*), and the *sub-genus Geochelone* (comprising *G. platynota and G. elegans*) should be retained in the genus *Geochelone*. These three species form a well-supported trichotomy based on mitochondrial genes, independent morphological data (Crumly 1982, 1984) and a combination of the data partitions. Furthermore, low levels of pairwise sequence divergence estimates between the taxa supported this relationship (less than 7%). This trichotomy was shown to be sister to the South American genus *Chelonoidis*, a relationship that has been supported by previous studies based on carpal elements and cranial osteology (Auffenberg 1966, Crumly 1982, Crumly 1984). Within the *Chelonoidis*, there is strong supporting evidence to suggest that *Chelonoidis (Geochelone) chilensis* is the closest living relative of the giant tortoises from the Galapagos Islands, *Chelonoidis (Geochelone) nigra* (Caccone et *al*. 1999a).

Stigmochelys (*Geochelone pardalis*) is not associated with the other African members of the *"Geochelone"* (*Centrochelys*) or the closely affiliated South American *"Geochelone" (Chelonoidis)*. Although it does share several features with *Geochelone elegans* of India, including the lack of a nuchal shield, small anals and a large supratympanic scale (Hewitt

1937), it is resolved as most closely related to the southern African species of the Testudinidae. This southern African association was rigorously tested using constraint analysis and was strongly supported in all molecular in this study. This novel relationship suggests that previous taxonomies based on morphological data for the Testudinidae may have been affected by incorrect polarities assumed for selected characters or alternatively by the complex retention of combinations of primitive features. This is particularly true in the designation of the genus *"Geochelone"*, which was based for the most part on large size (Auffenberg 1974, Crumly 1982, 1984).

Kinixys was resolved as a well-defined and well-supported monophyletic genus using all methods of inference, with all pairwise sequence divergence estimates within the clade less than 8%. The genus is defined on morphological grounds by a hinge in the carapace between the 4th and 5th costals and 7th and 8th peripherals (Gerlach 2001). There has been some debate as to the validity of the genus *Kinixys* within the Tribe Testudini (Crumly 1984). This is mainly because other members of this Tribe, which included all genera excluding *Manouria, Gopherus* and *"Geochelone"*, were united by the presence of a short trachea. *Kinixys* spp., however, are defined by a longer plesiomorphic trachea, which is shared with the Gopherinae (Crumly 1984). There was conflict with the placement of *Kinixys* in the Gopherinae, however, as it was also shown to share several characters with the genus *Indotestudo* (Crumly 1984). The placement of the African endemic *Kinixys* within the Geocheloninae was subsequently supported using molecular data and a combined analysis approach with the available morphological characters. The longer trachea probably developed secondarily in this genus and could be associated with the modified hinge on the carapace. Sequence divergence estimates between the *Kinixys* species, in combination with topological evidence suggest that *K. homeana* and *K. erosa* are ancestral members of the genus. They are northern African representatives and are associated with more mesic conditions (Ernst and Barbour 1989). There is a close relationship between the widespread *K. belliana* and the southern representatives *K. natalensis* and *K. b. spekii* (Boycott and Bourquin 1988, Broadley 1989, 1992, 1993). These derived species are more arid adapted in their respective ranges. As mentioned in Chapter 2, the status of several sub-species allocations within *K. belliana* should be re-evaluated. Sequence divergence estimates of approximately 8% were estimated between *K. b. spekli* and *K. belliana* individuals sampled in this study. Consequently, it is suggested that *K. b. spekii* be given valid species status pending further investigation.

The monophyly of the Indian Ocean Island endemics is strongly supported by this study and agrees closely with estimates obtained from Caccone et *al.* (1999b) and Palkovacs et *al.* (2002). *Asterochelys (Geochelone) radiata* and *Asterochelys (Geochelone) yniphora* are sister taxa and closely related to *Dipsochelys (Geochelone gigantea)* whereas previous estimates have shown a closer association with the two *Pyxis* species and *Dipsochelys* (Palkovacs et *al.* 2002).

Asterochelys radiata and *A. yniphora* have been shown in previous morphological studies to be very closely related (Auffenberg 1966, 1974) or not closely related at all (Crumly 1982). However, low levels of observed sequence divergence support a close affiliation between these two large Malagasy endemics and the suggested sister relationship with the giant *Dipsochelys,* which points to a common ancestor and a common evolutionary history. The inclusion of the *Pyxis* species within this Ocean Island clade is readily acceptable from a biogeographic perspective and the current phylogenetic estimate indicates a single colonisation event, although it is interesting to note the size differences between *Asterochelys, Dipsochelys* and the smaller *Pyxis* species (Ernst and Barbour 1989). Furthermore, morphological studies have indicated a strong association of these smaller *Pyxis* species with diminutive African genera including *Homopus, Chersina, Psammobates* and the European *Testudo* (Crumly 1984). This association is due to shared features that are based on smaller body size, such as reduced ossification near the fenestra prostotica and the presence of reduced trochlear processes (Crumly 1984). Considering the independent evolution of large body size in the African endemic, *Stigmochelys (Geochelone) pardalis,* in spite of overall smaller absolute adult size in remaining species in the southern African clade, it is not inconceivable that a reduction in body size could have occurred in the Island endemics (Auffenberg 1974, Caccone et *al.* 1999b, Austin and Arnold 2001) (see PART III: Biogeography). This would allow for support for the close association of *Pyxis* with the remaining Malagasy endemics.

The two *Pyxis* species, *P. arachnoides* and *P. planicauda*, have been placed in two monotypic genera (*Pyxis* and *Acinixys* respectively) based on differences in the plastral formulae and the morphology of the cervical vertebrae (Ernst and Barbour 1989). Alternatively, they have been assigned to the same genus (*Pyxis*) based on similarities in carapace color, morphology and skull osteology (Bour 1981, Ernst and Barbour 1989). Recently, the validity of *Pyxis* as a distinct genus rather than a sub-genus within *"Geochelone"* has been brought into question (Caccone et *al.* 1999b). However, taking into

account i) the sister relationship between *P. planicauda* and *P. acinixys* (less than 9% sequence divergence), ii) the high levels of divergence displayed between these two species and the Malagasy endemics *Asterochelys* and *Dipsochelys* (14 – 18% sequence divergence) and iii) the high pairwise divergence values displayed between the two *Pyxis* species and previously recognised sub-genera within the *"Geochelone"* (14 – 26%), the monophyly of this unique genus appears to supported.

i. Taxonomic Revision of southern African Testudinidae with descriptions of current Distribution Patterns (refer to distribution plates 6.1 – 6.14 on page 174-175)

Monophyly of African genera could not be supported as *Malacochersus tornieri*, *Geochelone sulcata* and the genus *Kinixys* could not be constrained to be included in a monophyletic African clade. This paraphyly could point to multiple invasions in the colonisation of Africa by the Testudinidae (see PART IV: Evolution of African Testudinidae). Furthermore, the monophyly of the southern African genera was not supported as *K. natalensis* and *K. b. spekii* were firmly embedded within a monophyletic *Kinixys*, which was closely affiliated with the South American endemics, Asian/ Indian endemics and the remaining African genera.

The Cape Province, South Africa is the richest in the world in terms of diversity of terrestrial tortoises that are either endemic to it or have this region falling within their natural distribution range (see PART IV: Evolution of African Testudinidae) (Boycott and Bourquin 1988). The five Cape genera seem to constitute a natural group based on head scaling patterns (Hewitt 1937) and include *Stigmochelys* (previously *Geochelone*) *pardalis*, *Homopus*, *Psammobates*, *Chersina* and the newly designated *Chersobius* Hewitt, 1937. Shared characteristics include a prominent or strongly projecting snout, with tubular nostrils, which are particularly pronounced in juveniles (Hewitt 1937). With tortoises specific to North Africa or Europe, however, the snout is not as prominent and is limited to perforations within a flattish membrane, which can be distinctly divided as in *Kinixys* (Hewitt 1937). The monophyly of the Cape genera is further supported by the molecular phylogenetic estimates reported in this study.

The monotypic genus *Chersina* displayed high levels of genetic diversity with sequence divergence estimates of 2.4% between the four individuals that were sampled for analysis. It is a distinctive genus in that it possesses a large undivided gular shield that is not present in any other members of the Family Testudinidae (Hewitt 1937). *Chersina angulata* is restricted to the coastal areas and central districts of the Cape Province (Boycott and Bourquin 1988). In the present study, it is closely associated with the genus *Homopus* and this relationship is supported by morphological characters as the two genera share similar nostril morphology (Hewitt 1937). *Chersina angulata* is a sister taxon to a monophyletic group consisting of *Homopus bergeri*, *H. boulengeri* and *H. signatus* spp. *Homopus areolatus* and *H. femoralis*

were not included in this assemblage, and instead were resolved in a clade which included the remaining Cape genera i.e. *Psammobates* and *Stigmochelys*.

Homopus species have a broad distribution within southern Africa, particularly in the Cape Province and the southern limits of Namibia (Boycott and Bourquin 1988). *Homopus femoralis, H. areolatus* and *H. boulengeri* are endemic to South Africa, whereas *H. signatus* spp. are endemic to the western/ northern Cape and southern Namibia (Boycott and Bourquin 1988). *Homopus signatus* and *H. boulengeri* are allopatric and have similar habitats in areas of rocky outcrops and low rainfall in the northern Cape Province (Greig and Burdett 1976). *Homopus areolatus* and *H. femoralis* occur in areas of high rainfall, with *H. areolatus* confined to coastal and indigenous Fynbos, although it has also been found inland at elevations of more than 900 meters (Greig and Burdett 1976). In some areas it overlaps with *H. femoralis*, which is restricted to higher plateaus of over 1800 metres (Greig and Burdett 1976). Therefore, despite the fact that the geographic distributions for these two species are sympatric, altitude and habitat separate them.

The paraphyletic status of the genus *Homopus* has been aluded to in previous investigations. Hewitt (1937) noticed two discernible groupings within the genus consisting of *H. areolatus* + *H. femoralis*, which he referred to as *Homopus* and a second containing *H. signatus* + *H. boulengeri* (+ *H. bergeri*), which he designated *Pseudomopus* and later renamed *Chersobius* (Hewitt 1937). Although there are appreciable skull differences between *H. areolatus* and *H. boulengeri*, Loveridge and Williams (1957) maintained that the two groups should be retained as assemblages within genus *Homopus*. This is mainly due to the distinctiveness of these five species in that they have four claws on the front and rear foot whilst the other African genera have five on the front and four on the rear. However, given the high levels of sequence divergence differences estimated between the two groups (27% between *H. femoralis* and *H. signatus* spp.), the close association of the monotypic genus *Chersina* with the "*H. signatus* + *H. boulengeri* (+ *H. bergeri*)" assemblage, and the results of the constraint analysis in which the monophyly of *Homopus* spp.was rejected, it is recommended that the genus *Chersobius* be reinstated.

The genus *Chersobius* would contain the currently recognised *Homopus* species, *H. signatus* spp., *H. bergeri* and *H. boulengeri*, excluding *H. areolatus* and *H. femoralis*, and be endemic to Namaqualand (*H. signatus*), southwestern Namibia (*H. bergeri*) and the dry central districts of the Cape (*H. boulengeri*) (Hewitt 1937). Furthermore, *H. bergeri*, in the southern

parts of Namibia should be maintained as a synonym (see Iverson 1997 and references therein) of *H. boulengeri* as these two taxa display limited sequence divergence (0.6% sequence divergence estimate). Furthermore, the validity of the sub-species status within *Homopus signatus* spp. was also investigated. *Homopus signatus signatus* and *Homopus signatus cafer* were separated by 0.9% sequence divergence. The two sub-species do display disjunct ranges with *H. s. signatus* restricted to the northwestern Cape Province and southern Namibia, whilst *H. s. cafer* is restricted to the Cape Province, particularly west of the Cedarberg Mountains (Boycott and Bourquin 1988). There is, however, a zone of intergradation between these two sub-species, which occurs along the escarpment and in the western Great Karoo (Boycott and Bourquin 1988).

The distinctiveness of the two remaining species within *Homopus*, *H. areolatus* and *H. femoralis* should be further investigated before they are retained together under the revised *Homopus*. Several reasons endorse this including the observation that pairwise sequence divergence estimates between the two species are very high (more than 20%). The two species are not supported as a monophyletic clade in all phylogenetic estimates obtained in this study and there are several differences in their morphological features. For example, *H. areolatus* has no scales present on the snout, whereas there are generally two to three present in *H. femoralis*, which also displays a particularly enlarged auditory bulla compared to *H. areolatus* (Hewitt 1937).

As already discussed the resolution of *Stigmochelys pardalis* within the African clade is an interesting result. The particularly close relationship of this species with *Psammobates* is also surprising although Hewitt (1937) suggested that the two genera could be each others closest relatives as the auditory bulla is compressed posteriorly from side to side in both groups. The sister relationship between the two genera was strongly supported by all methods of inference and data sets. *Stigmochelys* is the largest of the southern African tortoises with the widest distribution range and inhabits a variety of diverse habitat types ranging from arid Karoo, Kalahari thornveld, grasslands, savannah and tropical bush (Boycott and Bourquin 1988). It is the only southern African tortoise that lacks a nuchal shield (Boycott and Bourquin 1988).

The genus *Psammobates* is a monophyletic group and contains three distinct species, *P. geometricus, P. oculifer* and *P. tentorius*. *Psammobates geometricus* is endemic to the western Cape Province where it is confined to a specific vegetation type within the Cape

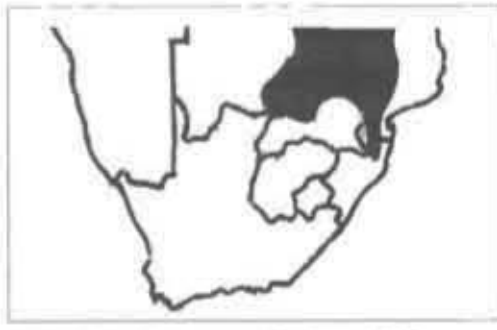
Floral Kingdom, the "Renosterveld" (Baard 1993). *Psammobates oculifer* is sister to *P. geometricus* and is distributed throughout the scrub desert and savannah regions of central southern Africa, never occurring south of the Orange River (Boycott and Bourquin 1988). The two sister species are allopatric and share morphological similarities based on nuchal size and plastral pattern (Loveridge and Williams 1957). The sister species relationship is confirmed by estimates of pairwise sequence divergence, which although relatively high (15%), can be explained by the increased rates of evolution that have been shown to be associated with this genus. All molecular estimates within the present study confirm this close association, particularly maximum parsimony inferences.

The third and most distinct of the three species under *Psammobates* is the highly variable *Psammobates tentorius*, which has been allocated 33 possible races or sub-types (Hewitt 1937, Loveridge and Williams 1957). *Psammobates tentorius* is the smallest of the three species united under *Psammobates* and displays high levels of within-species variation. This morphological variation is corroborated by high levels of pairwise sequence divergence estimates calculated for the *Psammobates tentorius* individuals sampled in this study (up to 5%). Within a phylogenetic context, three sub-species are recognised, *P. t. tentorius, P. t. trimeni* and *P. t. verroxii* (Greig and Burdett 1976). The three sub-species show complex patterns of intergradation over their ranges, particularly in the south western region of the Great Karoo (Boycott and Bourquin 1988) that could in part explain the large numbers of local races that have been recognised.

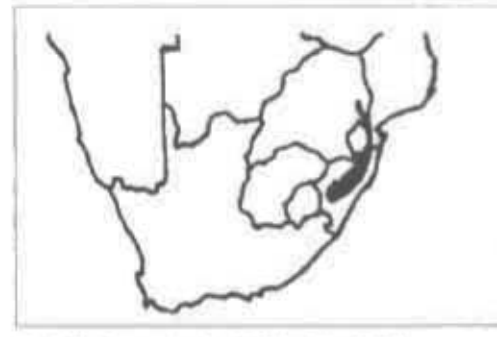
Three highly supported assemblages, each of which could potentially represent one of the currently defined sub-species within the *Psammobates tentorius* spp. complex, were resolved using parsimony analysis for the genus *Psammobates*. However, no specific conclusions could be drawn regarding the taxonomic associations of the sub-species, as the individuals received from the Chaffee Zoo Gardens had not been identified to sub-species level. The two *P. t. veroxii* individuals sampled from Tygerberg Zoo grouped together in a monophyletic assemblage. Another individual sampled from Tygerberg Zoo was identified as belonging to the *P. t. trimenII* sub-species (*P. tentorius*40C). This individual was associated with two of the five *P. tentorius* samples from Chaffee Zoo and the three samples together formed a monophyletic group that was sister to the positively identified *P. t. veroxii* sub-species. The remaining samples from Chaffee Zoo gardens formed a third clade that could tentatively be the *P. t. tentorius* sub-species. Further investigations regarding the taxonomy within the *Psammobates tentorius* complex will require additional sampling from across the

broad distribution zones that define this species and the positive identification of potential individuals at the sub-specific level.

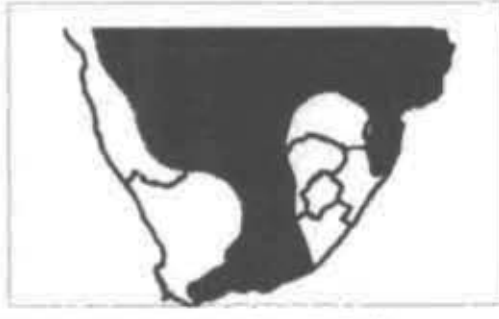
Psammobates tentorius spp. have a highly varied distribution ranging from the dry and arid environs of the West Coast and the Karoo, stretching to the semi-desert of the Little Karoo and the Great Karoo (Greig and Burdett 1976). The distribution of *P. tentorius* spp. separates the allopatric sister species, *P. geometricus* and *P. oculifer* (Boycott and Bourquin 1988, Baard and Mouton 1993). Baard and Mouton (1993) speculate that retreating ancestors of the latter two species may have been separated by the Pleistocene radiation of *P. tentorius* spp. (see PART IV: Evolution of African Testudinidae).



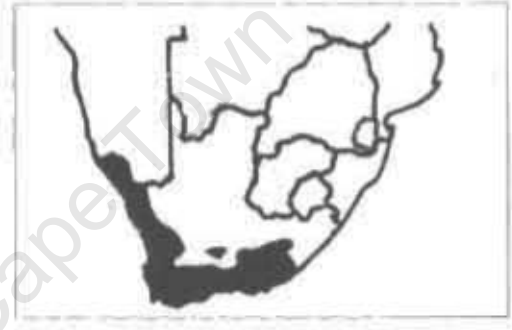
6.1: *Kinixys belliana spekii* (Gray, 1863)



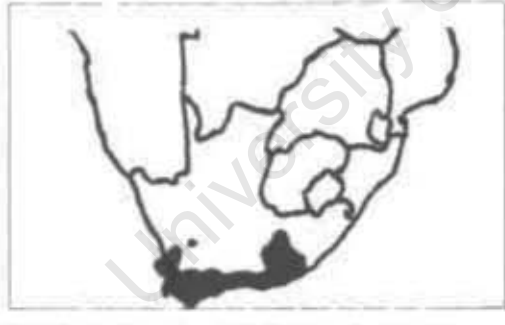
6.2: *Kinixys natalensis* (Hewitt, 1935)



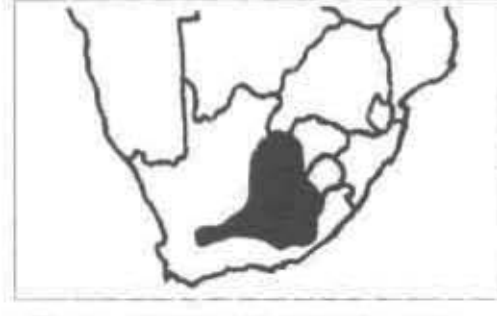
6.3: *Geochelone pardalis* (Bell, 1828)



6.4: *Chersina angulata* (Schweigger, 1812)

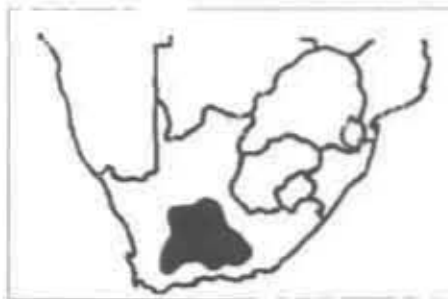


6.5: *Homopus areolatus* (Thunberg, 1787)

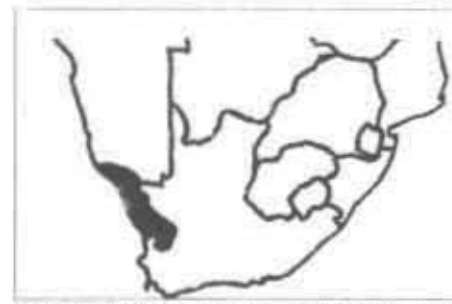


6.6: *Homopus femoralis* (Boulenger, 1888)

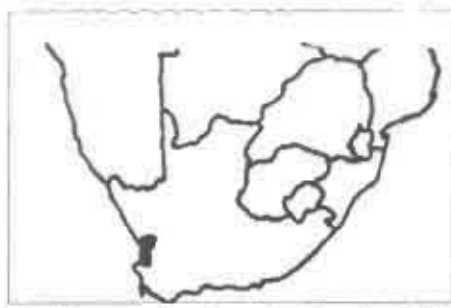
Plates 6.1 – 6.6: Distribution ranges of species from southern African representatives of the Family Testudinidae modified from Boycott and Bourquin 1988).



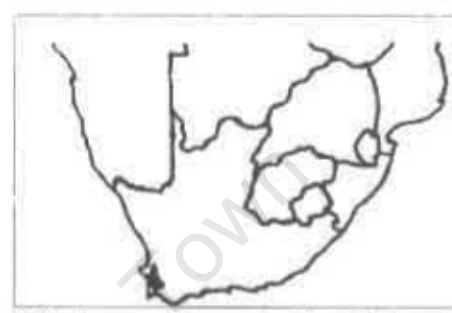
6.7: *Homopus boulengeri* (Duender, 1906)



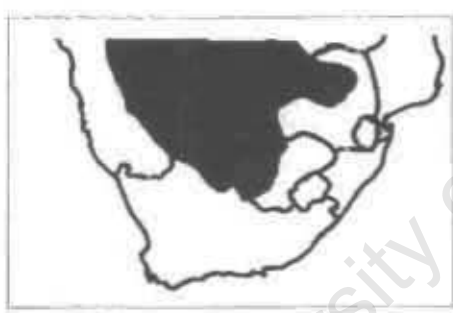
6.8: *Homopus signatus cafer* (Gmelin, 1789)



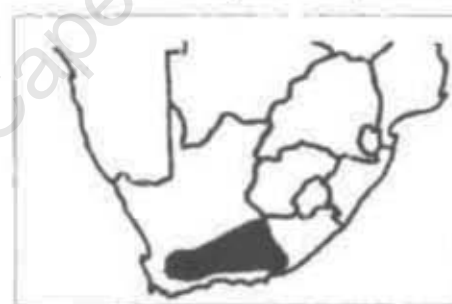
6.9: *Homopus signatus cafer* (Daudin, 1801)



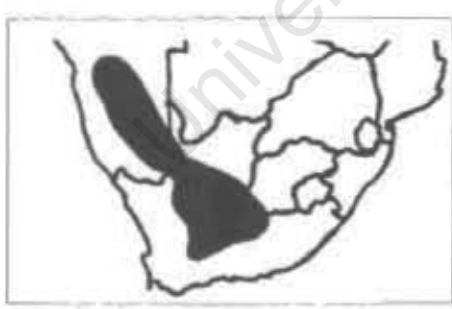
6.10: *Psammobates geometricus* (Linnaeus, 1758)



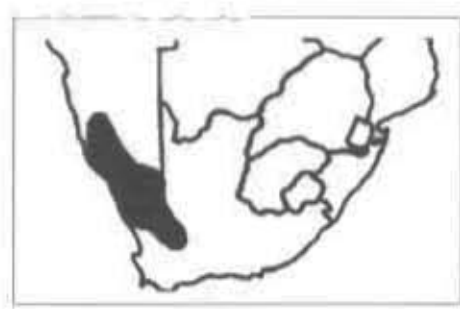
6.11: *Psammobates oculifer* (Kuhl, 1820)



6.12: *Psammobates tentorius tentorius* (Bell, 1828)



6.13: *Psammobates tentorius veroxii* (Smith, 1839)



6.14: *Psammobates tentorius trimeni* (Boulenger, 1836)

Plates 6.7 – 6.14: Distribution ranges of species from southern African representatives of the Family Testudinidae (modified from Boycott and Bourquin 1988).

PART III: BIOGEOGRAPHIC INFLUENCES ON THE FAMILY TESTUDINIDAE

To date, hypotheses on the biogeography of the Family Testudinidae have been limited by poor resolution of relationships among genera within this group. This is particularly true with respect to relationships between the Old and New World genera. This study provides a novel and well-resolved phylogeny of the group based on a combination of molecular and morphological data. It provides insight into historical radiations of the terrestrial Chelonians, which appear to be dominated by dispersal events as opposed to vicariant biogeography. The effects of vicariant biogeographical patterns can adequately explain the evolution of several reptile groups, for example Acrodont lizards (Macey et *al.* 2000), and is particularly parsimonious for the description of current distribution patterns for several plant groups (Darlington 1957, Peilou 1979, Prothero 1994). Distribution patterns within the Testudinidae, however, are more difficult to interpret as they show a post-Gondwanaland evolution and diversification (Crumly 1982, 1984).

The revised phylogenetic estimate for the Family Testudinidae is characterised by short internal branches and longer terminal branches, suggesting a rapid but ancient evolution for the group (Shaffer et *al.* 1997). This is corroborated by unresolved estimates, particularly for the Cyt *b* gene, using parsimony methods. Rapid and simultaneous radiations result in poorly resolved estimates, particularly with parsimony inference, resulting in polytomies or "star-burst" topologies (Shaffer et *al.* 1997). This is because there are an insufficient number of synapomorphies that are able to support deeper level relationships, due to the rapid accumulation of changes (Matthee and Davis 2001). These features also characterise phylogenies for the Bovidae (Matthee and Davis 2001), Saxifragales (Fishbein et *al.* 2001) and resolution of the Cryptodires (Shaffer et *al.* 1997), all of which are defined by rapid radiations.

In the Cryptodires, there is substantial fossil evidence to support a rapid series of cladogenic events approximately 100 MYA that established all the major clades within the group (Shaffer et *al.* 1997). Furthermore, major lineages within this group appeared over a brief period of approximately 30 million years or less and this hypothesis is supported by short internal branches and poor resolution achieved with the Cyt *b* gene (see Chapter 1, Shaffer et *al.* 1997). In Testudinidae, the dating of divergence events, represented by nodes on the revised phylogenetic estimate, suggest an old but rapid diversification within the group. This is supported by evidence from the fossil record, indicating the existence of all major genera in

the family by the mid-Oligocene, approximately 24 million years ago (Auffenberg 1974, de Lapparent de Broin 2000). The molecular clock was calibrated using two rate estimates, one of 0.6% per million years (Caccone et *al.* 1999a, 1999b) and a standard mitochondrial estimate of 2% per million years (MY) (Wilson et *al.* 1985). Although these estimates represent two extreme calibration rates, the results suggest that rates of molecular evolution within the Testudinidae are slower than that assumed for a universal molecular clock (Avise et *al.* 1992). A 2% per MY rate estimate for the Testudinidae places divergence of this family from ancestral Emydidae turtles, approximately 20 MYA. This represents a period extending well into the Neogene (mid-Miocene). However, the fossil record for terrestrial tortoises dates back to the early Paleogene at least, approximately 60 MYA (Auffenberg 1974, Crumly 1984). The former estimate, therefore, proved too conservative to accurately depict the point of divergence for the Testudinidae. Calibrating the molecular clock with an estimate of approximately 0.6% per MY dates this divergence event to approximately 69.5 MYA and, given the diverse and abundant fossil record for the Family Testudinidae during this period, is considered a more accurate estimate.

As discussed in Chapter 5, the Family Testudinidae was found to conform to a molecular clock only after exclusion of the genus *Psammobates*. This genus was characterised by increased rates of evolution, as indicated by relative rate tests, likelihood ratio tests and long branches in phylogenetic estimates, particularly with *Psammobates oculifer*. Palkovacs et *al.* (2002) found a similar result in their study on Indian Ocean Island endemics where *Pyxis* spp. displayed increased rates of molecular evolution using a portion of Cyt *b*, 12sRNA and 16sRNA. In this case, violation of rate homogeneity among sampled taxa was attributed to the hypothesis thatthe DNA mutation rate is proportional to metabolic rate and life history traits (Britten 1986). This was subsequently associated with the small size of *Pyxis* species with respect to the larger Malagasy endemics utilised in the study. However, Zhang and Ryder (1995) have shown that rate differences between taxa are not a simple function of size and metabolic rate. This idea has been further corroborated by this study and emphasises the dangers of assuming taxon and gene specific rate differences across studies. Furthermore, it provides additional support for the importance of complete, or adequate, taxon sampling. Within this study, *Pyxis* species complex did not violate molecular clock assumptions using the combined data set. The diminutive representatives of the southern African clade, including the smallest known Testudinid *Homopus signatus*, were also found to conform to rate homogeneity within lineages. The only exception was with *Psammobates* spp. and using an increased rate of evolution of 2% per MY, the diversification of this genus

was found to correspond with vicariant events and climate changes in the southern African region (see PART IV: Evolution of African Testudinidae).

Terrestrial tortoises are proposed to have originated in the Northern Hemisphere, specifically in North America, from an ancestral Testudinoidae stock (see Chapter 1). This is supported by a high diversity and abundance of fossil tortoise specimens on the continent (Brattstrom 1961, Auffenberg 1974, and Crumly 1984). The majority of these fossil species appear to be closely aligned with members of extant *Manouria* spp. and *Gopherus* spp, for example the fossil genus *Stylemus* (Auffenberg 1974, Crumly 1984). Fossil specimens of giant tortoises have been found as far North as Ellesmore Island, which is the northern most point in the Canadian Arctic. The presence of these fossils, in addition to fossils representing tropical plant species, lizards and alligators, suggests that early Paleogene (approximately 65 MYA) temperatures in the Northern Hemisphere were substantially warmer than current temperatures (Prothero 1994).

This Paleocene-Eocene (65-58 MYA) transition period was unusually warm and the global climate was tropical from the equator to high lattitudes (Prothero 1994). It is thought that major contributing factors to these warming global temperatures could have been a result of greenhouse gases erupting from mid-ocean volcanic ridges, the collision of Asia with India and the commencing of the closure of the Mediterranean Tethys sea (Prothero 1994). Regardless of the cause of this "hot house" effect, it provided an optimal environment for evolution and diversification within ectothermic Testudinidae. This appears to be supported by the divergence date of 69 MYA for terrestrial tortoises from the ancestral Emydidae.

The beginning of a "reverse greenhouse effect" seems to have occurred in the early-middle Eocene, approximately 50 MYA (Peilou 19779, Prothero 1994). This period defines the Eocene-Oligocene transition, 48-28 MYA, and could have initiated migration of the Testudinidae across the Bering land Bridge, connecting North America and Eurasia (Peilou 1979). This period coincides with discovery of mid-Eocene fossil tortoises in Europe, China and Mongolia that have been attributed to the genus *Stylemus* (closely affiliated with extant North American *Gopherus*) and *Manouria* (Auffenberg 1974, Crumly 1984, Ernst and Barbour 1989). Following this, there was the subsequent evolution and diversification of genus *Testudo* took place in Asia and northern Europe, approximately 48-40 MYA, as evidenced by the presence of *Testudo* fossils in these areas (Ernst and Barbour 1989, van der Kuyl et *al*. 2002). This process of diversification is supported within the framework of this

study by dating the split between the Gopherinae and the Geocheloninae at approximately 48 MYA (see Chapter 5). Furthermore, there was an initial radiation of Testudinidae into North Africa from Europe, probably due to the closing of the Tethys seaway (Peilou 1979, Macey et *al*. 2000), that facilitated the first wave of migration between the two continents in the Eocene. There is substantial evidence of fossil tortoises in North Africa that are associated with the genus *Geochelone* (sensu Auffenberg 1974) and *Testudo* (Auffenberg 1974).

It has been suggested that there was a concurrent dispersal of the Testudinidae from North America to South America during this early Eocene period (Crumly 1984). However, South America was still in close contact with both Antartica and Australia, and cooler temperatures were associated with these southern lattitudes due to the formation of mountain glaciers on the Antarctic peninsula (Prothero 1994). Furthermore, the distance between the northern and southern continents was far greater than that of today, with no evidence of a land connection between them (Peilou 1979). Given a lack of appropriate fossil evidence in Central America to support a possible ancestor from North America, and given that the fossil record in South America only commenced in the late Oligocene (approximately 24 MYA), it is doubtful that South American Testudinidae evolved from North American stock. Furthermore, molecular and combined morphological + molecular analysis presented within this thesis, indicates a strong association between South American *Chelonoidis* with endemic African and Asian *"Geochelone"* (Figure 5.14).

The Testudinidae show a substantial radiation in the Southern Hemisphere, commencing in the early Oligocene approximately 34 MYA. This includes radiations into Africa (see PART IV: Evolution of African Testudinidae), Southeast Asia, India, South America, Madagascar and the Galapagos Islands. This radiation coincided with Antarctic glaciation and extensive global cooling, particularly in the Arctic (Prothero 1994). The subsequent Oligocene-Miocene transition (23 MYA) was a result of Antarctic glaciation occurring at the end of the Oligocene and thought to be due to completion of the circum-Antarctic circulation through the Drake Passage between a separated South America and Antarctica. Global climates began to recover and temperatures became warmer during the early Miocene, with a concurrent increase in the diversity of organisms present (Prothero 1994).

Assuming the validity of the novel phylogenetic estimate presented here, the African, Asian, Indian, South American and Ocean Island endemics all appear to be derived from a common

Testudinid ancestor. This ancestral stock probably originated in Europe and subsequently migrated to North Africa during the first wave of migration. There were subsequent dispersal events from North Africa between the mid-Oligocene and start of the Miocene (30 – 24 MYA). These dispersal events resulted in the colonisation of South America (*Chelonoidis*) by Oceanic or waif dispersals and the invasion of Southeast Asia/ India through the Sahara and Arabian Peninsula (sub-genus *Geochelone*) after the collision of Afro-Arabia with Laurasia (18MYB) (Macey et *al.* 2000). In addition, there was a single colonisation event of Madagascar (*Asterochelys, Dipsochelys* and *Pyxis*), also by waif dispersal (Caccone et *al.* 1999a, Palkovacs et *al.* 2002). The Bufonidae show a similar pattern of distribution, with close affinities between the South American, African and Southeast Asian taxa and an ancestral European stock (Graybeal 1993).

Within terrestrial tortoises, there was continued diversification in Africa from the original ancestral stock, particularly into the late Miocene and Pliocene (15-4 MYA) (*Kinixys*, sub-genus *Centrochelys*) (see PART IV: Evolution in African Testudinidae). There were additional migrations from Northern Europe into Southeast Asia and India (extant *Indotestudo, Manouria* spp.), as well as into the Mediterranean Basin and Northern Asia (*Testudo horsfieldi, Malacochersus tornieri, T. graeca* spp. and *T. marginata*). These dispersal events culminated in the extensive speciation of the Family Testudinidae by the end of the Pliocene, approximately 1.3 MYA i.e. all extant species were represented.

Although a post Gondwanaland colonisation of South America from a North African ancestor appears a more complicated theory than a colonisation event from North America, there are several lines of evidence to support the South America-Africa connection. Firstly, the presence of Oligocene fossils in South America suggest that the continent was colonised by Testudinidae long before the great North-South American Interchange, which only commenced in the Pliocene. This followed the formation of a "stepping stone" land bridge between the two continents (Peilou 1979, Prothero 1994). Predating this land bridge there could have been migration across the Caribbean Plate, although there are no suitable fossils to support this (Burns 1998). Furthermore, long distance waif dispersal from Africa to the New World has been shown in several groups of organisms, including gekkonoid lizards (Carranza et *al.* 2000), scincid lizards (Carranza et *al.* 2001) and Caviomorph rodents (Lavocat 1993). This post Gondwanian Southern Continents Origins model is also associated with interpretation of the origins of New World primates, for which there is a strong fossil record in the Fayum deposits in Egypt (Aiello 1993). Despite the fact that the probability of

waif dispersal would decrease with increased time from the Cretaceous separation of Africa and South America, owing to increasing distance between them, the distance across the South Atlantic was not necessarily entirely open water. There were in fact shallow areas of water connecting West Africa to the northern Coast of South America, in the form of Ceara Rise and Sierra Leone Rise (Lavocat 1993). During periods of lowering sea levels, the most significant of which occurred in the Middle Oligocene (~30 MYA), parts of the mid-Atlantic rise were in fact areas of dry land. The prevailing oceanic currents would have aided dispersal from one "island" to another. These currents favored an East to West crossing from Africa to South America (Tarling 1980). Therefore, the possibility of waif dispersal in Testudinidae from North Africa to South America, as suggested by the "African" association obtained in the phylogenetic estimate, would have been very probable in the mid-Oligocene despite the greater distance that had to be traversed.

The Galapagos Islands have never been connected to mainland South America (in Caccone et *al.* 1999a). Terrestrial organisms, including terrestrial tortoises and Iguanas, colonised this Archipelago by waif dispersal and rafting (Sites et *al.* 1996), providing further evidence to support efficient trans-marine migration of reptiles.

The sister relationship between *Chelonoidis chilensis* and *Chelonoidis nigra* suggest that, despite its smaller size, the Chaco tortoise is the closest living relative to the Giant tortoises of the Galapagos Islands (also in Caccone et *al.* 1999a). Using molecular clock estimates, divergence between these two species is estimated at approximately 20.6 MYA. Caccone et *al.* (1999a) estimate the age of the current Galapagos Islands as significantly younger, approximately 5 MY old, and suggest a recent colonisation event from mainland South America, despite an older divergence indicated between the two sister species (~12 MYA). However, recent evidence suggests that a Galapagos "Hotspot" has been in existence for more than 80-90 MYA (Christie et *al.* 1992). There may have been an early split between the South American endemics, as suggested by the molecular data presented here and in Caccone et *al.* (1999a). The presence of giant tortoises on the current Galapagos Archipelago could therefore be explained by a colonisation event from an older now submerged Island. Conflicting divergence dates for the split between mainland and Island Iguanid genera and the subsequent colonisation patterns of this Family on the present Galapagos Islands, suggest a similar hypothesis (Carson 1992, Christie et *al.* 1992).

PART IV: EVOLUTION OF AFRICAN TESTUDINIDAE

Centres of Endemism

A central focus of biogeography and ecology is the development of hypotheses or theories to describe patterns and cause of species richness (Daniels et *al.* 1992, Kay et *al.* 1997, Chown and Gaston 2000, Jetz and Rahbek 2001, Richardson et *al.* 2001a). Of particular importance is the understanding of the evolutionary history of "Hotspots" or centres of endemism, where the generally accepted definition of an endemic area is "an area that has at least two endemic species" (Linder 2001). Africa, in particular, is represented by a number of localised species that have evolved and diversified within these "centres of endemism". These areas can be considered land-locked Islands and are distinct in the flora and fauna that characterise them (Kingdon 1989). These centres recognition as natural conservation areas, and the importance in the understanding of processes of evolution that govern them, are becoming increasingly recognised (Branch et *al.* 1995, Reyers et *al.* 2001). These centres are associated with areas of stability around which great climactic fluctuations exist, which in combination with disjunct distributions in terrain, provide localised pockets for the evolution of local species or sub-species (Prothero 1979, Kingdon 1989). Within the cosmopolitan distribution of Family Testudinidae, southern Africa and in particular the western Cape Province, contain more terrestrial Chelonians than is found anywhere else in the world (Crumly 1984, Boycott and Bourquin 1988). The majority of these species are endemic to the region (Boycott and Bourquin 1988). Furthermore, Islands closely associated with the African continent, for example Madagascar, also show high levels of endemism with regard to terrestrial tortoise diversity.

Africa

Africa has been described as one of the most "homogenous of continents" (Figure 6.15) (Kingdon 1989) and its flora and fauna can be partitioned into four major zones, including desert, savannah, forest and Highlands. The continent is governed by large climactic changes directly associated with a combination of prevailing winds and surrounding ocean currents. Repeated cycles of aridification of mesic habitat followed by their recovery and renewal, has resulted in repeated periods of isolation. These periods appear to have driven the evolutionary adaptation and diversification of the local flora and fauna that characterise them.

North Africa in particular is defined by isolated mountain ranges, plains, massifs and deserts and is a natural centre for selection, adaptive evolution and the divergence of species (Highfield 1990). This is particularly true for terrestrial tortoises, which are limited by their poor potential for efficient mobility, resulting in the isolation of localised populations and subsequent speciation and adaptation to localised environments. These high levels of diversity are also evident in North African and Saharan mammals that demonstrate a large amount of morphological divergence throughout their ranges (Kingdon 1989, Matthee and Davis 2001).

In the Testudinidae, fossil records suggest an initial migration of ancestral stock from the Eastern part of the Mediterranean basin in the early Oligocene (~36 MYA), as evidenced by the discovery of suitable fossils specimens from the Fayum deposits of Egypt (Highfield 1990, de Lapparent de Broin 2000). Furthermore, there was migration by the Asian members, more closely affiliated with *Manouria*-like representatives of the fossil genus *Hadrianus*, which would explain the paraphyletic origins of the African genera. Larger forms such as *Geochelone* (sub-genus *Centrochelys*) *sulcata* and *Kinixys* species are possibly associated with these ancestors, for example the large fossil form representing *Impregnochlys*. The southern African genera, including *Stigmochelys* (previously assigned to *"Geochelone"*) are more likely associated with a *Testudo*-like ancestor (de Lapparent de Broin 2000).

Habitat diversity coupled with the potential for limited mobility of terrestrial tortoises resulted in the long-term isolation of these populations, and in combination with more recent Pleistocene climactic changes, has resulted in a large diversity of tortoise species, the full extent of which remains unknown in North Africa (Highfield 1990). This is due to limited research in the Southern Hemisphere in general, and in particular in the Family Testudinidae. All current tortoise populations in Africa, north of the Sahara, are ascribed to the genus *Testudo*, particularly to the species *T. kleinmanni* and *T. g. graeca*, although this limited classification is possibly an underestimation of the species and even genera present within the region (Highfield 1990).

Following an initial wave of immigration, tortoises seem to be absent from the fossil record of North Africa until the mid-Miocene about 20 MYA (de Lapparent de Broin 2000). Fossil remains indicate that diversification of the extant genus *Kinixys* from Uganda and Kenya had occurred by 19 MYA. This is corroborated by estimation of divergence dates from the

phylogenetic estimate, placing the split of the extant *Kinixys* species from a common ancestor, also associated with *Chelonoidis* and *Geochelone,* at approximately 24 MYA. The appearance of *Geochelone* (sub-genus *Centrochelys*) *sulcata* and *Stigmochelys*, a possible ancestor for extant *S. pardalis*, was also evident in the fossil record of North Africa and Arabia during the early to middle Miocene (de Lapparent de Broin 2000). The subsequent evolution and diversification of Testudinidae in Africa appears to be a gradual progression associated with increasing aridity along the East coast of the continent, and culminating in a "burst" of speciation in the southern part of the continent, in the early Pliocene in particular (Figure 5.14, Table 5.13). Furthermore, high levels of variability are evident within the East Coast representatives of genus *Kinixys* (Iverson 1997), which has resulted in the recent allocation of several sub-species within the group. This could be a result of the aridification of East Africa due to the closing of the Indonesian seaway, commencing 5-2.5 MYA (Cane and Molnar 2001) and resulted in the rapid diversification and adaptation of this genus to a changing environment.

Fossil data estimates divergence of the major lineages of southern African genera at between 20-12 MYA, for example *Homopus*, *Chersina* and *Stigmochelys* (de Lapparent de Broin 2000), and extant species from 5 MYA to the present (Table 5.8). These estimates agree closely with i) major climate changes associated with the mid-Miocene and ii) aridification of the Cape Province in South Africa and the initiation of multiple Pleistocene glaciation events, a major contributing factor to refugia based hypotheses of diversification (Darlington 1957, Peilou 1974).

i. Diversity in Southern African Representatives of Testudinidae

The southern Africa subregion can be defined as the mainland region of the African continent south of the Cunene/ Zambezi rivers and its coastal waters, including Prince Edward Islands. It includes Namibia and the Caprivi Strip, Botswana, Zimbabwe, that part of Mozambique lying south of the Zambezi River, South Africa and the independent states within this countries borders (Skinner and Smithers 1990).

Southern Africa has the highest diversity of Chelonian fauna in the world with 13 species of terrestrial tortoises occurring within its range, ten of which are endemic to this subcontinent (Boycott and Bourquin 1988, Branch et *al.* 1995). These species represent 31% of the known living species, encompassing five of the 11 recognised genera (Branch et *al.* 1995), or given the reinstatement of the genus *Chersus*, six genera are represented. South Africa supports

11 species of which only two do not occur in the Cape Province. Three species are endemic to the western Cape Province (Boycott and Bourquin 1988), one is endemic to the Cape Province and adjacent Orange Free State and a further two are endemic to the Cape Province and southern Namibia (Plates 6.1 – 6.14) (Boycott and Bourquin 1988, Branch et *al.* 1995 and Iverson 1997).

One hypothesis to explain high levels of diversity and endemism of terrestrial tortoises in the Cape Province is the aridification of this region, which began approximately 14-11 MYA (Richardson et *al.* 2001b). This aridification has been ascribed to separation of South America from the Antarctic, which allowed for the development of a cold circum-Antarctic current, or Benguela current (Prothero 1994, Richardson et *al.* 2001b). The interaction of this icy Atlantic current with warm waters of an equatorial Mozambique current, flowing from the East coast, occurred at the southern most tip of the continent and may have been instrumental in the subsequent aridification of the west Coast of southern Africa (Kingdon 1989, Richardson et *al.* 2001b and references therein). This period is consistent with and potentially responsible for the development and diversification of the Cape Floral Kingdom approximately 8-7 MYA, with most of these unique floral areas diversifying even more recently (Figure 6.15) (Richardson et *al.* 2001b). In line with this and given the rapid diversification of the Cape genera of terrestrial tortoises during the Pliocene (~ 5 MYA)(Table 5.8), it stands to reason that the aridification of the southern African subcontinent, and the subsequent diversification of habitats in the Cape Province in particular, could have been responsible for the rapid diversification of the Testudinidae. Further corroboration for this hypothesis can be found in Crumly (1984), where the development of smaller, more derived forms of the Testudinidae i.e. the southern African genera, was concurrent with the evolutionary adaptation of the feeding apparatus to more xeric plant communities, which are typical of the region.

The progression of the terrestrial tortoises from the Northern parts of the continents to a more southern distribution meant that their increasing adaptation to more arid areas would also have allowed the Family to overcome barriers associated with crossing the Kalahari, Namib and Karoo (Plates 6.1 – 6.14, Figure 6.15). This aridity-associated limitation is one of the factors that led to the development of the "Island" style biogeography theory for the western Cape in particular (Kingdon 1989). It could be a reason for the high levels of endemism and subsequent diversification of many genera that are unique to this region, as it

resulted in the evolution of species that were able to overcome these aridity barriers and their subsequent expansion into available niches.

The effects of increasing aridity associated with the fauna of the southern African subcontinent are also evident in the size of many of the species that are endemic to its range. For example, South Africa has an endemic species of the Berg Adder, which is substantially smaller than its sister species that is widespread throughout Africa (Kingdon 1989). This is mirrored by species of Chameleon endemic to the Cape region, which are smaller than their tropical counterparts (Kingdon 1989). Within the Family Testudinidae, the arid adapted endemics associated with the Cape and southern parts of Namibia, for example *Homopus, Chersobius* and *Psammobates,* are smaller than the widespread generalist, *Stigmochelys pardalis*, and are the most diminutive representatives of the Family as a whole. High levels of variability define *Homopus* and *Psammobates tentorius* spp. in particular, and could be a result of rapid rates of diversification that could be associated with their arid and semi-arid distributions. The increased rates of evolution evident within *Psammobates oculifer*, could be a result of the effects of niche competition due to encroachment and diversification of *Psammobates tentorius* spp. into a retreating *Psammobates geometricus-P. oculifer* ancestor (Baard and Mouton 1993, also see Matthee and Flemming 2002). Further morphological adaptations to increased levels of aridity within the Family include i) the flattened forms of *Homopus* and *Chersobius*, a morphological characteristic also evident in scorpions and doormice endemic to the region (Hewitt 1937) and ii) inflated bullae, which are characteristic of *H. femoralis, H. areolatus* and many rodent species that occur in the Karroid areas of the Cape Province (Hewitt 1937).

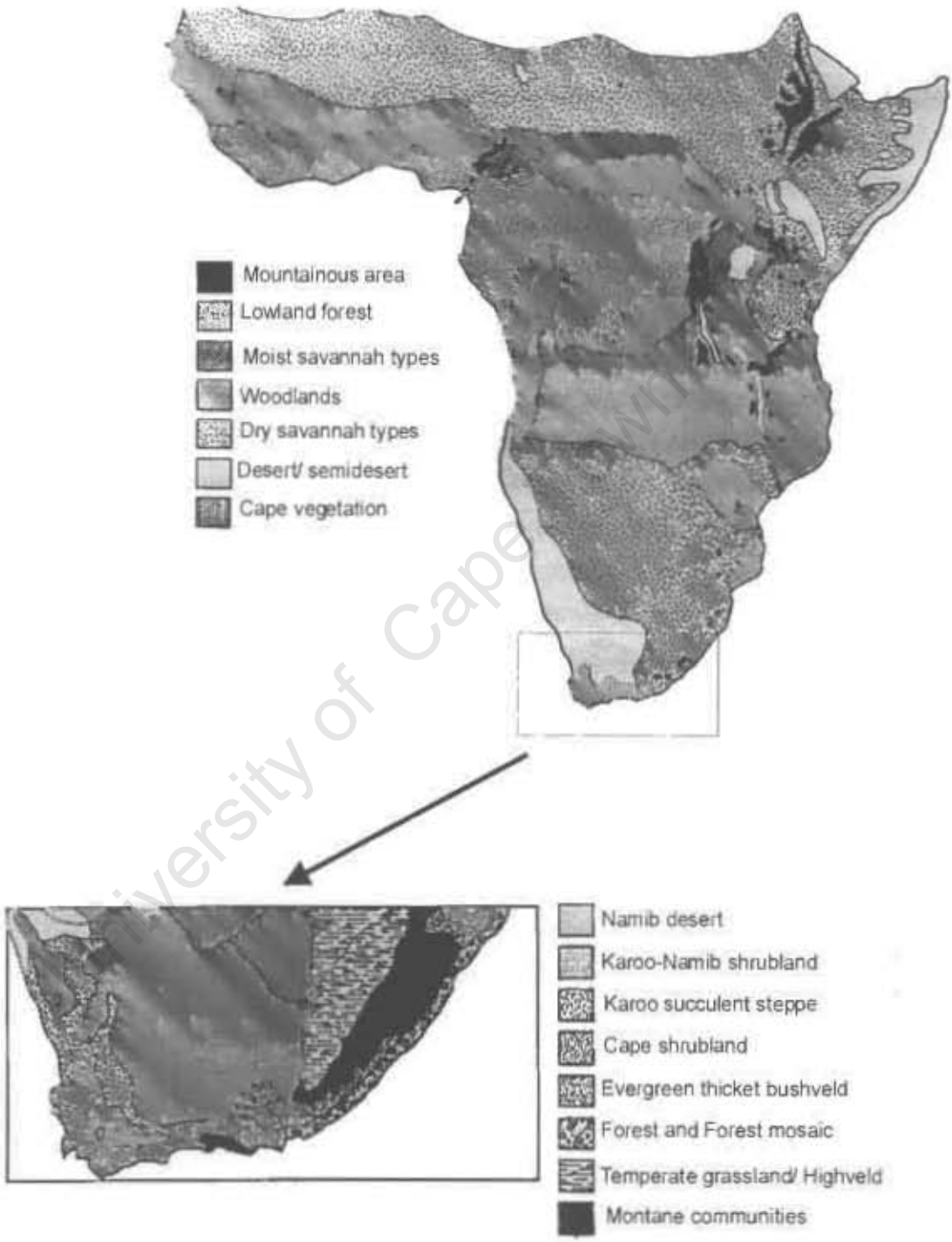


Figure 6.15: A map showing vegetation types associated with the African continent, south of the Sahara. The insert shows habitat types in southern Africa and in particular in the Western Cape region (modified from Kingdon 1989).

ii. Colonisation of Indian Ocean Islands

The colonisation of the Indian Ocean Islands by terrestrial Chelonians has been researched in some detail (Caccone et *al.* 1999b, Palkovacs et *al.* 2002). Magnetic anomaly data suggest that Madagascar and Greater India broke away from the African continent 165 MYA and Madagascar reached its current position by 121 MYA (in Raxworthy et *al.* 2002). Greater India subsequently separated from Madagascar 88 MYA and collided with Laurasia 50 MYA (in Macey et *al.* 2000, Raxworthy et *al.* 2002).

A novel Chameleon radiation from Madagascar to Africa has been shown to have occurred, refuting all previous ideas that the radiation of this Family involved an "out of Africa" radiation (Raxworthy et *al.* 2002). This novel hypothesis, however, can not be used to explain evolution in the Family Testudinidae because there is currently a lack of Testudinidae fossil evidence on this Indian Ocean Island that dates further than the late Pliocene (Caccone et *al.* 1999b). Furthermore, there are a plethora of early Oligocene fossils tortoises in Africa, which have been associated with extant Island Giants (de Lapparent de Broin 2000). Given these separate lines of evidence, tortoises must have reached Madagascar from the coast of East Africa by waif dispersal, and suggests a radiation from Madagascar to Africa is unlikely to have occurred. The current distribution of the Testudinidae is not the result of a vicariant event associated with a Gondwanaland distribution (Palkovacs et *al.* 2002). This is because the separation of Madagascar from Africa predates the evolution of the Family Testudinidae by almost 80 million years. Data from this study, in agreement with Palkovacs et *al.* (2002) and Caccone et *al.* (1999b), suggest a single colonisation event from Africa approximately 24 MYA, at the interface of the Oligocene-Miocene transition, followed by rapid diversification of tortoises on this Island with subsequent dispersal events to the Seychelles. As mentioned, Indian Ocean Island tortoises, particularly the giant representatives, show an affiliation with the African fossil form *Impregnochelys* from the North and East Coast of the African continent (de Lapparent de Broin 2000). This fossil form has also been affiliated with *Kinixys* spp. and *Geochelone* (sub-genus *Centrochelys*) *sulcata* and suggests that waif dispersal from Africa to Madagascar may have occurred from the East Coast of the continent and been aided by movement through the Mozambique channel (Caccone et *al.* 1999a, Palkovacs et *al.* 2002).

Concluding Remarks

The rich Chelonian diversity and high levels of endemism on the African continent, and in particular in southern Africa, is indicative of a process of evolution that is also evident on islands that are associated with the continent, for example Madagascar (Kingdon 1989). Branch et *al.* (1995) have shown that the majority of the southern African testudinid endemics are relatively well protected in existing reserves. All these tortoises have endangered and/ or vulnerable status and the diversification of the Cape genera of tortoises in particular, appear to be associated with the unique flora in the region, or at least the evolutionary processes that have been instrumental in its production.

Given the endangered status of many of the tortoises associated with the southern African region, combined with the growing concern of the impacts of climate change on the unique flora in the Cape Province (Midgley et *al.* 2001, Reyers et *al.* 2001), it is essential that the processes that govern the evolution and diversification of this unique centre are preserved.

CHAPTER 7

MICROSATELLITE DNA AND THE GEOMETRIC TORTOISE, *Psammobates geometricus*

INTRODUCTION

As discussed in Chapter Six, Southern Africa has the highest diversity of terrestrial Chelonians in the world, with a complement of 13 species representing five genera (Boycott and Bourquin 1989). Several of these species are endemic to the south-western Cape province, for example *Homopus areolatus* and *Psammobates geometricus*, and are all threatened by the effects of habitat loss through urban and agricultural development.

The Geometric tortoise, *Psammobates geometricus*, is one of three species comprising the southern African genus *Psammobates* (Boycott and Bourquin 1988, Iverson 1992) (see Chapter 6). It is considered one of the rarest land tortoises in Africa, and is listed in the IUCN Red List of Threatened Animals (Groombridge 1993). The protection and management of this species is a conservation priority in South Africa and is protected by the Nature and Environmental Conservation Ordinance, No. 19 of 1974 (Baard 1993a). *Psammobates geometricus* is limited to the extreme south-western part of the Western Cape Province of South Africa, where it is confined to an area of 4000 - 5000 hectares of an endangered and specific habitat type known as the renosterveld (Baard 1993b). The renosterveld forms part of the Fynbos Biome, a vegetation zone characterised by a Mediterranean style climate (Cowling and Richardson 1995). Suggestions for this renosterveld-habitat dependence by *P. geometricus* include specific dietary preferences and adaptations to topographical factors, since the species favors low-lying, well drained areas (Baard 1995a). The utilisation of the renosterveld, once ubiquitous throughout this region of the Cape Province, for agriculture has been the major factor responsible for the massive decline in tortoise numbers over the latter half of the last century (Baard, 1993b). This problem is compounded by the spread of alien vegetation, crop spraying and veld fires (Baard 1992). It is estimated that more than 96% of the preferred habitat of the Geometric tortoise, west of the Cape Fold Mountains, has been destroyed or irreversibly altered (Baard 1995a, McDowell and Moll 1992).

The species was originally thought to have become extinct by the late 1950's, with the last documented sighting in 1939. However, small populations were rediscovered in isolated areas of the renosterveld in the mid 1960's (Rau 1971). Six reserves have since been established in the south-western Cape Province, with the Elandsberg Private Nature Reserve containing the largest surviving natural population. The remaining populations occur in three areas that are separated from one another by localised peaks of the Cape Fold Mountains, approximately 750 – 1400 meters above sea level. These areas include the south-western coastal lowlands, where the Elandsberg Private Nature Reserve is situated, the Worcester/Tulbagh valley and the Ceres valley, all less than 40 Km apart (Figure 7.1).

In addition to the immediate effects of habitat reduction, small isolated populations are vulnerable to increased rates of allelic loss and a decrease in average heterozygosity over time. This loss of genetic variability is compounded by the effects of genetic drift, and acts as a contributing factor to local extinction (Primack 1993, Saccheri et *al.* 1998, Frankham and Ralls 1998, Westemeier et *al.* 1998). Population fragmentation of the Geometric tortoise populations across its range into small, isolated populations has raised the question as to whether there is a need to manage these fragments as separate conservation units (Baard, 1992, Moritz 1994). The Cape Fold Mountains are thought to have acted as a physical barrier to migration and subsequent gene flow, thus restricting movement of these tortoises between the remnant populations. This would result in the development of genetic differentiation among these populations. Alternatively, if there is migration, this could allow for the maintenance or even an increase in the overall genetic diversity in the population (Young et *al.* 1993).

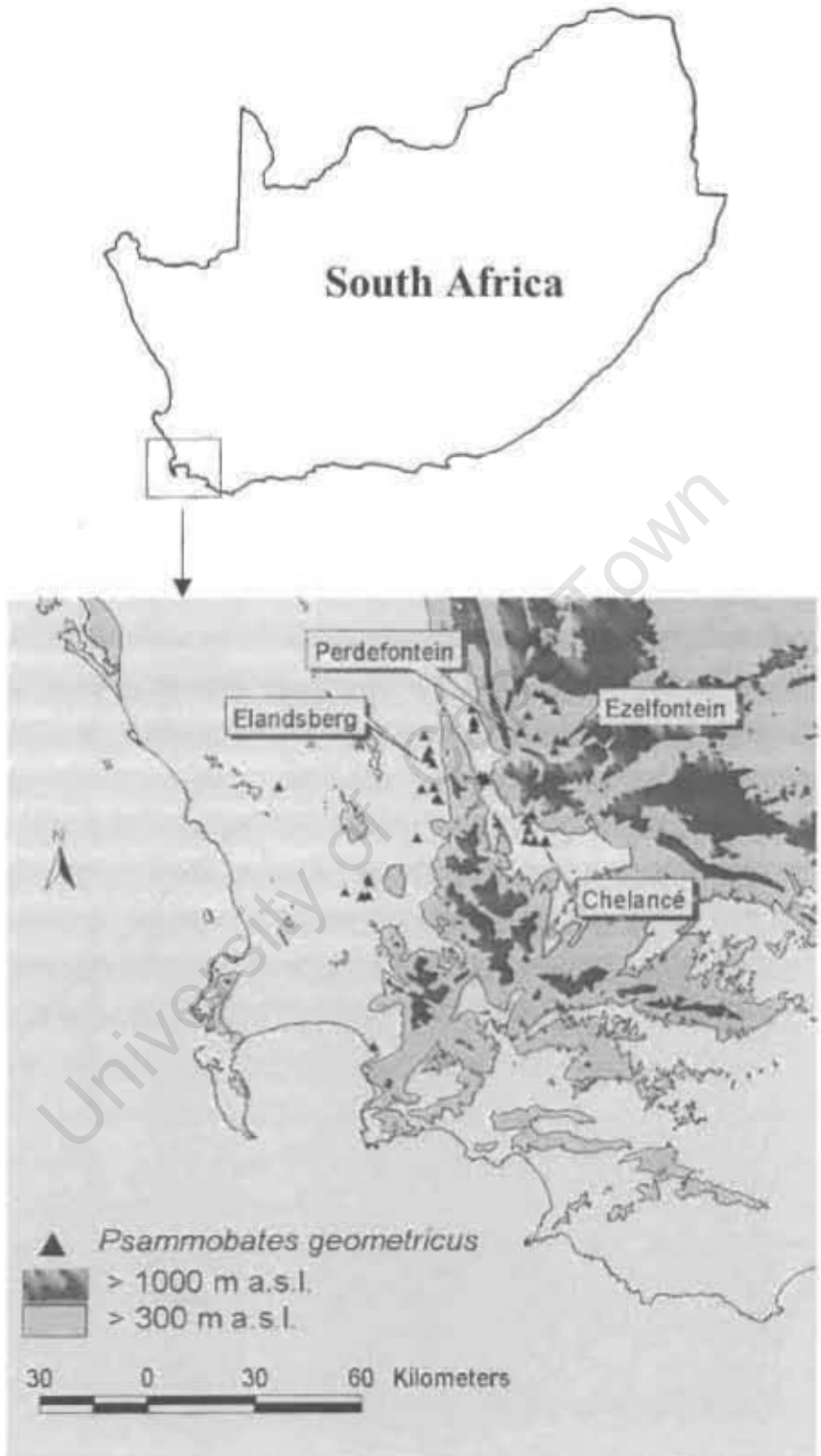


Figure 7.1: Map of the south-western Cape Province showing the remaining *Psammobates geometricus* populations

Conservation Genetics

The value of genetic data is becoming increasingly recognised in the development of conservation management programs (Smith and Wayne 1996). In this regard, microsatellite DNA can be used to quantify population structure (Bowcock et *al.* 1994) and to assess paternity or determine the effects of social structure and mating systems in natural or wild populations (Smith and Wayne 1996, Bishop 2002).

Microsatellite DNA is composed of tandemly repeated motifs (Tautz and Rens 1984, Bruford and Wayne 1993) from two to five nucleotides in length, and are among the fastest evolving DNA sequences recognised. Mutation rates range from 10^{-2} - 10^{-5} per generation (Edwards et *al.* 1992, Weber and Wong 1993). The main mechanism for the generation of new length alleles at microsatellite loci is thought to be due to slippage during replication, particularly at pure repeat units, for example $(CA)_n$ (Weber and Wong 1993). There is, however, evidence to suggest that there may be selective or mechanical constraints limiting the upper and lower lengths achievable within microsatellite arrays (Orti et *al.* 1997, Weirdl et *al.* 1997).

Increased mutation rates at microsatellite loci compared to other parts of the genome result in differences in length variation of alleles and potentially high levels of variability. These allele and genotype frequencies generated by microsatellite markers are amenable to a wide range of robust statistical methodologies and, as such, are widely used in the field of conservation biology. One limitation to the use of microsatellite markers, however, is that species specific primers need to be developed for each new group studied, which is both an expensive and time consuming procedure. In response to this, cross-species testing has become common practice (Moore et *al.* 1991, Blanquer-Maumont and Crouau-Roy, 1995, FitzSimmons et *al.* 1995). Primers developed in one group of organisms are tested for positive amplification of the locus, conservation of the repeat unit and allelic variability in populations of closely related species. For example, horse primers have been used to successfully amplify polytypic loci in zebra (Y. Moodley *pers. comm.*), whilst primers developed for the odontocetid whale have amplified polytypic loci in other toothed whales and in baleen whales, indicating conservation of the repeat unit and primer binding site over 35 - 40 million years of evolution (Schlotterer et *al.* 1991).

Research Aims

The main objective of this part of the study was to quantify genetic relationships between extant populations of *P. geometricus*, using microsatellite DNA markers originally developed in the Galapagos tortoise, *Geochelone nigra* (E. Louis *pers. comm.*). Microsatellite markers are known to be highly variable and were selected after mitochondrial DNA sequencing (see Chapter 6) failed to reveal significant differentiation amongst selected individuals, representing the geographically separated range for *P. geometricus*. This pilot study included sequencing approximately 340 bp of control region (D-loop) mitochondrial DNA (results not shown). The *Psammobates* populations selected for the study were representative of three localities separated from one another by the Cape Fold mountain ranges and included individuals sampled from farms in the Ceres Valley, Elandsberg Private Nature Reserve and a population in the Worcester/ Tulbagh Valley (Figure 7.1). In addition to providing information on the levels of structuring observed among the populations, genetic data may provide insight into the response of these populations to habitat fragmentation, range restriction and potential migratory routes (if any) between the restricted habitats. This information will be valuable for the management of this endangered species, and specifically for the establishment of captive breeding programs for the reintroduction of individual tortoises into areas of suitable habitat or the translocation of individuals from populations of non-viable size to more suitable areas.

Furthermore, microsatellite loci originally developed in *Geochelone nigra* were tested for the amplification of polytypic loci in the remaining nine genera within the Family Testudinidae. As discussed in Chapter 1, a major limitation to the implementation of successful management and conservation strategies for terrestrial tortoises, is the limited baseline genetic and behavioral data available for the group. This is the case despite the fact that a high proportion of terrestrial Chelonians remain severely threatened by habitat degradation and human exploitation (see Chapter 1). The successful amplification of variable microsatellite loci, using cross-species primers, would negate the need for loci to be developed for each new species of tortoise studied. This information, in combination with the revised taxonomic status outlined in the phylogenetic study of the Family Testudinidae (see Chapter 6), together can provide a guideline for future population genetic studies within the group. The data will be invaluable in the delimitation and identification of conservation units and evolutionary processes for the future management of terrestrial tortoises as well as elucidating the mechanism of mutation and generation of alleles at microsatellite loci.

MATERIALS AND METHODS

Sampling of *Psammobates geometricus*

Approximately 100µl of blood was sampled from individual tortoises from the three geographically separated areas representing the remaining habitat localities for *Psammobates geometricus*. Twenty-eight samples were collected from the Elandsberg Private Nature reserve, 26 samples from the Ceres valley and 25 samples from the Worcester/Tulbagh valley (Farm Chelance). The samples from the Ceres valley were collected from two farms adjacent to one another (Farm Ezelfontein and Farm Perdefontein). These two populations displayed no significant structuring or differentiation, using tests of genetic differentiation described below (results not shown) and so were treated as a single sample for the remainder of the study.

DNA Extraction and Cross-Species Amplification of Microsatellite Loci

Total DNA was extracted from nucleated red blood cells by proteinase K digestion (0.1mg/ml) at 65^{0}C for 1 hour in lysis buffer (50mM Tris-HCl pH 7.5; 400mM NaCl, 5mM EDTA pH 7.4, 0.5% SDS). This was followed by precipitation of DNA in the presence of 2M ammonium acetate after addition of 2 volumes of 100% ethanol (Sambrook et *al.* 1989). The resulting DNA pellets were air dried and resuspended in 100µl TE, pH 8. The genomic DNA was then diluted to a final concentration of between 10 - 50ng/µl for Polymerase Chain Reaction (PCR) amplifications. The primers used to amplify microsatellite loci in the Family Testudinidae (Table 2.1) were first described in the Galapagos tortoise, *Geochelone nigra* (E. Louis, in press). The forward primer of each primer set was end-labeled with ?- ^{32} P - ATP (O'Ryan et *al.* 1998). PCR was then performed in 10µl reaction volumes using the following reaction conditions: 25?M of reverse primer, 5mM dNTP's, 1.5mM $MgCl_2$ and 0.5U Taq polymerase (Bioline). Cycling parameters for PCR amplifications were as follows: a one minute denaturing step at 94°C, one minute at the annealing temperature (50°C to 62°C) to maximise the specificity of the hybridisation and a 45 second extension step at 72°C. The amplified product was then electrophoresed on a 6% denaturing polyacrylamide gel. Genotypes were visualised using x-ray plates after 24-hour exposure.

For cross-species amplification of the loci in the Family Testudinidae, DNA extracted from samples as outlined in Chapter 4 (Table 2.1) were used. A minimum of two individuals from each species were used to determine whether the loci were polytypic and subsequently sequenced to determine whether the microsatellite repeat unit was conserved across members of the Family and selected outgroup taxa. Genotypes were scored from the

autoradiographs and allele lengths (in base pairs) determined using a sequenced size ladder of M13 ssDNA. Thirteen sets of *Geochelone nigra* primers were chosen for amplification, of which eight amplified polytypic loci in *Psammobates geometricus*. These eight loci were utilised in the subsequent population genetic study for this endangered species.

Microsatellite Motif Conservation across Taxa

Two *Geochelone nigra* microsatellite repeat loci, Galap9 and Galap11, were selected for sequencing analysis to determine whether or not the microsatellite motif was conserved across the Family Testudinidae. This was to ensure that the amplified products in the cross-species testing were not a result of non-specific binding of the *Geochelone nigra* primers. PCR reactions were repeated as outlined previously but with no radioactive labeling and 10µl of each of the resulting products were run on a 2% agarose gel (Type D1-LE, Whitehead Scientific) to check for positive amplification. A positive control, *Geochelone nigra*, and a blank were included in all PCR reactions. The bands were visualised on the agarose by staining with Ethidium bromide ([EtBr] = 10ug/ml).

Positive PCR products were ligated into a TA pCR® vector using the TA Cloning® Kit following the manufacturer's instructions (Invitrogen™). Ligations were subsequently transformed into competent INVαF' One-Shot® cells using a heat-shock protocol (Invitrogen™ product guide). Transformed cells were transferred to 200µl SOC medium (Invitrogen™ product guide) and recovered by incubating at 37°C for 1 hour, with agitation. Following incubation, approximately 100µl of the transformation mix was plated onto Luria broth selection media (with Agar), containing 50µg/ml ampicillin and 40µl X-Gal (10mg/ml). Plates were incubated at 37°C for 18 hours and then transferred to 4°C for 2-3 hours for maximum color development. Recombinant cells, containing DNA inserts, were identified using insertional inactivation of the β-galactosidase gene i.e. white colonies verify the presence of the target insert DNA. Positive clones were then picked from the agar plates and incubated in 200µl of Luria broth containing 50µg/ml ampicillin at 37 °C for 90 minutes.

Target DNA was purified from the bacterial cultures using a QIAPrep® Spin Miniprep Kit (Qiagen™) as per the manufacturer's instructions. The microsatellite motif i.e. the insert DNA was then amplified using pUCM13 Forward and Reverse primers and standard PCR cycling parameters (see Invitrogen™ product guide). PCR products were checked for positive

amplification of the target region by running 10µl of the reaction on a 1.2% agarose gel (Type D1-LE, Whitehead Scientific).

PCR product (approximately 90µl) for cloning and sequencing was purified using QIAquick® PCR Purification Columns (Qiagen™ Inc., Chatsworth, CA), following manufacturer's instructions, and eluted in 50µl of sterile distilled water, pH 8. This process removes any excess primers, nucleotides, polymerases and mineral oil that could interfere with subsequent enzymatic reactions. Following purification, 5µl of the product was run on a 1.2% agarose gel (Type D1-LE, Whitehead Scientific) and compared with the intensity of 5µl of a PGEM® - 3ZF(+) double stranded DNA control template (0.2g/L) (the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction kit v1.0, Applied Biosystems, Perkin Elmer, Forster City, USA) in order to estimate the concentration of the PCR fragments. All cycle sequencing reactions were performed using an ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction kit v1.0 (Applied Biosystems, Perkin Elmer, Forster City, USA). Reactions were performed in 10µl volumes that contained approximately 100ng of the purified PCR product, 4µl of the termination mix, 10pmol/µl of the specified sequencing primers (pUC18-M13 forward primer and pUC18-M13 reverse primer) and made up to a final volume with sterile distilled water, pH 8.

The following cycling parameters were used in the sequencing reaction: 96°C denaturation (30 seconds), 50°C annealing (15 seconds) and 60°C extension (4 minutes), for a total of 25 cycles. Forward and reverse sequences were obtained for each clone. Cycle sequencing products were purified of salts, polymerases and excess dye terminators using a G-50 Sephadex matrix (0.05g in 800µl sterile water) in Centri-Step spin columns (Princeton Separations, Inc., Adelphia, NJ). Samples were dried in a Speed-Vac and stored at -70°C. Sequenced products were run on a 96 well ABI Prism 377 Automated Sequencer (Applied Biosystems, Perkin Elmer, Forster, CA). Forward and reverse sequences for each amplified clone were aligned using Sequencher v3.0™ (GeneCodes Corp.).

Statistical Analysis for *Psammobates geometricus*

i. Genetic Variability Measures

Genetic variation within the sampled populations of the Geometric tortoise was quantified using the average number of alleles per locus (A), observed heterozygosity (H_o) and Hardy-Weinberg expected heterozygosities (H_e) (Nei 1973). These were calculated for each locus

for each population, and averaged over all loci using the Biosys-1 package (Swofford and Selander 1981). Deviations from Hardy-Weinberg expectations were determined by chi-square analysis. The inbreeding estimator, F_{is}, (Weir and Cockerham 1984) for each locus was calculated for each population using the software package GENEPOP v1.2 (Raymond and Rousset 1995), with estimation of Hardy-Weinberg exact probabilities using the Markov chain method (Guo and Thompson 1992). Tests for linkage disequilibrium, to confirm that allelic states at one locus were independent from allelic states at all other loci, were conducted using GENEPOP v1.2 (Raymond and Rousset 1995).

ii. Tests for Detecting Recent Reductions in Population Size

Tests to detect recent reductions in population size were calculated using two programs, BOTTLENECK 1.2.02 (Piry et *al.* 1999) and Garza and Williamson's (2001) "M" statistic implemented in Agarst (Harley 2001). Populations that have undergone a reduction in effective population size show a reduction in allelic diversity and a concurrent reduction in the associated levels of observed heterozygosity (Piry et *al.* 1999). Allelic diversity is reduced at a faster rate than heterozygosity and, as a result, observed heterozygosity is larger than expected than if the population were in mutation-drift equilibrium (Piry et *al.* 1999) (but see caveats in Cornuet and Luikart 1996). Under the assumption of mutation-drift equilibrium, the program BOTTLENECK computes the distribution of heterozygosity that would be expected given an observed number of alleles (k), with sample size (n), for each population sample and for each locus. This distribution is generated by simulating the coalescent process of n genes under the two possible mutation models for microsatellite DNA: the Infinite Allele Model (IAM) and the Step Wise Mutation model (SMM). The SMM is simulated using a bayesian approach (see Cornuet and Luikart 1996). Average expected heterozygosity is then calculated from the distribution and compared to the observed heterozygosity to establish whether a heterozygosity excess or deficiency is present within the data set. Once all loci under study have been processed, three statistical tests are performed for both the IAM and the SMM mutation model to determine whether allelic frequency distributions are approximately L-shaped, as would be expected under mutation-drift equilibrium. If the distribution is not L-shaped, it indicates a mode-shift in the allelic frequency distribution and therefore, a recent population bottleneck (Cornuet and Luikart 1996 and Luikart et *al.* 1998).

Garza and Williamson's (2001) 'M' value, implemented in Agarst (Harley 2001) was also calculated. This test relates the mean number of alleles present in the data set to the total range in allele size. The test is able to distinguish between populations that have been

reduced in size from those that have been small for long periods of time and are in mutation-drift equilibrium. It achieves this by comparing an observed allelic frequency distribution to that obtained under a simulation based on a specified mutation model. The ratio "M" is essentially a measure of the number of unoccupied potential allelic states between the largest and smallest allele size within a sample compared to that obtained from the simulated distribution. Given the effects of genetic drift, a large number of unoccupied states would be expected after a recent reduction in population size and the value of "M" will decrease accordingly (Garza and Williamson 2001). The method, however, is particularly sensitive to the type of mutation model assumed. For microsatellite data, the SMM model is implemented as most appropriate in describing the generation of new alleles. In permanently reduced or small populations the value of "M" will partially recover within the simulation procedure whilst allelic diversity does not. This indicates that the measure can be used to distinguish between populations that have undergone a recent reduction in size, from those that have been small for a long period of time (Garza and Williamson 2001).

iii. Measures of Population Differentiation

Comparisons of geographic heterogeneity in allele frequency distributions, between all pairs of populations for each locus, were assessed using a Monte Carlo procedure (Roff and Bentzen 1989). Probability values were calculated after 1000 replicates and corrected for multiple analysis errors using the Sequential Bonferroni test (Rice 1989). Levels of population differentiation were calculated using F-statistics from the programme FSTAT v2.8 (Goudet 1995). *Rho* estimates were calculated using Goodman's RstCalc (1997). In addition, Goldstein's $d\mu^2$ (Goldstein *et al.* 1995) was used to generate pairwise genetic distance estimates and is implemented in the RstCalc package (Goodman 1997). This measure was used as it was specifically developed for microsatellite evolution assuming the SMM (Kimura and Crow 1964), taking into account the high variance associated with microsatellite data, and is robust to differences in sample size (Goldstein and Pollock 1997).

iv. Principal Component Analysis

Principal component analysis (PCA) was performed on allele frequency data using PCA-GEN v1.2 (Goudet 1999). PCA is a multivariate statistical method that uses as few factors as possible to explain the majority of the total variation in a data set and effectively reduces the dimensionality of the data set. If the data set is highly structured, the first two principal components should account for a large majority of this variation (> 50%) (Everitt and Dunn 1991). Component scores are obtained for each sample within the data set and reflect the

relationship between each variable and each principle component. In the analysis of the allele frequency data, a relationship, in the form of a correlation, should exist between the locus-specific allele frequencies of individuals within populations and thereby supports a graphical representation of the relationship when plotted on a dispersion matrix.

The program PCA-GEN v1.2 (Goudet 1999) associates this form of multivariate analysis to Wright's F_{st} (1965), which is a known measure of population differentiation. This is achieved by associating the alleles that display the majority of the differentiation with each of the principal component axes i.e. reducing the dimensionality of the original data set. The technique has been shown to demonstrate a consistent relationship between F_{st} and the graphical representation of the PCA (Goudet 1999). The technique allows for the visualisation of the association between the apportionment of genetic variation within principal component space and the geographical apportionment of genetic variation within the sampled populations.

v. Assignment Tests

Assignment tests were implemented to determine whether sampled individuals could be assigned to their respective populations based on a locus specific identity. These tests compare the product of allele frequencies in individuals across loci to the population mean values and were carried out on all individuals in each population, using the program WHICHRUN v3.2 (Banks and Eichert 2000). WHICHRUN is a computer simulation program that utilises multilocus genotypic data to allocate individuals to their most likely source population based on the similarity between the observed allele frequency distributions of the populations and the genotypic profiles of each of the individuals. The program requires baseline genotypic data for all potential source populations and assumes that the genetic loci employed are independent. The likelihood that an individual sample may come from either of the source populations is presumed to be equivalent to the Hardy-Weinberg frequency of that genotype at each locus in each population. Likelihood values from each locus for each individual are then multiplied to give an indication of the likelihood of each of the individual samples originating one of the source populations under consideration. When there is sufficient population differentiation, which generates significantly different allele frequency distributions, the assignment test can provide estimates of potential "dispersal rates" between the populations (Paetkau et *al.* 1995).

There are however several considerations that should be taken into account to ensure the accuracy of the subsequent likelihood calculations and assignment estimates. For example, there should be reasonable amounts of differentiation between the populations under study and the rates of DNA mutation at the selected loci should be closely correlated with divergence estimates. Consequently, when analysing populations that have been separated for long periods of time, markers that are prone to homoplasy would not be suitable for use in the calculation of the likelihood estimates. In addition, a large number of independent loci should be selected and sample sizes should be equal amongst the sampled populations. This is to reduce potential bias that could affect the frequency distributions and consequently the assignment estimates (Banks and Eichert 2000).

RESULTS

PART I: MICROSATELLITE DNA

i. Cross-species Amplification of *Geochelone nigra* Microsatellite Primers in the Family Testudinidae: (refer to Table I and Table II at the end of the Chapter)

Seven *Geochelone nigra* primers were tested for amplification of polytypic loci in the Family Testudinidae. Primers from two turtle species, *C. caretta* and *C. mydas* were also included in the panel (Table I). The Galapagos primers (Galap2, Galap4, Galap5, Galap9, Galap10, Galap11 and Galap13) showed extensive cross-species amplification in all genera within the Family Testudinidae. The repeats are classified as either pure repeat units or compound repeat units depending on the sequence obtained from the clones isolated in the source species, *G. nigra*. Although only a limited number of individuals were available for each species of terrestrial tortoise, the primers were polytypic for the majority of sample sets tested.

The compound repeat unit, Galap2, amplified products that ranged in size from 171bp - 257bp with a total of 48 alleles for 104 individuals tested. It did not amplify the target locus in *Chersina angulata* and appeared monotypic in all individuals of *Geochelone radiata* (176bp allele). Galap2 displayed high levels of variability and observed heterozygosity in several of the species tested for example; a total of eight alleles were identified in six genotyped individuals of *Geochelone pardalis* and nine alleles were identified in the nine individuals representing *H. areolatus*. Galap2, however, did not amplify polytypic loci in the Turtles, *Terrapene* and *Dermatemys* (Table I).

Galap4 occurs as a pure repeat motif in *G. nigra* with 34 alleles identified in 110 individuals. The alleles ranged in size from 103bp - 251bp. The largest allele occurred in the outgroup representative, *C. insculpta* (251bp). The locus appeared to be monotypic in *K. belliana* (125bp allele represented in all four individuals). Galap5, a pure $(CA)_n$ motif in the source species *G. nigra*, displayed low levels of observed heterozygosity and was monotypic in most species tested, including *Gopherus* spp., *H. areolatus*, *Psammobates* spp, *Kinixys* spp. and *Testudo* spp. The locus displayed low allelic diversity with 18 alleles across 114 individuals. Allele size ranged from 98bp *in L. kempii* to 265bp in *G. pardalis*.

Galap9 and Galap10 displayed high levels of allelic diversity and observed heterozygosity with few exceptions. Galap9 was isolated as a compound repeat motif from *G. nigra*. The locus displayed a range in allele size from 106bp in *T. triunguis* to 256bp in *H. areolatus*. It did not amplify any product in *M. tornieri*. The locus displayed high levels of allelic diversity with 51 alleles representing 111 individuals.

Galap10, a pure $(CA)_n$ repeat motif, ranged in size from 104bp in *L. kempii* to 222bp in *P. geometricus*. The locus did not amplify any product in *G. pardalis* nor in *Terrapene triunguis*. Galap10 had a complement of 31 alleles for 105 individuals tested.

Galap11, a pure repeat in *G. nigra*, had 35 alleles for 110 individuals tested. The locus was monotypic in *H. areolatus* and ranged in size from 131bp to 182bp for the remainder of the species tested.

Galap13, a compound repeat unit in the source species, was found to show reduced levels of allelic diversity in all genera tested, although higher levels were displayed for the larger study samples for *P. geometricus* and *G. nigra* (Table 7.1). This locus contained a small microsatellite array ranging in size from 81bp in *G. platynota* to 164bp in *M. tornieri*. The locus was monotypic in all *Gopherus* species (89bp) allele and was not successfully amplified in several of the *Testudo* spp. Galap13 displayed low levels of allelic diversity compared to the other loci with 20 alleles across 101 individuals genotyped.

The two turtle primers tested for amplification, Caretta and Cmydas (courtesy of R. Heubinger, Henry Doorly Zoo, NE), showed low levels of allelic diversity in the cross-species tests for the Family Testudinidae. The primer isolated in *C. caretta* was particularly uninformative and displayed only seven alleles across 121 individuals sampled with a lack of observed heterozygote individuals genotyped at this locus.

ii. Sequence Analysis of Two Microsatellite Loci, Galap9 and Galap11, Isolated from the Galapagos Tortoise, *Geochelone nigra*

Positive microsatellite amplification products for Galap9 and Galap11 were cloned and sequenced to determine the extent to which the microsatellite motif was conserved across the Family Testudinidae and selected outgroup taxa represented by the Family Emydidae (*Terrapene*). This was carried out in order to obtain information on the process by which the

microsatellite locus mutates with respect to the flanking regions and the rest of the nuclear genome. Both the $(CA)_n$ repeat at Galap11 and the compound repeat unit $(CT)_n$ $(CA)_n$ at Galap9 were conserved across the Testudinidae and the Emydidae. Both motifs were characterised by mutational events that changed the nature of the motif from that of the original, cloned from *G. nigra* (Table II). Overall, Galap9, $(CT)_n$ $(CA)_n$, showed greater susceptibility to mutational events within the motif, particularly transversion events that were more characteristic in the $(CT)_n$ region, for example in *I. elongata* and *P. tentorius* (Table II). Consequently, a proportion of the variability at Galap9 was not evident unless the alleles had been sequenced. Galap11 on the other hand, was found to be more conserved in the sequence of the repeat unit across the Family Testudinidae. The majority of the variation attributable to this locus was a direct result of a change in the number of repeat units associated with the tandem repeat motif itself and was therefore evident by simple genotyping of the locus. In cases where there were mutations in the sequence comprising the repeat unit, these were observed to be transitional events, for example in *G. pardalis* and *G. carbonaria.* Transversions within this microsatellite locus were only observed in a limited number of species, for example *G. radiata* and *G. sulcata.*

Sequencing the flanking regions of Galap9 and Galap11 across 11 genera within the Family Testudinidae and the selected outgroups, revealed sequence variability that was specific to particular genera and in some cases to populations within species (the aligned sequence data file is not displayed but is available on request). Substitutions in the flanking regions have only been reported if the change was observed in all species within a species complex. Point mutations not shared by other individuals within the species group or genus have not been reported.

At Galap9, the aligned sequence lengths for all species sequenced at this locus was 256bp including gaps, the repeat region and primer binding sites. Informative sites within Galap9 included position 22 where *Testudo* spp. and *M. tornieri* showed a transition event from a G ? A. Another transition event from a C ? T occurred in *Gopherus* spp. *Manouria* spp., *M. tornieri* and *T. horsfieldi* at position 23. *Geochelone pardalis* was defined by a G ? A transition at position 38 and an A ? T transversion at position 215 of the aligned sequence for Galap9. The *Testudo* spp. were identified by a transition event from a C ? T at position 41 and the *Gopherus* spp. and *Manouria* spp. identified by an A ? G transition at position 86. *Gopherus, T. kleinmanni, T. marginata* and *the Terrapene* showed a T ? C transition at position 94 whilst *Gopherus* and *Terrapene* shared an A ? G substitution at position 220. A

phylogenetically informative character was also identified at position 207 of the aligned sequence where a C ? T transition was shared by *Manouria* spp., *Gopherus* spp., *Testudo* spp., *M. tornieri* and the turtle representatives of the *Terrapene*. Across the total aligned sequence, excluding the microsatellite repeat, the *Terrapene* showed approximately 20 – 25% sequence divergence when compared with other members of the Family Testudinidae.

Sequence variability at Galap11 was lower, with fewer phylogenetically informative sites, than that of Galap9. Total sequence length was approximately 182bp. A transition event from a C ? T defined the *Kinixys* spp. at position 113 of the aligned sequences. Furthermore, *H. areolatus* spp. were identified by a T ? C substitution at position 96. *Geochelone gigantea (Dipsochelys)* and *G. pardalis* spp. shared a G ? A transition at position 154 whilst *M. tornieri* and the *Terrapene* shared a G ? A at position 153.

Within the genus *Psammobates*, several individuals of the *Psammobates geometricus* species were sequenced for Galap11. These species were representative of the geographically separated populations analysed within this study i.e. two individuals from the Elandsberg population, two individuals from the Ceres population and one individual from the Worcester population. All members of *P. geometricus* showed a transversion event from an A ? C at position 108 of the aligned sequences, while individuals from the Ceres and Elandsberg populations had an additional transition substitution from a G ? A at position 109.

PART II: The Geometric Tortoise, *Psammobates geometricus*

i. Genetic Variability Measures

A total of 79 *P. geometricus* individuals were genotyped at eight polytypic loci, supporting a total of 83 alleles. All loci displayed high levels of allelic diversity with 19 alleles at the most variable locus (Galap11), four alleles at the least variable locus (Galap4) and a mean of 10.3 alleles per locus (Table 7.1). The allelic diversity measures and allele size ranges in the *Psammobates geometricus* populations were compared to those found in the *Geochelone nigra* populations in which the microsatellites were originally isolated. Galap2 had higher allelic diversity in the Geometric tortoise compared to the Galapagos tortoise populations (number of alleles = 11 and 7 respectively) despite the fact that the number of samples in the Galapagos sample study was substantially higher (n = 260). This trend was true for Galap9 and Galap10 (Table 7.1). For Galap4, Galap9, Galap10 and Galap13 the allele size ranges between the two species were non-overlapping and for Galap4, Galap9 and Galap10 the alleles were larger in *P. geometricus* than was found in the *Geochelone nigra* source species (Table 7.1). The average number of alleles per locus was 15.3 for the Galapagos study with as many as 39 alleles present at both Galap6 and Galap11.

Observed heterozygosity values were consistently high in *P. geometricus* and ranged from 0.35 at Galap6 to 0.82 at Galap9 (Table 7.2). The average observed heterozygosity over all populations for all loci was 0.66. Observed (H_o) and expected (H_e) heterozygosity values over all loci were consistent between populations and ranged from 0.69 and 0.68 in Elandsberg, to 0.62 and 0.66 in Ceres (Table 7.2). Despite the high levels of allelic diversity at Galap11, the heterozygosity levels were found to be lower than that at Galap2 and Galap4 which were found to support fewer alleles than the Galap11 locus (Table 7.2). Observed heterozygosity values for the *Geochelone nigra* species ranged from 0.90 at Galap6 (39 alleles were identified at this locus) to 0.22 at Galap9 (5 alleles identified) (Table 7.2).

Table 7.1: Table showing details for eight microsatellite loci, their size range and respective number of alleles detected across populations of *Psammobates geometricus* at Elandsberg, Ceres and Worcester (n = 79 individuals). Locus specific statistics for the source species for populations of *Geochelone nigra* (n = 260 individuals) from the Galapagos Islands are also shown (*Geochelone* data courtesy of E. Louis, Omaha Henry Doorly Zoo, NE).

Locus	Size Range (bp) in *P. geometricus*	No. of alleles in *P. geometricus*	Size Range (bp) in *G. nigra*	No. of alleles in *G. nigra*
Galap2	176 - 234	11	191 - 203	7
Galap3	168 - 176	5	171 - 173	4
Galap4	156 - 176	4	142 - 154	5
Galap6	178 - 186	6	162 - 192	39
Galap9	218 - 254	16	195 - 201	5
Galap10	180 - 238	14	160 - 176	9
Galap11	142 -180	19	140 - 222	39
Galap13	101 - 121	8	122 - 154	15

Table 7.2: Observed (H_o) and expected (H_e) heterozygosity values for all eight loci for *Psammobates geometricus* populations and mean observed heterozygosity for the microsatellite source species, *Geochelone nigra* (*Geochelone* data courtesy of E. Louis, Omaha Henry Doorly Zoo, NE).

Locus	Elandsberg	Ceres	Worcester	Mean	Mean H_o for source species, *G. nigra*
Galap2					
H_o	0.893	0.720	0.731	0.785	0.473
H_e	0.742	0.747	0.791	0.786	
Galap3					
H_o	0.481	0.583	0.520	0.526	0.251
H_e	0.389	0.460	0.500	0.445	
Galap4					
H_o	0.714	0.640	0.769	0.709	0.520
H_e	0.668	0.621	0.681	0.657	
Galap6					
H_o	0.321	0.261	0.478	0.351	0.902
H_e	0.323	0.237	0.426	0.366	
Galap9					
H_o	0.852**	0.800*	0.800	0.818	0.224
H_e	0.913	0.859	0.899	0.916	
Galap10					
H_o	0.704	0.760	0.731	0.731***	0.590
H_e	0.781	0.776	0.894	0.842	
Galap11					
H_o	0.750	0.440***	0.654***	0.620***	0.832
H_e	0.913	0.842	0.820	0.891	
Galap13					
H_o	0.786	0.760	0.615	0.722	0.788
H_e	0.733	0.744	0.547	0.697	
Average					
Ho	0.688	0.621***	0.662		
He	0.683	0.661	0.695		

Values found to be significantly different from Hardy–Weinberg expectations using chi-squared tests are represented with an asterix, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Diversity within populations was variable, with the mean number of alleles per locus (A) ranging from 8.1 in Elandsberg to 6.6 in the Ceres population. This range is due largely to a decrease in the number of rare alleles present in the smaller populations of Ceres and Worcester. A rare allele is defined as an allele occurring at a frequency of less than 5%. Elandsberg was found to support the largest number of rare alleles and a total of 10 rare alleles were identified, with only four and six in the Ceres and Worcester respectively (Figure 7.2).

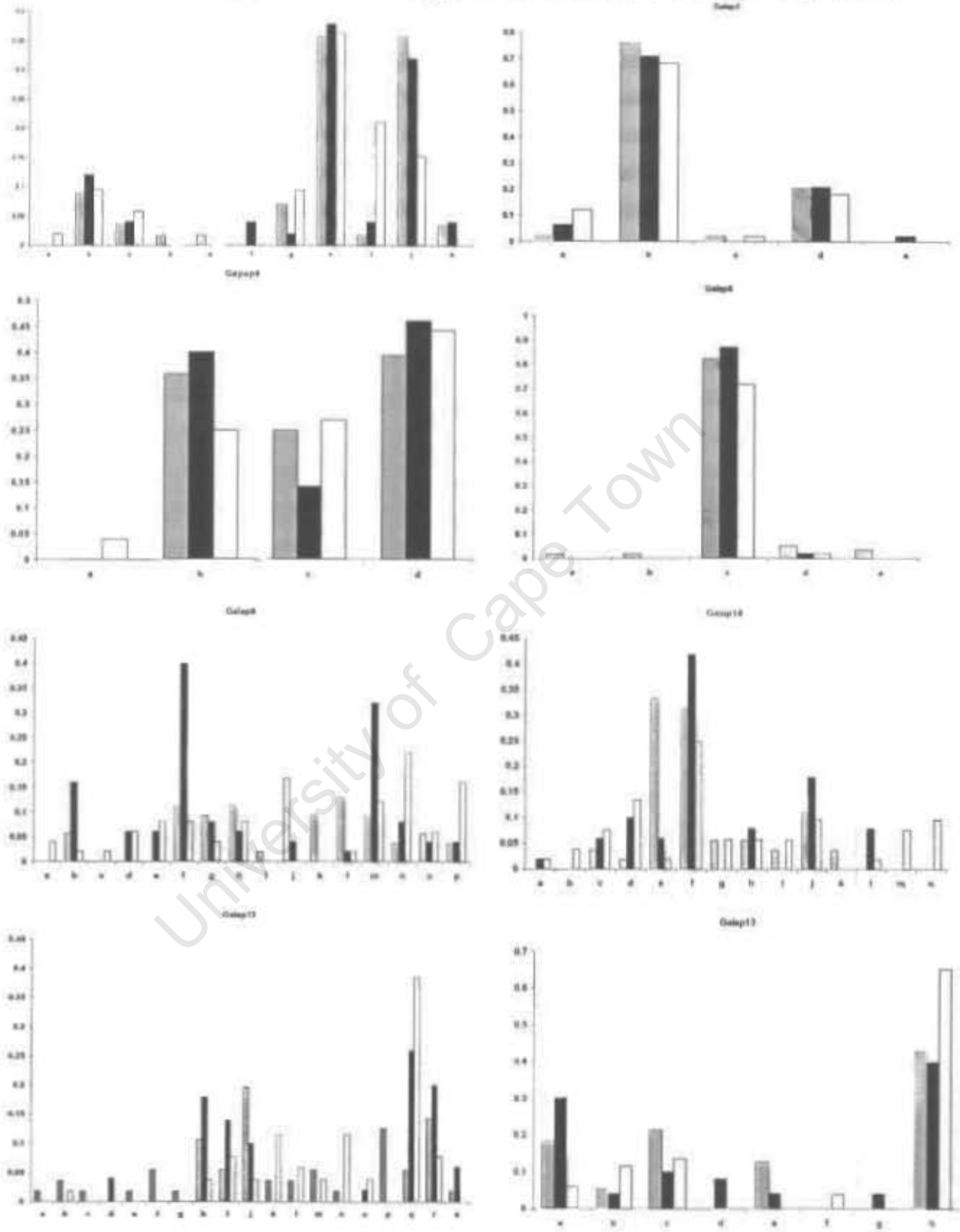


Figure 7.2: Histograms showing allele frequency distributions at all loci for Elandsberg (grey bar), Ceres (black bar) and the Worcester (white bar) populations of *P. geometricus*. Allelic designation on the x-axis with allele frequency on the y-axis.

ii. Genotypic Disequilibrium and Hardy-Weinberg Equilibrium

A total of 84 pairwise tests across all loci for each population produced only two significant p-values for genotypic linkage ($p < 0.05$). However, these probabilities were not significant after a Sequential Bonferronni test (Rice 1989), suggesting that the loci provide independent information.

Only four out of 32 comparisons indicated significant deviations from Hardy-Weinberg expectations at the 95% confidence interval using chi-squared analysis (Table 7.2). The associated positive F_{is} values, based on Weir and Cockerhams' inbreeding estimator (1984), showed that these deviations were due to a significant excess of homozygote individuals at these loci (Galap9 in Ceres, and Galap11 in Ceres and Worcester) (Table7.3). An excess of heterozygotes evident at one locus, Galap2 in Elandsberg, was represented by a significantly negative F_{is} value of -0.209 ($P < 0.05$). But this locus was not shown to deviate significantly from Hardy-Weinberg expectations (Table 7.2). For both the Ceres and Worcester populations, Galap11 showed strongly positive F_{is} values of 0.482 and 0.206 respectively (Table 7.3). The overall F_{is} value over all loci for all three populations was significantly positive. This bias was due largely to the high positive value of Galap11 and when this locus was excluded from the analysis the resulting F_{is} value was no longer significant at the 95% confidence interval.

Table 7.3: F_{is} values (Weir and Cockerham 1984) for each polytypic locus per population, and among all populations for *Psammobates geometricus*

Locus	Elandsberg	Ceres	Worcester	Over all fragments
Galap2	-0.209*	+0.037	+0.0780	-0.023
Galap3	-0.0245	-0.275	-0.04	-0.184
Galap4	-0.071	-0.031	-0.133	-0.079
Galap6	+0.006	-0.105	-0.126	-0.046
Galap9	+0.068	+0.070*	+0.112	+0.108**
Galap10	+0.101	+0.021	+0.185	+0.133*
Galap11	+0.181	+0.482***	+0.206*	+0.306***
Galap13	-0.073	-0.022	-0.128	-0.035
Over all loci	-0.030	+0.022***	+0.019*	+0.0225***

Estimation of exact p–values by the Markov chain method, *p< 0.05, **p < 0.01, ***p < 0.001

iii. Tests to Detect Recent Population Reduction

Neither of the two tests performed in the BOTTLENECK program supported the hypothesis that there had been a recent reduction in size for the tortoise populations, in spite of the reduction in habitat (Baard 1993b). There was no indication of a significant heterozygote excess in any of the populations and no evidence of a mode shift from an approximate L-shaped distribution (expected under mutation-drift equilibrium), as would be expected in a recently reduced population (Cornuet and Luikart 1996). Garza and Williamson's M-statistic, however, did support the idea that bottleneck events in all three populations may have occurred. The M-statistic, which can demonstrate persistently low values after a bottleneck event for over 100 generations, produced values below those expected from normal outbred populations (0.7) (Garza and Williamson 2001). The values obtained for Elandsberg, Ceres and Worcester were 0.57, 0.50 and 0.54 respectively.

iv. Levels of Population Differentiation

Monte Carlo estimates of genetic heterogeneity indicated that the greatest variation both between and across the three populations occurred at Galap9, Galap10, Galap11 and Galap13. These differences were significant at the 95% confidence interval (data not shown). The *Rho* estimate (RstCalc, Goodman 1997) over all populations for all loci was 0.018, ($p < 0.05$). Overall F_{st} (FSTAT v2.8, Goudet 1995) was slightly higher than that of *Rho* ($F_{st} = 0.031$). Pairwise population comparisons showed that the greatest level of population differentiation, calculated using $d\mu^2$, *Rho* and F_{st}, occurred between the Elandsberg and Worcester populations (Table 7.4).

Table 7.4: Estimates of genetic differentiation: *Rho* (Goodman 1997), F_{st} (Goudet 1995) and $d\mu^2$ estimates (Goldstein et *al.* 1995).

	Rho	F_{st}	$d\mu^2$
Elandsberg vs Ceres	0.007*	0.018	0.040
Elandsberg vs Worcester	0.039*	0.042	1.29
Ceres vs Worcester	0.018**	0.030	0.81

*$p < 0.05$, **$p < 0.005$

v. Principal Component Analysis

Principal component analysis (PCA) of *Psammobates geometricus* differentiated the three populations within the dispersion matrix according to their geographic location and with no overlap (Figure 7.3). PC1 and PC2 accounted for 66% of the variation in the allele frequencies between the three study populations, indicating some degree of structure in the allele frequency data. This corresponded to a global F_{st} of 0.03.

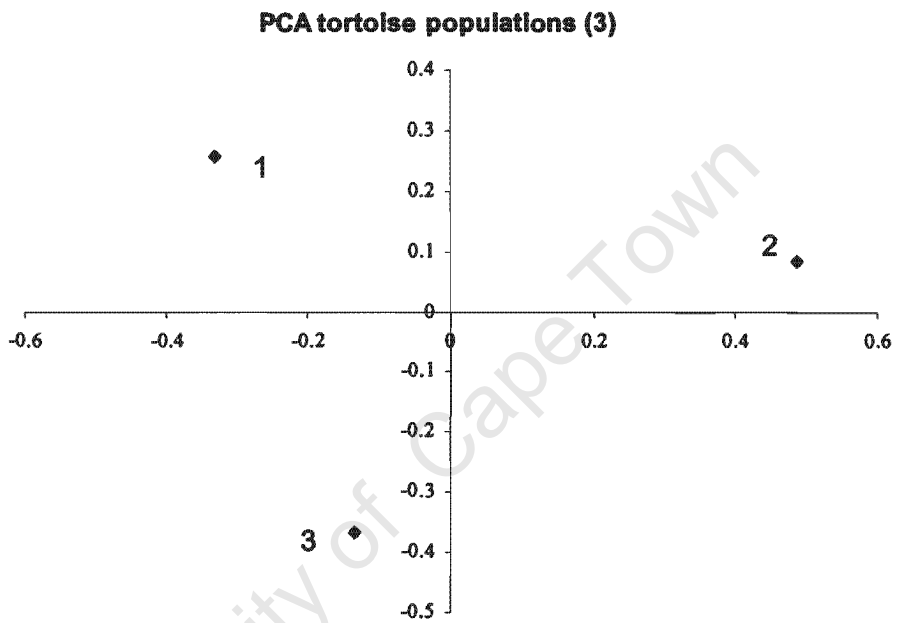


Figure 7.3: Factor map of the two main axes of the Principal Component Analysis of *P. geometricus* genotypes from the Elandsberg (1), Ceres (2) and Worcester (3) populations.

vi. Assignment Tests

Assignment test likelihood ratio values obtained from WHICHRUN v3.2 (Banks and Eichert 2000) (data not shown) indicated that 68% of individuals in the Elandsberg population were correctly assigned to that population with 100% confidence i.e. all other populations were excluded as possible sources at p=0.01. The remainder of the Elandsberg samples were assigned with greatest likelihood to the Ceres Valley, except for one individual which was assigned to Worcester population. The Ceres population had 72% of individuals correctly assigned (at p=0.01), whilst the remainder were assigned to either the Elandsberg or the Worcester populations. The Worcester population had 87% of individuals correctly assigned, with the next most likely population for the incorrectly assigned individuals identified as Ceres. The low log likelihood values associated with the assignment data and the low percentage of individuals assigned with a high degree of confidence is potentially a result of the low levels of differentiation observed between the populations (Table 7.4).

DISCUSSION

PART I: Cross-Species Microsatellite Amplification and Evolution of Repeat Units in the Family Testudinidae

Cross-species amplification of microsatellite loci developed in the Galapagos tortoise, *Geochelone nigra*, was extensive in species representative of the 11 genera in the Family Testudinidae. The majority of the loci showed high levels of heterozygosity and allelic diversity, with longer alleles present in non-source species, despite observations that these should decrease with increasing evolutionary distance from source species (Ellergren et *al.* 1995, Primmer et *al.* 1996, Ellergren et *al.* 1997). This effect has been attributed to ascertainment bias, which is introduced during the isolation of loci from source species. There is an inherent propensity for longer repeat units to be selected during the isolation procedure. As a result, the length of these alleles in the source species may be longer than those amplified in closely related lineages (FitzSimmons et *al.* 1995 but see Crawford et *al.* 1998, Zhu et *al.* 2000). This trend, however, was not evident from cross-species amplification using the Galap primers. Alleles were often longer in individuals of *Psammobates geometricus,* and observed heterozygosity values were often higher than those reported for the source species, *Geochelone nigra*. This may be a result of the recent population crash in these Island endemics Furthermore, there did not appear to be an obvious relationship between allelic diversity and observed levels of heterozygosity in either of the two study species.

Cross-species amplification using the turtle primers, Caretta and Cmydas1, within the terrestrial tortoises was not successful, with both primer sets amplifying monotypic loci in all species genotyped. Increasing evolutionary distance from the source species has been shown to decrease the utility of primers that amplify polymorphic loci in related organisms (Schlotterer et *al.* 1991, Ellergren et *al.* 1995). This is a likely explanation for the poor amplification of Galap primers within the turtle outgroups, and was verified by the low success rate of turtle primers that amplified polytypic loci in the Family Testudinidae. The evolutionary time scale that defines the separation of these two groups has been estimated at between 65-100 million years (see Chapter 6). Although the repeat unit itself has been maintained, there appears to be some mechanism that has resulted in the loci becoming fixed with little or no variability present.

The sequencing of the two microsatellite loci, Galap9 and Galap11, indicated that the repeat region was highly conserved across both Families. Although the basic repeat structure was

shown to be conserved, particularly in the pure $(CA)_n$ repeat unit, the motifs at both loci were punctuated with mutations that appeared to have some influence on the overall levels of observed allelic diversity. However, there did not appear to be any relationship between these interruptions and phylogenetic distance from the source species (as calculated in Chapter 6). For example, Galap9 in the more derived *Psammobates* group was characterised by high levels of allelic diversity and heterozygosity, and on sequencing was shown to contain a transition substitution within the first repeat motif at this compound locus. *Manouria impressa,* however, which has been shown to be the most basal of the extant tortoises (Chapter 6) was shown to contain a pure compound repeat unit with no transition mutations in the motif. *Geochelone nigra* is more closely related to the *Psammobates* genus than the *Manouria* spp. using genetic distance data based on mitochondrial DNA sequence.

Overall, pure repeat loci did not appear to display higher levels of heterozygosity and polymorphism than impure loci as has been shown in other published studies (Primmer et *al.* 1998). It has been proposed that longer and less degenerate microsatellite loci display higher levels of allelic diversity, thereby inferring a greater rate of mutation (Rose and Falush 1998). This has been attributed to the fact that longer repeats may be more susceptible to slipped strand mis-pairing, whereas point mutations may interrupt repeat sequences and reduce slippage (Kruglyak et *al.* 1998). Slippage is thought to be the predominant method whereby microsatellites mutate, resulting in the generation of new length alleles during DNA replication (Schlotterer and Tautz 1992).

In agreement with the study by Zhu et *al.* (2000), changes in the motif repeat numbers for Galap9 and Galap11 evolved more rapidly than other changes in the repeat region i.e. substitutions or insertions/ deletions. These changes did seem consistent with the step-wise mutation model, which is based on an increase or decrease in allele length equal to the size of the repeat motif or a multiple thereof. However, it may be necessary to increase the sample size of each species represented in the panel to further investigate the mode of evolution and directionality of these mutations within a phylogenetic context. Furthermore, this information may offer insight into the effect of repeat length on mutation rate, allelic diversity and heterozygosity within non-source populations.

Analysis of the sequenced regions for the Galap9 and Galap11 microsatellite loci indicated three mechanisms that are implicated in the generation of allelic variation at these loci. These include i) addition and deletion of repeat units ii) substitutions and indels in the

flanking regions and iii) interruptions and mutations within the repeat unit. Mutations in the flanking regions were phylogenetically informative, particularly in the older members of the group. For example the basal *Manouria* spp, *Gopherus* spp. and the *Testudo* spp., *Indotestudo* spp. and *Malacochersus* shared several phylogenetically informative sites that were absent in the other more derived genera. In addition, *Manouria* and *Gopherus* shared parsimony informative sites with turtle species, *Terrapene*, included in the study.

Intra-specific variation was also indicated. *Psammobates geometricus* sequences displayed mutations in the flanking regions of the Galap11 locus that distinguished this genus from the remaining genera within the family. Additionally, a second substitution adjacent to this pointed to some differentiation between the Ceres-Elandsberg individuals from the Worcester populations that would not have been distinguishable from size homoplasy in the genotypes (Taylor et *al.* 1999).

Based on this preliminary analysis of cross-species amplification, a panel of polymorphic markers is available for future population level studies on all species of terrestrial tortoises, although further investigation is required to develop an understanding of the process by which these loci have evolved in the Family Testudinidae. This requires additional sequencing of repeat motifs and flanking regions at the loci, and genotyping further samples for each species in order to get an indication of levels of heterozygosity, allelic diversity and the directionality of mutation, as well as the subsequent limitations on maximum and minimum allele length.

PART II: The Geometric Tortoise, *Psammobates geometricus*

The Geometric tortoise has undergone a severe decline in population numbers since the turn of the 19th century. This decline is closely correlated with the disappearance of its natural habitat - the critically endangered renosterveld (Greig and Boycott 1977). Based on current densities reported for Geometric tortoises, it is estimated that 150 000 animals have been lost over the last 200 years along with a 97% loss of their natural habitat (Baard 1993b). It is estimated that there are only between 3500 - 5000 Geometric tortoises left in the wild. Renosterveld habitat is still declining at an alarming rate despite concerted efforts for its rehabilitation and conservation. The conversion of natural renosterveld to cultivated land remains the biggest threat to the tortoises' survival. The uncontrolled spread of invasive vegetation, overgrazing, and the high frequency of fires in renosterveld remnants are further threatening the survival of many of the remaining populations.

i. Levels of Genetic Variability

Despite the great reduction in numbers for this tortoise species, they have retained appreciable levels of diversity, particularly within the remaining fragmented populations. Sampled populations displayed relatively high levels of heterozygosity and high allelic diversity across all populations. Assumptions about inbreeding and/or random mating in a population can be made if the majority of loci give consistent results across the population. However, in this study only two of the eight loci deviated significantly from Hardy-Weinberg expectations across all three populations. The deficiency of heterozygotes at these two loci could indicate the presence of non-amplifying or null alleles (Pemberton et *al.* 1995). The fact that the primers used in this study were developed in an evolutionarily distant species of Testudinidae, *Geochelone nigra*, suggests that null alleles, rather than any form of population level structuring may be responsible for the excess of homozygotes at these loci. Sequencing of the microsatellite loci at Galap9 and Galap11 did indicate a limited degree of mutational events in the primer sequences of non-source species, adding credence to the possibility of mutations in flanking regions preventing efficient binding of the primers and therefore potentially causing allelic drop-out.

The extent to which genetic variability is lost in a population undergoing a population bottleneck is complex and is affected by a number of factors. These include (i) the scale and pattern of the loss of habitat (ii) the duration of the bottleneck (iii) population numbers (iv) the presence of fine-scale genetic structure (v) time since the bottleneck and (vi) generation time (Frankel and Soulé 1981). Over longer periods of evolutionary time, the combined effects of

isolation and reduced migration would further influence this genetic variability. Garza's M statistic supports the historical evidence for a bottleneck in all three populations of Geometric tortoise. The failure of the two estimates implemented in BOTTLENECK can be attributed to their reduced ability to demonstrate an effect beyond a few generations after a bottleneck event (Luikart and Cornuet 1998). Assuming populations are in mutation/ drift equilibrium, population size estimates can be calculated from allelic data. Using $(1/(1-Hexp)^2-1)/(8*\mu)$ (Rooney et *al* 1999) and assuming a mutation rate, μ, as 2.05E-4 (Lehmann et *al* 1998), population sizes would be estimated as 5009, 4292, and 5163 individuals for Elandsberg, Ceres, and Worcester populations respectively. Since these figures are in excess of current estimates of population sizes, particularly in the Ceres and Worcester populations, it implies that the populations are not in mutation-drift equilibrium. Thus, continued reduction in genetic diversity could be expected whilst population sizes remain below these values.

ii. Population differentiation

Four alternate measures of population differentiation were utilised: a test for allele frequency heterogeneity (Roff and Bentzen 1989), *Rho* (Goodman 1997), F_{st} (Weir and Cockerham 1984) and PCA (Goudet 1999). Levels of allelic heterogeneity were significantly different at four of the eight loci studied suggesting some differentiation.

R_{st} (Stepwise Mutation Model, Kimura and Ohta 1978) and F_{st} (Infinite Allele Model, Kimura and Crow 1964) estimates assume different mutational models. R_{st} (Slatkin 1995) compares variance in allelic sizes rather than frequency and should therefore be a more appropriate measure over a longer time frame when new mutations influence the statistic. F_{st}, however, may be more reliable than R_{st} for detecting local differentiation and sub-division in recently diverged populations, where migration and genetic drift, as opposed to new mutational events, play the major role in generating population level differentiation (Slatkin 1995, Balloux and Lugon-Moulin 2002). F_{st} and *Rho* estimates were consistent in all pairwise population comparisons. In each case, F_{st} gave higher values than *Rho* with the majority of the differentiation occurring between the Elandsberg population and the Worcester population. This was verified using the genetic distance measure, $d\mu^2$. There was no significant differentiation between the Elandsberg and Ceres populations, implying high rates of historical gene flow and limited sub-population structuring. Worcester appears to be the most genetically differentiated population and was most closely associated with the Ceres population as indicated by all the population differentiation estimates.

PCA correlation gave some indication of population spatial relationships inherent in the data set as represented by allele frequency data, despite the low values obtained for population differentiation using both F_{st} and R_{st} . This spatial resolution could be a result of the differences in the variability across the microsatellite loci. For example, Galap9, Galap10, Galap11 and Galap13 showed locus specific R_{st} values of up to 0.07 with corresponding high levels of heterogeneity in frequency distributions using a Monte Carlo Simulation (Roff and Bentzen 1989). The remaining loci, however, gave low estimates of differentiation (data not shown). PCA would be sensitive to these extremes in the allele frequency data range at the respective loci and this is represented in the specific apportionment of the genetic variation and subsequent spatial arrangement in the data set.

Mitochondrial sequencing data corroborated these low levels of differentiation. However, sequencing of highly variable microsatellite loci in *P. geometricus* did imply a limited degree of differentiation between the Worcester population and that of the Ceres and Elandsberg populations, as indicated by a single transition substitution in the flanking regions of the Galap11 locus. Furthermore, limited population structuring between the three geographically separated areas was observed using Principal Component Analysis.

Geometric tortoises are known to favour low altitudes and the Cape Fold mountain ranges are thought to act as a natural barrier to migration and the associated gene flow. However, evidence from the pairwise differentiation measures, as well as the assignment test values, provide support for the hypothesis that there could be directional gene flow occurring between these fragmented populations. Alternatively, the currently observed low levels of population differentiation may be a consequence of the recent fragmentation of a historically continuous population of Geometric tortoises. These low levels of observed differentiation may also be a result of the slower rates of molecular evolution demonstrated for the Family Testudinidae (Avise et *al.* 1992).

Based on the assumption that migration may be the major contributing factor to the homogenisation of these populations, high rates of gene flow are implied between the Ceres and Worcester population and the Ceres and Elandsberg populations. This is indicated by assignment tests and *Rho*/$d\mu^2$ estimates, with significantly less migration between Worcester and Elandsberg. Evidence presented here suggests that the Ceres population may act as a natural 'source' population for the migration of individuals between the remaining fragments and, assuming the Cape Fold Mountains are not necessarily acting as a barrier to gene flow,

may be responsible for the observed levels of differentiation. Juvik (1971) reported that the Geometric tortoise populations occurring in the Ceres valley might have originated from the release of a pet population of a few individuals, and potentially introduced from elsewhere. In agreement with Baard and Mouton (1993a) the predominance of renosterveld in this region, combined with the populations high levels of observed heterozygosity and allelic diversity observed in this population, suggests that a founder event from a recent introduction is unlikely (Broders et *al.* 1999, O'Ryan et *al.* 1998).

On initial observation, the levels of differentiation between these three populations are consistent with limited structuring and, therefore, potentially high rates of gene flow. However, the presence of a large number of low frequency alleles, combined with the widespread sharing of common alleles, suggest that these populations have undergone a recent separation. Since F_{st} values are higher than R_{st}, and considering the limited mobility of the species, the minor degree of observed population differentiation is likely the result of genetic drift and recently restricted migration (Balloux and Lugon-Moulin 2002). Low rates of DNA evolution in the Testudinidae (Avise et *al.* 1992), together with the recent and extensive reduction in habitat and population numbers, would account for the low levels of structuring observed between the geographic units. Furthermore, these findings are consistent with a once continuous distribution of the species, as supported by historical records, which suggests that the renosterveld was once ubiquitous throughout the Western Cape Province. Similar results have been reported for the Amazon River turtle, *Podocnemis expansa* (Sites et *al.* 1999), where microsatellite analysis revealed high levels of heterozygosity, allelic diversity and low levels of genetic differentiation among rookeries (less than 250 Km apart), indicative of the geographic scale over which these populations are defined.

iii. Concluding remarks

Little is known about the migratory routes and behavior of *Psammobates geometricus*. Despite the fragmentation of extant populations, the species does not demonstrate a significant degree of between population differentiation to justify consideration of the remaining fragments as separate conservation units. The continued and unsustainable exploitation of the renosterveld suggests that the development of larger areas or reserves for the long-term management of this species may be required. To this end information on genetic relationships between extant populations could allow for the simulation of "natural" gene flow, or allow for the more accurate assignment of individual tortoises to suitable populations or sample groups. This would prove to be an invaluable aid in reintroduction and translocation programs, particularly in the establishment of captive breeding programs. Data on the genetic structure present in the extant populations can be used to protect against the potential hazards of inbreeding, and/or outbreeding depression, which can arise when individuals are either too closely related or alternatively share excess allelic identity by descent. These factors may have a direct impact on populations by potentially reducing reproductive fitness, which in turn could lead to widespread population crashes and local extinctions.

Table I: Cross-species amplification using microsatellite primers isolated from *Geochelone nigra*, *C. caretta* and *C. mydas*. Polymerase Chain Reactions were conducted at between 50°C - 60°C depending on the primer sequence. All alleles are scored in base pairs (n = 121). The Galap markers were classified as either a pure repeat (e.g. $(CA)_n$) motif or compound repeat motif (e.g. $(CA)_n$ $(TG)_n$) according to the sequences isolated from the source species, *G. nigra*.

ID	Species	Galap2 Compound	Galap4 Pure	Galap5 Pure	Galap9 Compound	Galap10 Pure	Galap11 Pure	Galap13 Compound	caretta	cmydas
1A	*G. gigantea*	179/179	157/157	173/173	198/198	160/172	164/166	97/97	181/181	139/139
1B	*G. gigantea*	175/175	155/157	173/173	198/200	160/172	164/166	97/97	181/181	139/149
2A	*C. angulata*	-	-	183/183	198/206	178/178	138/206	97/97	-	-
2B	*C. angulata*	-	-	173/173	198/200	162/170	144/156	103/113	-	-
2E	*C. angulata*	-	228/228	196/200	196/214	176/204	146/150	107/107	-	-
2F	*C. angulata*	-	208/208	182/192	200/202	174/202	152/166	107/107	-	-
3A	*G. carbonaria*	205/221	155/155	165/165	220/234	186/186	157/159	103/107	181/181	157/171
3B	*G. carbonaria*	221/221	145/153	165/165	198/200	168/168	157/167	-	181/181	145/157
4A	*G. chilensis*	181/191	152/152	169/173	204/210	164/182	164/168	95/109	-	-
4B	*G. chilensis*	183/197	150/152	171/175	206/206	172/172	154/162	105/107	-	-
5A	*G. denticulata*	185/193	157/157	167/167	203/219	168/174	148/158	95/95	181/181	139/145
5B	*G. denticulata*	203/213	149/151	165/165	203/225	162/174	152/154	119/119	181/181	143/143
7A	*G. elegans*	189/189	159/159	171/171	200/200	164/172	133/139	113/113	181/181	149/153
7C	*G. elegans*	185/191	157/159	169/171	220/226	166/174	135/137	-	181/181	147/147
8	*G. nigra*	193/193	151/155	181/181	203/203	164/176	154/164	137/137	181/181	145/145
9A	*G. pardalis*	185/203	151/153	185/199	210/210	-	156/178	99/99	181/181	145/145
9B	*G. pardalis*	183/205	153/155	211/265	202/212	-	156/172	99/99	181/181	145/145
9C	*G. pardalis*	175/209	153/153	181/199	202/204	-	164/166	97/97	181/181	145/145
9D	*G. pardalis*	171/175	153/155	179/223	204/206	-	162/164	97/99	181/181	145/145
9G	*G. p. babcocki*	177/185	153/153	-	202/202	-	162/168	99/119	-	-
9H	*G. p. pardalis*	183/183	151/153	181/185	200/202	154/170	174/180	99/99	-	-
10A	*G. platynota*	180/186	161/167	181/193	206/206	168/170	135/135	99/99	181/181	-
10B	*G. platynota*	184/188	161/163	183/183	206/208	166/170	137/139	81/87	-	-
11A	*G. radiata*	-	175/175	171/173	209/221	174/176	160/168	111/111	181/181	145/145
11C	*G. radiata*	-	171/173	173/173	207/215	168/178	164/166	105/111	-	-

11D	*G. radiata*	176/176	179/187	175/175	204/222	174/176	164/166	-	-	-
11E	*G. radiata*	176/176	175/187	173/175	204/224	180/206	154/168	105/105	-	-
11F	*G. radiata*	176/176	175/191	173/173	208/222	188/222	150/150	105/111	-	-
11G	*G. radiata*	176/176	175/181	173/173	194/210	168/168	150/150	-	-	-
12A	*G. sulcata*	187/191	147/149	191/195	216/230	162/166	144/144	109/109	181/181	135/135
12B	*G. sulcata*	191/191	149/149	193/193	230/230	166/166	144/144	107/109	181/181	135/135
13A	*G. yniphora*	201/201	153/157	169/171	210/210	172/174	154/158	105/105	181/181	145/145
13B	*G. yniphora*	195/199	153/157	167/167	206/208	170/172	154/158	105/105	-	-
14A	*G. elongata*	185/223	145/151	167/167	204/204	168/170	132/156	107/107	181/181	145/149
15A	*G. agassizi*	181/211	165/165	171/171	204/204	170/178	160/164	-	185/185	133/145
15B	*G. agassizi*	177/179	165/165	171/171	204/204	170/178	158/164	-	185/185	139/145
16	*G. berlanderi*	239/253	129/129	171/171	202/202	194/194	144/144	89/89	185/185	139/145
16B	*G. berlanderi*	249/251	129/129	171/171	212/214	182/198	146/146	89/89	185/185	139/145
16C	*G. berlanderi*	183/183	129/129	171/171	208/210	182/198	138/146	89/89	185/185	133/133
17A	*G. flavomarginatus*	195/195	151/171	171/171	208/208	165/181	139/143	89/89	-	-
17B	*G. flavomarginatus*	195/195	169/179	171/171	204/204	165/165	141/141	89/89	-	-
18A	*G. polyphemus*	-	149/169	171/171	202/210	164/164	144/144	89/89	185/185	139/145
18B	*G. polyphemus*	-	149/169	171/171	220/224	176/176	144/144	89/89	185/185	139/145
19B	*H. areolatus*	207/213	155/155	173/173	215/225	172/172	137/137	89/109	181/181	149/149
19C	*H. areolatus*	191/213	161/165	173/173	234/256	180/180	137/137	109/109	181/181	139/145
19E	*H. areolatus*	201/201	165/165	173/173	215/225	180/180	137/137	109/111	181/181	143/143
19F	*H. areolatus*	193/201	153/185	173/173	214/214	-	137/137	109/119	-	-
19G	*H. areolatus*	193/205	153/165	173/173	220/224	173/181	137/137	109/119	-	-
19H	*H. areolatus*	193/203	179/185	173/173	215/225	181/181	137/137	109/119	-	-
19I	*H. areolatus*	193/205	163/165	173/173	214/220	172/180	137/137	109/111	-	-
19K	*H. areolatus*	173/177	153/153	173/173	214/224	167/167	137/137	119/119	-	-
20A	*H. bergeri*	171/171	153/153	167/169	206/224	162/162	150/172	111/111	181/181	117/117
21A	*H. boulengeri*	193/213	153/155	171/173	234/238	180/180	168/170	109/109	181/181	143/143
22A	*H. femoralis*	175/175	155/163	171/183	204/218	158/162	137/201	121/121	181/181	149/149
22B	*H. femoralis*	175/175	155/159	171/171	194/194	172/180	139/217	113/113	181/181	149/149
23A	*H. signatus cafe*	177/181	151/151	195/195	208/208	158/162	145/147	121/121	181/181	147/149
23B	*H. signatus cafe*	179/183	151/153	179/185	-	158/162	-	109/109	181/181	145/153
24A	*H. signatus sign*	179/189	151/155	185/185	200/200	162/168	-	121/121	181/181	143/143
24B	*H. signatus sign*	179/179	151/153	185/185	238/244	162/178	154/154	119/119	181/181	139/151
25A	*I. Travancorica*	187/207	143/153	165/167	200/200	166/168	154/160	-	181/181	149/153
26A	*I. Forsteni*	219/229	151/151	165/167	202/202	164/164	139/139	95/109	187/187	158/155
26B	*I. Forsteni*	-	-	-	218/236	-	139/155	-	-	-
27B	*K. belliana*	199/199	125/125	181/181	-	162/162	154/156	-	187/187	147/149

27C	*K. belliana*	191/191	125/125	181/181	206/228	162/162	156/168	123/123	187/187	139/147
27D	*K. belliana*	191/199	125/125	181/181	206/206	162/162	156/168	123/123	187/187	147/147
29A	*K. belliana*	151/189	155/155	-	202/202	170/170	154/154	119/119	187/187	-
30A	*K. b. spekii*	197/205	-	-	202/218	164/170	187/187	119/119	187/187	-
31A	*K. erosa*	179/195	157/157	-	249/253	162/162	150/150	109/109	187/187	117/117
32A	*K. homeana*	195/235	123/127	177/177	200/216	164/164	150/156	121/121	187/187	145/145
32B	*K. homeana*	207/227	-	177/177	200/200	164/164	140/182	89/121	187/187	-
33A	*K. natalensis*	191/195	127/127	175/175	200/200	168/168	132/160	-	187/187	149/149
34A	*M. tornieri*	179/181	155/183	164/164	-	168/168	131/139	164/164	181/181	145/145
34B	*M. tornieri*	175/179	165/185	164/164	-	162/162	133/133	162/162	181/181	143/145
34D	*M. tornieri*	179/179	159/163	164/164	-	-	133/133	-	-	-
36A	*M. emys emys*	197/197	103/107	181/181	204/217	164/166	158/170	89/99	159/159	155/179
36C	*M. emys emys*	179/179	103/107	169/169	216/230	166/166	154/172	99/99	159/159	147/147
37A	*P. geometricus*	183/197	155/169	169/169	-	170/174	154/164	99/99	-	161/161
37B	*P. geometricus*	197/197	155/155	169/169	224/236	168/168	170/182	-	-	145/145
37C	*P. geometricus*	175/187	161/165	169/169	226/226	204/222	164/182	99/117	-	155/161
37D	*P. geometricus*	233/233	-	169/169	226/240	204/220	170/172	99/121	-	-
37E	*P. geometricus*	179/233	167/169	169/169	214/244	218/218	182/182	115/119	-	-
37F	*P. geometricus*	185/235	157/171	169/169	216/216	194/218	150/150	99/119	-	-
37G	*P. geometricus*	179/183	159/169	169/169	232/234	200/222	162/164	109/119	-	-
37H	*P. geometricus*	179/185	155/155	169/169	200/224	198/202	164/182	99/115	-	-
38A	*P. oculiferus*	181/233	157/157	169/169	224/224	204/204	140/148	97/97	-	153/153
38B	*P. oculiferus*	183/183	169/169	169/169	198/226	184/186	146/146	97/97	187/187	153/153
39A	*P. tentorious* spp.	173/173	169/169	169/169	198/198	184/184	148/148	101/103	187/187	155/155
39B	*P. tentorious* spp.	171/171	151/173	169/169	202/202	178/200	154/168	103/109	187/187	157/157
39C	*P. tentorious* spp.	189/199	165/183	169/169	196/196	170/170	158/164	103/109	187/187	143/157
39D	*P. tentorious* spp.	193/201	165/175	169/169	192/200	160/162	144/156	103/103	187/187	157/157
39E	*P. tentorious* spp.	-	167/183	169/169	188/192	166/166	152/164	103/103	-	-
40A	*P. t. verroxii*	181/195	183/189	169/169	188/188	168/168	150/172	95/95	187/187	157/157
40B	*P. t. verroxii*	193/193	183/189	169/169	202/202	166/166	158/164	95/95	187/187	157/157
40C	*P. t. verroxii/*	243/243	-	169/169	190/190	170/170	150/150	119/119	-	-
42A	*P. arachnoides*	181/195	156/158	172/172	190/214	160/184	146/148	-	181/181	155/155
42B	*P. arachnoides*	201/201	-	172/172	186/190	172/192	154/176	-	-	
43A	*P. planicauda*	197/197	153/153	179/179	198/198	160/170	148/148	111/111	181/181	131/155
43B	*P. planicauda*	-	-	179/179	198/198	197/197	148/148	111/111	-	-
44A	*T. g. graeca*	189/193	151/151	167/167	210/210	180/196	145/147	-	181/181	151/173
46A	*T. g. terristris*	163/163	151/151	167/167	204/204	164/164	159/171	-	181/181	159/173
47A	*T. hermanni*	197/203	147/151	167/167	204/204	164/164	137/137	109/111	181/181	171/171

47B	*T. hermanni*	185/185	151/155	167/167	202/220	170/178	135/137	109/111	181/181	-
48A	*T. horsfieldi*	178/198	147/147	167/167	164/164	158/166	135/137	109/111	181/181	137/145
49A	*T. kleinmanni*	191/191	153/157	167/167	-	164/166	156/156	-	181/181	137/137
49B	*T. kleinmanni*	201/201	163/163	167/167	192/192	164/164	154/160	-	181/181	137/137
49C	*T. kleinmanni*	-	169/179	167/167	-	-	-	-	181/181	155/155
50A	*T. marginata*	191/191	159/159	167/167	186/192	162/162	154/160	-	181/181	141/145
50B	*T. marginata*	193/193	157/157	-	-	-	-	-	-	-
50C	*T. marginata*	191/191	151/151	167/167	156/160	162/162	-	-	181/181	159/173
51A	*C. insculpta*	-	251/251	181/187	205/205	-	165/165	-	-	161/161
52A	*D. mawii*	189/191	145/145	181/187	159/163	178/178	139/139	101/101	103/103	163/163
53A	*L. kempi*	177/177	141/149	98/98	157/159	104/104	139/139	97/97	201/201	133/133
54A	*T. ornata*	-	-	181/181	126/136	-	150/152	97/97	209/209	141/155
55A	*T. coahuila*	189/189	151/151	181/181	-	-	148/152	95/97	209/209	149/149
55C	*T. coahuila*	189/193	131/153	-	126/142	124/166	-	97/97	209/209	129/143
56A	*T. triunguis*	-	123/127	173/173	106/110	-	152/152	120/120	209/209	133/141
56B	*T. triunguis*	-	-	174/174	206/206	-	148/148	120/120	-	-
60A	*M. impressa*	219/221	145/145	189/189	205/205	145/167	154/160	98/98	243/243	133/133
60B	*M. impressa*	215/237	145/145	189/189	185/185	161/167	-	98/98	243/243	
61A	*C. caretta*	179/179	141/157	189/193	198/200	-	-	97/97	165/165	165/169

- not scored

Table II: The conservation of the microsatellite repeat unit across representatives of species from the Testudinidae and the Emydidae. Galap9 was isolated from *Geochelone nigra* as a compound repeat locus $(CT)_n(CA)_n$, whilst Galap11 was isolated as a pure $(CA)_n$ repeat unit. The repeat unit itself was conserved across individuals of the same species and as a result only a single motif sequence for each species is reported.

Species ID	Species Name	Galap9 repeat unit	Galap11 repeat unit
1A	*G. gigantea*	$(CT)_9$ $(CA)_{10}$	$(CA)_{22}$
2A	*C. angulata*	$(CT)_9$ $(CA)_{13}$	$(CA)_{16}$
3A	*G. carbonaria*	$(CT)_4$ CG $(CT)_4$ CG $(CT)_4$ $(CA)_8$	$(CA)_8$ TA $(CA)_{12}$
4A	*G. chilensis*	$(CT)_{13}$ $(CA)_{12}$	$(CA)_{19}$
5A	*G. denticulata*	$(CT)_7$ CC $(CT)_8$ $(CA)_{17}$	$(CA)_{14}$
7A	*G. elegans*	$(CT)_{10}$ TT (CT) $(CA)_9$	$(CA)_{10}$
8	*G. nigra*	$(CT)_8$ $(CA)_9$	$(CA)_{22}$
9B	*G. pardalis*	$(CT)_{12}$ (CA) CC $(CA)_{11}$	$(CA)_5$ $(CG)_2$ $(CA)_{21}$
10A	*G. platynota*	$(CT)_9$ TT (CT) $(CA)_7$ TA $(CA)_5$	$(CA)_8$
11A	*G. radiata*	$(CT)_{12}$ $(CA)_{17}$	$(CA)_{21}$ GA
12A	*G. sulcata*	$(CT)_9$ $(CA)_{21}$	$(CA)_2$ CT $(CA)_9$
14A	*I. elongata*	$(CT)_3$ GT $(CT)_3$ GT $(CT)_8$ $(CA)_8$	$(CA)_{26}$
15A	*G. agassizi*	$(CT)_6$ $(CA)_{36}$ TACAAG $(CA)_4$	$(CA)_{20}$
16B	*G. berlanderi*	$(CT)_7$ GTCG $(CA)_8$ TACAAG $(CA)_4$	$(CA)_{13}$
17B	*G. flavomarginatus*	$(CT)_{10}$ CG $(CA)_8$ CG $(CA)_4$	$(CA)_{11}$
18A	*G. polyphemus*	$(CT)_{11}$ CG $(CA)_8$ CG $(CA)_4$	$(CA)_{11}$
19B	*H. areolatus*	$(CT)_{14}$ $(CA)_{13}$	$(CA)_4$ TA $(CA)_4$
21A	*H. boulengeri*	$(CT)_{14}$ $(CA)_{22}$	$(CA)_{15}$ CG (CA) CG $(CA)_6$
22B	*H. femoralis*	$(CT)_6$ CA CTCACT $(CA)_8$	$(CA)_7$ GA $(CA)_2$
23A	*H. signatus cafer*	$(CT)_4$ C $(CT)_{12}$ $(CA)_{26}$	$(CA)_{28}$
24B	*H. signatus signatus*	$(CT)_{22}$ $(CA)_{14}$ TA $(CA)_2$	$(CA)_{10}$ CG $(CA)_5$
25A	*I. travancore*	$(CT)_3$ GT $(CT)_8$ $(CA)_8$	-
26C	*I. Forstenil*	$(CT)_3$ GT $(CT)_7$ $(CA)_8$	$(CA)_9$ GA
27B	*K. belliana*	$(CT)_{17}$ $(CA)_{12}$ ACA	$(CA)_{17}$
32A	*K. homeana*	-	(TA) $(CA)_{12}$
34A	*M. tornieri*	-	$(CA)_{10}$
36A	*M. emys emys*	$(CT)_{11}$ $(CA)_{17}$	$(CA)_8$ TA
37E	*P. geometricus*	$(CT)_{16}$ TT $(CA)_{19}$	$(CA)_{19}$
37H	*P. geometricus*	$(CT)_{18}$ TT $(CA)_{13}$	$(CA)_{26}$
38B	*P. oculiferus*	$(CT)_{12}$ $(CA)_{21}$	$(CA)_{13}$
39B	*P. tentorius*	$(CT)_5$ GT $(CT)_3$ GT $(CT)_4$ $(CA)_7$	$(CA)_{17}$
42A	*P. arachnoides*	$(CT)_2$ $(CA)_{10}$	$(CA)_{13}$
43B	*P. planicauda*	$(CT)_8$ $(CA)_{11}$	$(CA)_{14}$
46A	*T. graeca terristris*	$(CT)_{12}$ CA CTCACT $(CA)_6$	$(CA)_{19}$
47A	*T. hermanni*	$(CT)_3$ GT $(CT)_3$ GT $(CT)_7$ $(CA)_7$	$(CA)_8$
48C	*T. horsfieldi*	$(CT)_3$ GT $(CT)_9$ GT $(CT)_6$ TT $(CA)_8$	$(CA)_8$
49A	*T. kleinmanii*	$(CT)_6$ $(CA)_{29}$	$(CA)_7$ TA $(CA)_{11}$
50A	*T. marginata*	$(CT)_4$ $(CA)_{11}$	$(CA)_{11}$ CGCAGA
54A	*T. ornata*	(TA) $(CA)_2$ TA $(CA)_5$	$(CA)_{10}$ TA $(CA)_8$
55A	*T. coahuila*	-	$(CA)_{10}$ TA $(CA)_{11}$
60A	*M. impressa*	$(CT)_9$ $(CA)_9$ TA $(CA)_4$	$(CA)_8$ TA

CHAPTER 8

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