The phylogeography of the southern rock agama (*Agama atra*) in the Cape Fold Mountains, South Africa

by

Belinda Swart

Department of Zoology, Stellenbosch University, South Africa

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Supervisor: Prof. C.A. Matthee

Co-supervisor: Dr. K.A. Tolley

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature: .....…..... Date: .....

Abstract

An understanding of the phylogeography and evolutionary processes involved in speciation is essential for the conservation and management of any particular species. To investigate the phylogeographic patterns in *Agama atra* from the Cape Fold Mountains (CFM), 98 individuals from 38 geographically close localities were analysed. In addition, to understand the phylogeographic associations between the CFM populations and the rest of Southern Africa, 18 specimens from 12 localities outside the CFM were also included. A total of 988 characters derived from two mitochondrial DNA fragments (control region and ND2) revealed 59 distinct haplotypes in the CFM. Parsimony, Bayesian and maximum likelihood analyses revealed four distinct clades associated with geography within the CFM. These clades were supported by a haplotype network and were defined as the Cape Peninsula clade, the Limietberg clade, the northern CFM clade and the central CFM clade. Analysis of molecular variance confirmed the high degree of genetic structure within the CFM, with more than 75% of genetic variation found among the geographic areas. SAMOVA and nested clade analysis (NCA) suggest that the central CFM clade may be more diverse than detected by the networks and the phylogenetic analyses. The processes that caused the four distinct genetic groups in the CFM are not yet clear. Using a speculative molecular clock estimate, the main cladogenesis of *A. atra* within the CFM took place, approximately ~6.5 - 9 MYA. This dating coincides well with the documented Miocene-Pliocene climate fluctuations which might have contributed towards the isolation among lineages. The genetic structure found in *A. atra* is also markedly congruent with what has been found in other taxa such as

Mesamphisopus spesies, *Potamonautes brincki,* and *Pedioplanis burchelli* and this would further support vicariance as a main isolating factor here.

Opsomming

'n Goeie begrip van die filogeografie en die evolusionêre gebeurtenisse wat verband hou met spesiasie is belangrik vir die bewaring en bestuur van enige spesie. Om die filogeografiese patrone in *Agama atra* van die Kaapse Plooiberge (KPB) te ontleed, was 98 individue van 38 nabygeleë lokaliteite geanaliseer. Tesame met bogenoemde monsters was 18 individue van 12 lokaliteite van buite die KPB ook geanaliseer om die filogeografiese verwantskappe tussen die KPB bevolkings en die res van Suidelike Afrika te ondersoek. Uit 'n totaal van 988 karakters verkry uit twee mitochondriale DNS fragmente (die kontrole gebied en ND2) is 59 haplotipes gevind. Parsimonie en modelgebaseerde filogenetiese analises dui daarop dat vier groepe geassosieer met geografie binne die KPB voorkom. Die groepe word geondersteun deur 'n haplotipe netwerk en word soos volg gedefinieer: 'n Kaapse Peninsula groep, 'n Limietberg groep, 'n noordelike KPB groep en 'n sentrale KPB groep. Analises van molekulêre variansie (AMOVA) bevestig die hoë graad van genetiese struktuur binne die KPB, met meer as 75% genetiese variasie gevind tussen die geografiese areas. SAMOVA en gesetelde groep analises ("NCA") stel voor dat die sentrale KPB groep dalk meer variasie vertoon as wat die netwerk en filogenetiese analises vertoon. Die prosesse wat die vier genetiese groepe tot stand gebring het is nog nie bekend nie. Volgens 'n spekulatiewe molekulêre klok berekening het die hoof kladogenese van *A. atra* binne die KPB ongeveer ~6.5 - 9 miljoen jaar (MJ) gelede plaasgevind. Hierdie datering stem goed ooreen met die gedokumenteerde Mioseen-Plioseen klimaat veranderinge wat isolasie van die groepe kon bewerkstellig het. Die genetiese struktuur van *A. atra* in the KPB is ook gevind in ander taksa soos

Mesamphisopus spesies, *Potamonautes brincki,* en *Pedioplanis burchelli* en bevestig dus dat vikariansie hier die hoof faktor vir isolasie is.

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List of Contents

	Page
Abstract	2
Opsomming	4
Acknowledgments	6
List of Contents	7
List of Tables	9
List of Figures	11
Chapter 1: Introduction	15
1.1 Phylogeography	15
1.2 Molecular markers	17
1.3 *Agama atra* natural history	18
1.4 *Agama atra* phylogeography within Southern Africa	20
1.5 Fine scale population structure of *Agama atra* in the Cape Fold Mountains	21
1.6 Molecular markers used in the present study	23
1.7 Aims	24
Chapter 2: Materials and methods	25
2.1 Samples	25
2.2 Molecular techniques	25
2.3 Data analyses	28
2.3.1 Haplotype networks	28
2.3.2 AMOVA analyses	29

2.3.3 SAMOVA 30

2.3.4 Isolation by distance 31

2.3.5 Nested clade analysis 31

2.4 Estimation of divergence times 32

2.5 Phylogenetic methods 33

Chapter 3: Results 35

3.1 Control region 35

3.2 ND2 39

3.3 Combined mtDNA data set 42

3.4 SAMOVA analysis and isolation by distance 45

3.5 Nested clade analysis 48

3.6 Estimation of divergence times 50

3.7 The CFM association with the rest of Southern Africa 51

Chapter 4. Discussion 56

4.1 Comparisons of mtDNA regions 56

4.2 *Atra* phylogeography 57

4.3 High genetic diversification 64

4.4 Conservation implications 65

References 67

Appendix A 84

Appendix B 87

List of Tables

Page

Table 1 The average uncorrected sequence divergences among the four mtDNA clades, Pretoria, the northern-central Southern Africa and *A. knobeli* for CR (below diagonal) and ND2 (above diagonal). Standard errors are given in brackets. 37

Table 2. AMOVA results of the CR. 38

Table 3 F_{ST} estimates (below diagonal) and Φ_{ST} values (above diagonal) among the four *A. atra* clades for the CR. Significance values (*p*) are given in brackets. 38

Table 4 The molecular diversity indices of the four clades for CR. Standard errors are given in brackets. 39

Table 5. AMOVA results of ND2. 41

Table 6 F_{ST} estimates (below diagonal) and Φ_{ST} values (above diagonal) among the four *A. atra* clades for ND2. Significance values (*p*) are given in brackets. 41

Table 7 The molecular diversity indices of the four clades for ND2. 95% CI are given in brackets.

41

Table 8 AMOVA results of the combined dataset.

44

Table 9 F_{ST} estimates (below diagonal) and Φ_{ST} values (above diagonal) among the four *A. atra* clades for the combine dataset. Significance values (*p*) are given in brackets.

44

Table 10 The molecular diversity indices of the four clades for the combined dataset. Standard errors are given in brackets.

44

Table 11 Results of SAMOVA analyses. Significance based on 100 simulations, where $*p < 0.05$. The geographic partitioning which show the largest increase in F_{CT} is indicated in bold.

46-47

Table 12 Inference chain based on results of geographical dispersion analysis. Only those clades that resulted in a rejection of the null hypothesis are included in this table.

49

Table 13 Estimates of divergence times for three different evolutionary rates. The percentage pairwise genetic deference among the four major *A. atra* clades is given in the first column.

50

List of Figures

Page

Figure 1 The distribution map of *A. knobeli* and the two distinct *A. atra* groupings found by Matthee & Flemming 2002. (Reproduced directly from Matthee & Flemming 2002).

21

Figure 2 Map of sampling localities of *Agama atra* and *A.knobeli* used in this study. *A. knobeli* was sampled at a = Aus and *A. atra* individuals outside the CFM were sampled at b = Eksteenfontein, c = Augrabies, d = Postmansburg, e = Vaalputs, f = Nieuwoudtville, g = Grahamstown, h = Transkei, i = Bloemfontein, j = Qwa-Qwa, k = Pretoria, l = Beaufort West. The CFM is indicated by the black box and the numbers herein represent: 1 = northern Cederberg, 2 = Cederberg, Sneeukop, 3 = southern Cederberg, 4 = Turretpeak, 5 = Kaggakamma, 6 = Groot winterhoek, 7 = Waboomsberg, 8 = Thumas hut, 9 = Bainskloof, 10 = Limietberg, 11 = Jonkershoek 12 = Gordons Bay, 13 = Devilspeak, 14 = Silvermine, 15 = Scarborough, 16 = Saldomsdam, 17 = Steenboksberg, 18 = Riviersonderendberg, 19 = Robertson, 20 = Keeromsberg, 21 = Witteberg, 22 = Anysberg, 23 = Tradouw Pass, 24 = Klein Swartberg, 25= Die Hel, 26 =

Attakwa/Robertsonspas, 27 = Hartenbos, 28 = Outeniqua, 29 = Millwood, 30 = Tsitsikamma, 31= Baviaanskloof, 32 = Hudsonvale, 33 = Kareedouw, 34 = Cockscomb, 35 = Elandsberg, 36 = Lady Slipper, 37 = Port Elizabeth, 38 = Suurberg.

26

Figure 3 Control region median-joining network obtained for 36 *A. atra* haplotypes. The four *A. atra* clades, Cape Peninsula clade, northern CFM clade, Limietberg clade and central CFM clade, are indicated by the broken lines. Branch lengths longer than one step are indicated on the branches and red circles indicate intermediate missing haplotypes as suggested by Network

36

Figure 4 Geographic distribution of the four *A. atra* clades within the CFM. The colours correspond to those in Fig. 3 with blue squares indicating the Cape Peninsula clade, the yellow dots represent the Central Cape Fold clade, the green squares correspond to the Northern Cape Fold clade and the pink squares show the localities of the Limietberg clade.

36

Figure 5 ND2 median-joining network obtained for 45 *A. atra* haplotypes. The four *A. atra* clades, Cape Peninsula clade,

northern CFM clade, Limietberg clade and central CFM clade, are indicated by the broken lines. Branch lengths longer than one step are indicated on the branches and red circles indicate intermediate missing haplotypes as suggested by Network.

40

Figure 6 Combined median-joining network obtained for 45 *A. atra* haplotypes. The four *A. atra* clades, Cape Peninsula clade, northern CFM clade, Limietberg clade and central CFM clade, are indicated by the broken lines. Branch lengths longer than one step are indicated on the branches and red circles indicate intermediate missing haplotypes as suggested by Network.

43

Figure 7 Geographic distribution of the six *A. atra* populations identified by SAMOVA within the CFM.

47

Figure 8 Scatter plot showing the lack of isolation by distance among the CFM *A. atra* sampling sites.

48

Figure 9 The nesting design inferred only from the central CFM network for *A. atra*. Each line in the network represents one mutational change. Filled circles represent missing

haplotypes. The number inside each block indicates the nesting level.

49

Figure 10 A parsimony phylogram for the 61 *A. atra* haplotypes and the additional samples from the rest of Southern Africa. Individuals outside the CFM and still within the Southern-eastern Southern African clade are indicated by a black square. Bootstrap support values are indicated above nodes.

53

Figure 11 Bayesian topology for Southern Africa *A. atra*. Individuals outside the CFM and still within the Southern-eastern Southern African clade are indicated by a black square. Significantly supported nodes ($\geq$0.95 posterior probability) are indicated by the an asterisk.

54

Figure 12 A maximum likelihood phylogram for *A. atra*. Individuals outside the CFM and still within the Southern-eastern Southern African clade are indicated by a black square. The values above the branches indicate bootstrap support higher than 50% for the respective nodes.

55

Chapter 1: Introduction

1.1 Phylogeography

Identifying patterns of genetic variation within and among populations, and subsequently attempting to explain the variation, is of great importance to the global conservation of biodiversity. In identifying the processes influencing the evolution of taxa in a certain geographical area, hypotheses can be formulated regarding the patterns of genetic diversity. These hypotheses can be tested and the results obtained from these studies will ultimately lead to a better understanding of how certain historic events may have influenced populations (Nielson *et al.* 2001). Ultimately this information will enhance our ability to make predictions regarding natural ecosystem functioning and this in turn will allow for more efficient conservation planning in future.

Contemporary and historical processes of genetic drift, gene flow, and migration are known to determine the distribution of genetic variation among populations within species (Slatkin 1987). Phylogeography integrates information about the genealogies and their geographical distribution to make conclusions about historical patterns of gene flow (Avise 1994). Phylogeographic studies can thus greatly enhance our present understanding of these historical processes. In addition co-distributed species often reflect similar phylogeographic structures (Avise 2000; Ditchfield 2000; Sullivan *et al.* 2000; Willis & Whittaker 2000; Arbogast & Kenagy 2001; Branch *et al.* 2003). These congruent phylogeographic structures have been documented in Southern Africa in the arid north-western region of South Africa where at least four studies indicated similar disconcordant

patterns in genetic diversity across the Knersvlakte: *A. atra* (Matthee & Flemming 2002), *Pronolagus rupestris* (Matthee & Robinson 1996), *Pachydactylus* species (Lamb & Bauer 2000) and *Miniopterus schreibersii* (Miller-Butterworth *et al.* 2003). Because congruent phylogeographic patterns among multiple taxa (with different life histories) might be indicative of large scale genetic breaks it has important conservation implications for this region.

Five general phylogeographic categories were proposed by Avise *et al.* (1987):

1) genetically distinct populations associated with separate geographic regions;
2) discontinuities in gene phylogeny that are not associated with spatial separation;
3) continuity in gene phylogeny with spatial separation;
4) continuity in gene phylogeny with no spatial separation; and
5) continuity in gene phylogeny with partial spatial separation.

Although these categories are probably too exact, and in a way outdated in the modern era of statistical phylogeography (Knowles & Maddison 2002; Knowles 2004), they do provide a rough description that might be valuable for comparisons among taxa. It is thought that category 1 occurs more commonly in nature than the others (Avise *et al.* 1987). For *Agama* this pattern can also be seen in *A. atra* mentioned above (Matthee & Flemming 2002) and *A. impalearis* found throughout Morocco (Brown *et al.* 2002). The Atlas Mountains divide *A. impalearis* populations into two distinct clades; a north-west clade and a south-east clade. These clades are morphologically indistinguishable due to the ecologically similar habitats occurring on both sides of the Atlas Mountains.

There are many factors that can contribute to population subdivision, amongst others: strong territoriality in social structure – Matthee & Robinson 1999; presence of physical or ecological barriers – Gifford *et al.* 2004; habitat choice - Branch *et al.* 2003; gender-biased patterns of dispersal - Rassmann *et al.* 1997; Kerth *et al.* 2002. These aside, Avise (1994) regarded the mobility of the organism, fragmentation of the environment, and long-term separation of historical populations as perhaps the most important influences on phylogeographic patterns. It is thus likely that local adaptations occur in populations exhibiting minimal migration among populations/regions (Schluter 2000). For example, species with low mobility, such as some reptiles, generally have a well-defined spatial genetic structure (Poulakakis *et al.* 2003; Crochet 2004), whereas highly mobile species, like some mammals, generally show little spatial differentiation (Burland & Worthington 2001; Newton *et al.* 2003). In addition restriction to certain substrates may give rise to geographical isolation of populations, resulting in high species diversity (Poynton & Broadley 1978).

1.2 Molecular markers

In sexually reproducing organisms, genomes can be regarded as permanent records containing information that will reflect vicariance events, population size changes, and current and historic gene flow patterns among individuals of a population/s (Avise 1994; Hillis *et al.* 1996).

Mitochondrial DNA (mtDNA) was the initial, and still is the most frequently used, molecular marker in phylogeographic studies. Although mtDNA is a singly-linked gene system, it has several advantages over nuclear loci: they are

maternally inherited with a lack of recombination and have a simple sequence organization (Macaulay *et al.* 1999). In addition, nucleotide substitutions occur at a rapid pace, and in the absence of recombination, allow for the detection of recently diverged lineages (Avise *et al.* 1987). The popularity as an evolutionary marker for phylogeographic studies is enforced by the fact that the analyses of mtDNA sequence data are better understood than those of alternative nuclear markers, such as data derived from microsatellites. In addition, mitochondrial DNA genes/fragments show extensive variations in evolutionary rates, making it useful over a large span of divergences (Lopez *et al.* 1997; Pesole *et al.* 1999; Avise 2000).

Statistical analysis of mtDNA data has led to several advantages within population genetic studies: a) it provides good resolution to detect population differentiation (Costello *et al.* 2003; Small *et al.* 2003); b) it can be used to distinguish between habitat-dependent selection regimes and historical fragmentation, isolation-by-distance and colonisation effects (Macey *et al.* 1998; Hurwood & Hughes 2001; Branco *et al.* 2002); and c) in many instances the data can be used to estimate rough divergence times among lineages/clades and also to infer a timescale on the coalescence of haplotypes (Griswold & Baker 2002; Kotlik & Berrebi 2002).

1.3 *Agama atra* natural history

The southern rock agama is endemic to Southern Africa and has a widespread distribution from the Cape, northwards to southern Namibia, and east to KwaZulu- Natal, and extending across the Great Escarpment into Mpumalanga

(Branch 1998; Fig 1). *Agama atra* shows a well-developed social structure (Bruton 1977) with hierarchies formed by both sexes. Female territories are smaller than that of males and several female territories are overlapping with the larger male territories. The species generally occurs on rocky areas throughout its range and is not found in areas with dense vegetation (Burrage 1974).

Habitat-specific animals such as the saxicolous species might be restricted in their movement due to unsuitable habitat between two rocky outcrops. The patchiness of the rocky areas may isolate populations and consequently result in high genetic differentiation among geographically close populations. Rocky outcrops have frequently been documented as refuges to mountain-dwelling species (Crochet *et al.* 2004) and in Southern Africa several studies have found distinct evolutionary lineages for vertebrates occurring on disjunct rocky habitats (*e.g*. Prinsloo & Robinson 1992; Jacobsen 1994; Matthee & Robinson 1996; Bauer 1999; Matthee & Flemming 2002). Bauer (1999) suggested that the pattern of cladogenesis of Southern African rock-dwelling geckos has been strongly influenced by limited dispersal among the fragmented mountain habitats. However, most often the mountain habitat by itself is much older than the separation among clades/populations (Prinsloo & Robinson 1992; Matthee & Robinson 1996; Matthee & Flemming 2002; Crochet *et al.* 2004). The isolation among lineages is thus often the result of a combination of factors, such as limited dispersal coupled to palaeo-climatic climate changes, that caused animals to be confined to refugia (Prinsloo & Robinson 1992; Matthee & Robinson 1996; Matthee & Flemming 2002; Crochet *et al.* 2004; Daniels *et al.* 2004).

1.4 *Agama atra* phylogeography within Southern Africa

Based on size and reproduction attributes *A. atra* can be divided into two geographical groupings (Mouton & Herselman 1994). The first group is found in the north-western region of the Northern Cape Province and comprises larger size lizards, with a long breeding season. The second group occurs in the southern and eastern regions of South Africa and the lizards are smaller, with a shorter breeding season (Flemming 1996). The distributions of *A. atra* subspecies, *A. a. knobeli* and *A. a. atra* correspond loosely with these two groupings (Branch 1998). In a previous taxonomic investigation into the phylogeographic structure of *A. atra* the existence of these groupings were confirmed and in fact three mtDNA clades were detected (Matthee & Flemming 2002). The first clade extends over southern Namibia, the second is restricted to the arid north and central regions of South Africa and the third occurs in southern and eastern regions of South Africa (see Fig. 1). The authors suggested that the southern Namibian clade be recognized as a distinct species (*A. knobeli*) and it will be treated as such in the present study. The taxonomic rank of the north-central clade and the south-eastern clade is not yet clear, but haplotypes belonging to these clades are reciprocally monophyletic. A congruent pattern with other saxicolous taxa such as *Pronolagus* (Matthee & Robinson 1996) and *Pachydactylus* (Lamb & Bauer 2000) suggests vicariance as the main driving force behind the genetic isolation.

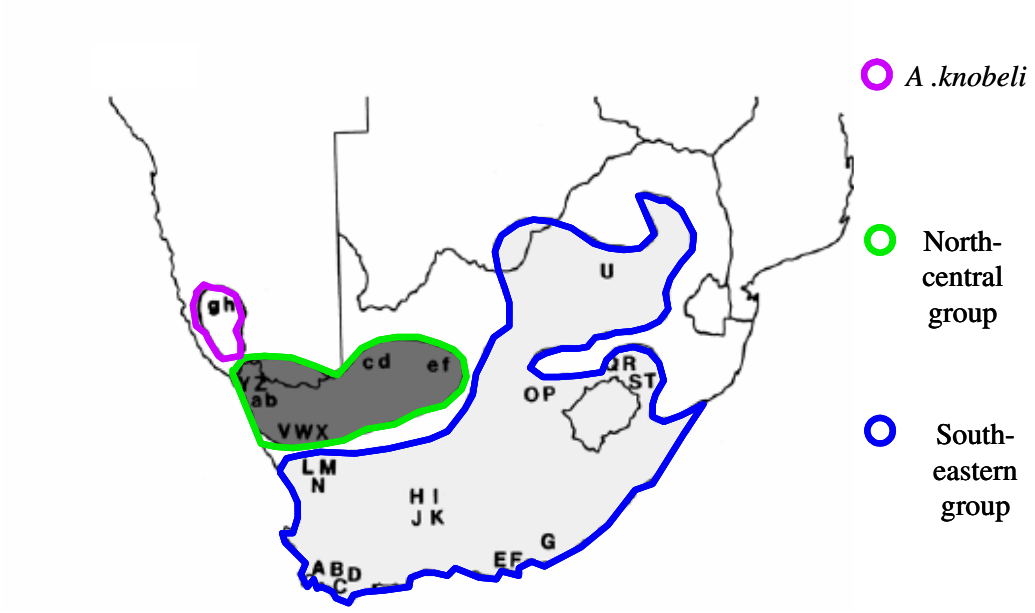


Figure 1. The distribution map of *A. knobeli* and the two distinct *A. atra* groupings found by Matthee & Flemming (2002). (Reproduced directly from Matthee & Flemming 2002).

1.5 Fine scale population structure of *Agama atra* in the Cape Fold Mountains

A previous study by Matthee and Flemming (2002) was based on a few populations sampled throughout the range. It was thus not possible for these authors to identify any fine-scale structuring influencing genetic isolation among lineages. In an attempt to better identify factors driving the evolution of the species, a more dense sampling approach was required. The present study thus only focused on individuals belonging to the south-eastern clade as described by Matthee and Flemming (2002). In this clade the authors indicated that populations showed isolation by distance among sampling areas but, more importantly, there were no shared haplotypes among populations.

The south eastern clade mainly span the Cape Floristic Region (CFR) which is one of six floral kingdoms and thus an important global "repository of biodiversity" (Cowling & Holmes 1992; Myers *et al.* 2000). This region has the highest known levels of local plant endemism outside tropical forests (Cowling & Holmes 1992; Myers *et al.* 2000). Linder (2003) suggests that the high degree of floral endemism in this region is the consequence of unique island-style habitat patches. These patches also differ in amongst other aspects, climate, soil and topography, from the rest of Southern Africa. Furthermore, floral speciation and differentiation in this region can be attributed to habitat fragmentation and restriction associated with climatic fluctuation during the Pliocene and Pleistocene (Linder *et al.* 1992; Midgley *et al.* 2001; Richardson *et al.* 2001; Linder 2003). Despite the great interest in the flora of the CFR, many aspects of the biogeography and evolution of the region's fauna remain poorly understood (Deacon 1983; Linder 2003).

The Cape Fold Mountains (CFM) represents a series of mountain belts that extend parallel to the continental margin, and are an integral element of the CFR. The CFM is the centre of endemic mammal richness within South Africa and also has the highest restricted range of species richness (Gelderblom & Bronner 1995). This mountain range is also inhabited by a unique collection of herpetofauna, comprising no fewer than 186 currently recognized species (28% of which are endemic, Baard *et al.* 1999). The high level of endemism coupled to the complex topography provide a potentially valuable system for studying evolutionary processes driving genetic differentiation in Southern Africa (see Linder 2003). Although the spatial patterns of genetic and phenotypic variation within and

among reptiles in the CFM are not well documented, fragmented distributions have thus far been recorded in taxa such as *Arthroleptella* species (Channing *et al.* 1994), *Pedioplanis burchelli* (Makokha 2004) and *Bradypodion* species (Tolley *et al.* 2004).

1.6 Molecular markers used in the present study

To explore the fine-scale phylogeographical patterns within *A. atra* from the CFM, two genes were sequenced: the mitochondrial gene ND2 (subunit 2 of NADH dehydrogenase), and the hypervariable region I of the mitochondrial control region (CR). The ND2 gene has proven useful for resolving the phylogeographical relationships among lizard species (e.g. Brown *et al.* 2002; Townsend & Larson 2002; Tolley *et al.* 2004; Matthee *et al.* 2004) and primers were based on Macey *et al.* (1997 a, b). When first described, the CR was believed to illustrate the fastest rate of evolution in the mitochondrial genome (Brown *et al.* 1979) and it is thus widely used for intraspecific studies (*e.g.* Hirota *et al.* 2004; Ravaoarimanana *et al.* 2004; Winney *et al.* 2004). Although very few studies on lizards have used CR, Brehm (2003) suggested that CR could be useful to resolve intraspecific phylogenies and this marker was thus also included.

1.8 Aims

The main aim of this study was to expand the published work of Matthee and Flemming (2002) and to investigate the fine scale phylogeographic structure of *A. atra* within the CFM. Apart from only focusing on the CFM, some additional *A. atra* representatives from outside the CFM were included to act as reference for the evolutionary interpretation. Knowledge obtained from this will potentially enhance our current understanding of the population genetic processes driving evolution in this taxon and the results obtained from this study will also provide additional evidence of potential vicariance in the region.

Chapter 2: Materials and methods

2.1 Samples

Tissue samples from 98 *A. atra* were collected from 38 localities spread throughout the CFM (Fig. 2). Tail clippings were preserved in 96% ethanol for DNA extraction. To provide a phylogenetic context between *A. atra* from the CFM and the rest of Southern Africa, 16 samples from 12 localities throughout Southern Africa were also included (Appendix A). The closely related sister taxon, A. *knobeli* was used to root the trees in the phylogenetic analyses.

2.2 Molecular techniques

Total genomic DNA was extracted following standard procedures with a proteinase K digestion, phenol/chloroform purification and ethanol precipitation (Sambrook *et al.* 1989). The ND2 gene was amplified and sequenced using primers L4437 (Macey *et al.* 1997a) and H5934 (Macey *et al.* 1997b), using standard PCR procedures and an annealing temperature of 53 ^{o}C. Agama-specific CR primers were designed using primer walking. The initial amplification was done using the L15162 Cyt b primer (Palumbi & Kessing 1991) and a newly designed 12S rRNA primer (H1204 – 5' ACA AGC CTA TAC ATG CAA GC 3') that was available in the laboratory. This ~2600 bp region spans the entire control region and was sequenced to design the CR primers (L15850 – 5' TAC TGC CTC TAA CCT CAA CC 3' and H698 – 5' GCT TGC ATG TAT AGG CTT GT 3'). Primer3 software (Rozen & Skaletsky 2000) was used and primer names correspond to positions on the human mitochondrial genome (Anderson *et al.* 1981). In some instances amplification was problematic and to eliminate all

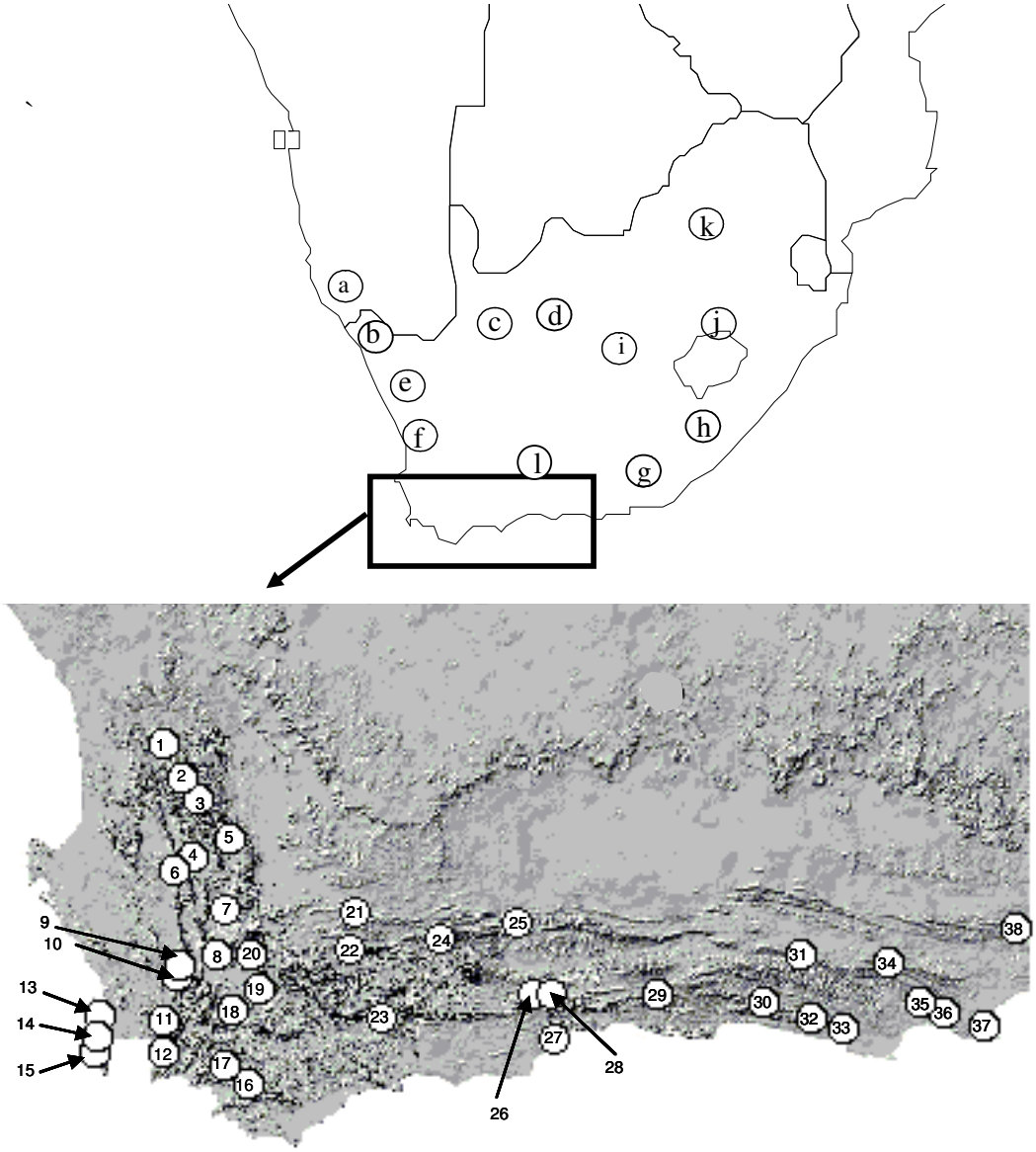


Figure 2. Map of sampling localities of *Agama atra* and *A.knobeli* used in this study. *A. knobeli* was sampled at a = Aus and *A. atra* individuals outside the CFM were sampled at b = Eksteenfontein, c = Augrabies, d = Postmansburg, e = Vaalputs, f = Nieuwoudtville, g = Grahamstown, h = Transkei, i = Bloemfontein, j = Qwa-Qwa, k = Pretoria, l = Beaufort West. The CFM is indicated by the black box and the numbers herein represent: 1 = northern Cederberg, 2 = Cederberg, Sneeukop, 3 = southern Cederberg, 4 = Turretpeak, 5 = Kaggakamma, 6 = Groot winterhoek, 7 = Waboomsberg, 8 = Thumas hut, 9 = Bainskloof, 10 = Limietberg, 11 = Jonkershoek 12 = Gordons Bay, 13 = Devilspeak, 14 = Silvermine, 15 = Scarborough, 16 = Saldomsdam, 17 = Steenboksberg, 18 = Riviersonderendberg, 19 = Robertson, 20 = Keeromsberg, 21 = Witteberg, 22 = Anysberg, 23 = Tradouw Pass, 24 = Klein Swartberg, 25= Die Hel, 26 = Attakwa/Robertsonspas, 27 = Hartenbos, 28 = Outeniqua, 29 = Millwood, 30 = Tsitsikamma, 31= Baviaanskloof, 32 = Hudsonvale, 33 = Kareedouw, 34 = Cockscomb, 35 = Elandsberg, 36 = Lady Slipper, 37 = Port Elizabeth, 38 = Suurberg.

missing data two internal primers were designed, forward primer L15895 (5'-AGC TTA ATA CAA AGC GCA GT-3') and reverse primer H592 (5'-CAC ATG ATC TTT CCA AGA CC -3').

The PCR reactions were performed in 50-µl volumes containing ≅ 25 ng genomic DNA, 0.2 µM of each primer, 0.2 mM dNTPs, 1.5-2.5 mM $MgCl_2$, 5 µL of reaction buffer and 0.5 units BioT*aq* DNA polymerase (Bioline). PCR amplification was performed under the following conditions: 1 min denaturing at 95 ^{o}C, followed by 35 cycles of denaturation (35 s at 95 ^{o}C), annealing for 30 s (at 53 ^{o}C for ND2 and 50 ^{o}C for control region) and extension (45 s at 72 ^{o}C), with a final extension at 72 ^{o}C for 3 min, using GeneAmp PCR system 2700 (Perkin-Elmer). Negative controls (template-free PCR reactions) were included each time.

PCR products were separated and visualized through 0.8% agarose gels containing ethidium bromide. Gel purification was done using the Wizard gel extraction kit (Promega). The purified products were cycle sequenced using the BigDye terminator kit v3.0 (Applied Biosystems) and analysed on a 3100 ABI automated sequencer. Sequences were edited with Sequence Navigator v1.01 (Perkin Elmer). Sequence alignment was initially performed in Clustal X (Thompson *et al.* 1997) using default parameters. To ensure optimal alignment the aligned sequences were manually adjusted in MacClade version 4.0 (Maddison & Maddison 1992).

2.3 Data analyses

2.3.1 Haplotype networks

Traditionally phylogeography was founded on tree-based reconstructed genealogies of individuals sampled from different populations (Althoff & Pellmyr 2002). These methods are, however, often inadequate to draw conclusions on fine-scale population structure because geographically neighbouring individuals generally share a close evolutionary origin. The amount of genetic divergence is thus often too low to generate meaningful resolution as the pattern of divergence is not bifurcating. Unrooted networks are more sensitive to resolve the relationships among closely related haplotypes (Excoffier *et al.* 1992) because they assess the distribution and relationship of haplotypes among the localities without assuming bifurcation events. Several haplotypes/individuals can thus be joined by a single node, which at the population level is clearly a more accurate way of reflecting the relatedness among maternal lineages.

To investigate genetic relationships among haplotypes from the CFM, median-joining networks were constructed using Network version 4.1 (Bandelt *et al.* 1999). Networks were drawn separately for the two markers used and included all individuals (n = 98) for ND2 (549 bp) and CR (439 bp). A median-joining network was also constructed using a combined dataset.

Uncorrected p-distances between groups were calculated using MEGA 2.1 (Kumar *et al.* 2001) and haplotype diversity (h) and nucleotide diversity (π) among groups were calculated in Arlequin ver2.0 (Schneider *et al.* 2000). The

latter was performed to assess the genetic variability within each clade identified in the network.

2.3.2 AMOVA analyses

The distribution of mitochondrial variation within and between assemblages was investigated by an analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) as implemented in Arlequin ver 2.0 (Schneider *et al.* 2000).

AMOVA analysis was performed on the separate and the combined datasets. Two hierarchical levels were considered: (i) an overall AMOVA incorporating all of the major mtDNA lineages, and (ii) pair-wise AMOVAs between each of the major mtDNA lineages. Due to small sample sizes from many localities, individuals were assigned to groups on the basis of their phylogenetic relatedness. These "groups" were identified by the median-joining network. An advantage of this subjective pooling method is that it avoids difficulties relating to the sometimes ambiguous group assignments based solely on geography. This approach offers a means of assessing the degree of genetic differentiation among mtDNA lineages/groups. Consequently, these data can support other quantitative measures (e.g. bootstrapping and average genetic distances).

To assess the genetic divergence among these major mtDNA lineages, F_{ST} and Φ_{ST} were estimated. F_{ST} takes into account only the differences in haplotype frequencies observed in different populations, while Φ_{ST} takes into account both the haplotype frequencies and the nucleotide diversity (Weir & Cockerham 1984; Hurwood & Hughes 1998; Beheregaray & Sunnucks 2001). The Tamura-Nei model was used to construct a distance matrix (Tamura & Nei 1993), the

corresponding gamma shape distribution parameter ($\alpha = 0.678$) was calculated using maximum likelihood in PAUP* 4.0b10, and 10 000 permutations were used to test the significance of F and Φ-statistics. The Tamura-Nei model was chosen as it accounts for substitutional rate differences between nucleotides, inequality of nucleotide frequencies, and distinguishes between transition and transversion frequencies.

2.3.3 SAMOVA

To *a posteriori* identify genetically distinct geographical groupings that might represent populations SAMOVA version 1.0 (spatial analysis of molecular variance; Dupanloup *et al.* 2002) was used on the combined data set. SAMOVA uses geographical information and the sequence data to identify groups of populations that are geographically homogeneous and maximally separated from each other. The program aims to maximize the proportion of total genetic variation due to differences between groups of populations based on a simulated annealing procedure. It also incorporates traditional F-statistics (F_{CT}, F_{SC}, F_{ST}) in recognising population substructure. F_{CT} is the proportion of total genetic variance due to the differences between groups of populations; F_{SC} reveals the degree of differentiation between populations within groups; F_{ST} shows the genetic variation between subpopulations relative to the total population. One hundred simulated annealing processes were performed for each possible number of populations, ranging from two through to ten populations for the combined dataset (ND2 + CR).

2.3.4 Isolation by distance

To determine whether phylogeographic patterns correlated with geographic distance, the Mantel test (Mantel 1967) was performed in MANTEL (available from http://life.bio.sunysb.edu/morph/). The data were grouped *a priori* using the most well differentiated sets of sampling localities suggested by the SAMOVA. From the sequence data the F_{ST} values between these groupings were calculated. The geographic distances (straight line distances) were estimated in ArcView GIS 3.2 using the central point in each geographic region. Given the mountainous habitat where *A. atra* is found, this distance estimate is certainly an underestimate of the "real" geographic distances among localities. However, in the absence of an appropriate model to correct for these biases and in support of the simplified technique used in this thesis, the CFM are generally considered to form part of the mountain complex known as the Great Escarpment (DEAT 1997) and it is thus reasonable to suggest that a roughly similar underestimate is applicable to most distance measurements.

2.3.5 Nested clade analysis

To gain further insight into the demographic history of *A. atra*, a nested clade analysis (NCA) was run on the combined dataset. NCA uses the protocol and nesting rules outlined by Templeton *et al.* (1987) and Templeton and Sing (1993), to transform a haplotype tree into a hierarchical set of nested clades. TCS version 1.02 (Clement *et al.* 2000) was used to determine whether the number of mutations between some of the clades could be unambiguously connected. In instances where the connection exceeded the 10-step limit in our study it is advisable to analyse these clades separately. The clades were nested using the

rules described by Templeton *et al.* (1987), and Geodis 2.0 (Posada *et al.* 2000) was used to test for significant associations between the nested clades and geographic location. Geodis 2.0 (Posada *et al.* 2000) tests three parameters simultaneously: 1) The average clade distance (D_C) measures for all individuals or haplotypes within a particular nesting group, the average distance between haplotypes and the estimated geographical center; 2) Nesting clade distance (D_N) measures, for all haplotypes or groups within the next highest nesting level, the average distance of individuals or haplotypes from the estimated geographical center; 3) Interior-tip distances measures the relative geographical spread of younger groups (tips) to older groups (interior), compared to other groups within the same nesting group. Using the updated inference key provided in Templeton (2004), biological inferences were made for groups that were statistically significantly associated with geography.

2.4 Estimation of divergence times

The constant molecular clock assumption has been extensively used in phylogeographical studies for estimation of the divergence times for mtDNA data. However, the divergence times inferred from DNA should be interpreted with caution as lineage-specific substitution rate variation and differences in substitution rates between genes appear to be rather common phenomena (Bromham *et al.* 1996; Graur & Martin 2004).

Because no calibration point was available for *A. atra*, estimates of divergence times were done under the constant molecular clock assumption. Estimation of divergence times among *A. atra* clades was calculated using the ND2 gene and

were obtained by applying a range of absolute substitution rates for agamid lizards. Divergence times were first estimated based on the expected evolutionary rate of 0.65% sequence divergence per lineage per million years, obtained from agamid lizards (Macey *et al.* 1998). This rate has been used for the ND2 region in studies on other lizards' species (Glor *et al.* 2001; Daniels *et al.* 2004; Gifford *et al.* 2004) and especially for another agama species *Agama impalearis* (Brown *et al.* 2002). However, for a more comprehensive analysis, two other calibrated Acrodonta mtDNA molecular clocks (0.6% and 0.4% sequence divergence per million years) were also suggested (Raxworthy *et al.* 2002). Uncorrected pairwise distances were obtained using only the ND2 sequences in MEGA 2.1 (Kumar *et al.* 2001), as explained above.

2.5 Phylogenetic analyses.

As mentioned above, traditional bifurcating phylogenetic analyses are often inadequate for phylogeographical studies. On the other hand, these methods can be useful where complete lineage sorting has occurred. To explore the relationships among *A. atra* within the CFM and other populations within Southern Africa (16 samples of 12 localities), three methods of phylogenetic reconstruction were implemented on the combined dataset: parsimony, maximum likelihood and Bayesian inference.

Parsimony was implemented in PAUP 4.0b10 (Swofford 2002). Unweighted parsimony analyses were done using the heuristic search option with TBR branch swapping and 100 random additions of taxa. Nodal support was assessed by 1000 bootstraps (Felsenstein 1985). The hierarchical likelihood ratio test (hLRT;

Huelsenbeck and Crandall 1997) employed in Modeltest 3.06 (Posada & Crandall 1998) was used to determine the optimal model of nucleotide substitution for each gene and the combined data set. Maximum likelihood was implemented in PHYML (Guindon & Gascuel 2003), and nodal support was assessed by 1000 bootstraps. A consensus tree with the resulting 1000 PHYML trees was produced by CONSENSE in PHYLIP (Felsenstein 1993), and visualized with TREEVIEW version 1.6.6 (Page 1996). Bayesian analyses were performed using MRBAYES version 3.1.1 (Huelsenbeck & Ronquist 2001) and six rate categories with uniform priors for the gamma distribution and invariable sites were specified. Four independent searches were performed for the combined dataset. Four Markov chains (one cold, 3 heated) initiated from random trees for 5 000 000 generations were run with trees saved every 100 generations. The burn-in was determined and the first 20 000 trees were excluded by examination of log-probabilities in Microsoft ® Excel (2002). The remaining trees were used to construct 50% majority rule consensus trees in PAUP 4.0b10 indicating the posterior probabilities for nodes.

Chapter 3: Results

3.1 Control region

The analysis of the mtDNA control region fragment derived from 98 CFM individuals were based on a total number of 439 bp of sequence and, of these, 44 sites were polymorphic and 30 were parsimony informative. The ratios of the A:C:G:T nucleotides were 0.300: 0.233: 0.116: 0.351. A total of 36 CR mtDNA haplotypes were recovered ($h = 0.944 \pm 0.011$; $\pi = 0.0181 \pm 0.009$). The median-joining network revealed four distinct mtDNA clades (Fig. 3), corresponding to different geographic locations (Fig. 4). These groupings can be defined as a Cape Peninsula clade (localities 13, 14 and 15), a northern CFM clade (localities 1, 2, 3, 4, 6), a Limietberg clade (localities 9 and 10) and a central CFM clade (made up of individuals that are distributed throughout the remainder of the CFM; Fig. 2). No haplotypes were shared between regions which is indicative of the absence of any recent female gene flow among the assemblages.

The average uncorrected sequence divergences between haplotypes in the four CFM clades are given in Table 1. The highest average pairwise nucleotide divergence was between the Limietberg clade and the central CFM clade (4.47% ± 0.94%). Within each of these clades, average sequence divergence was below 1%.

AMOVA analysis was conducted on the four clades detected in the haplotype network. These clades revealed a high degree of genetic subdivision with more

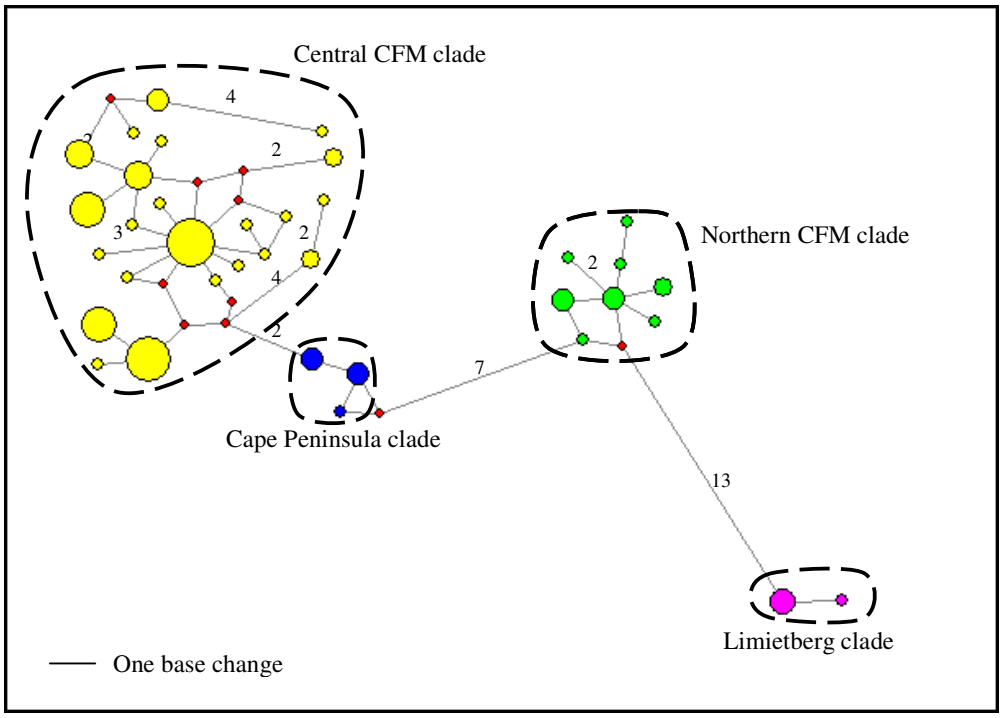


Figure 3. Control region median-joining network obtained for 36 *A. atra* haplotypes. The four *A. atra* clades, Cape Peninsula clade, northern CFM clade, Limietberg clade and central CFM clade, are indicated by the broken lines. Branch lengths longer than one step are indicated on the branches and red circles indicate intermediate missing haplotypes as suggested by Network.

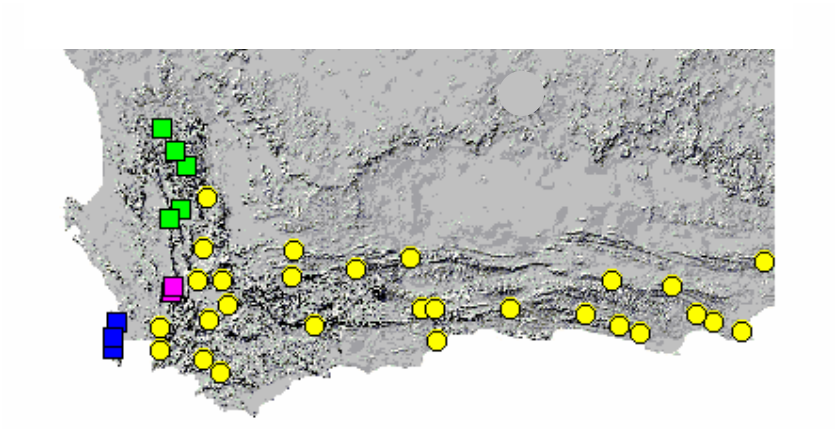


Figure 4. Geographic distribution of the four *A. atra* clades within the CFM. The colours correspond to those in Fig. 3 with blue squares indicating the Cape Peninsula clade, the yellow dots represent the Central Cape Fold clade, the green squares correspond to the Northern Cape Fold clade and the pink squares show the localities of the Limietberg clade.

Table 1. The average uncorrected sequence divergences among the four mtDNA clades, Pretoria, the northern-central Southern Africa and *A. knobeli* for CR (below diagonal) and ND2 (above diagonal). Standard errors are given in brackets.

	Northern CFM	Limietberg	Cape Peninsula	Central CFM	Pretoria	Northern-central southern African	*A. knobeli*
Northern CFM	—	4.53% (0.84)	4.55% (0.86)	5.34% (0.88)	6.70% (0.93)	8.59% (1.03)	9.25% (1.32)
Limietberg	3.54% (0.81)	—	4.70% (0.83)	4.18% (0.08)	5.34% (0.117)	8.89% (1.11)	10.08% (1.32)
Cape Peninsula	2.17% (0.58)	4.07% (0.91)	—	4.44% (0.79)	5.68% (0.91)	8.99% (1.08)	10.05% (1.27)
Central CFM	3.13% (0.70)	4.47% (0.94)	1.12% (0.39)	—	6.20% (0.94)	8.42% (1.07)	10.01% (1.32)
Pretoria	3.67% (0.83)	4.87% (1.01)	3.48% (0.86)	2.58% (0.87)	—	9.54% (1.09)	10.51% (1.34)
Northern-central Southern African	4.86% (0.74)	6.04% (0.94)	4.03% (0.69)	4.19% (0.68)	4.80% (0.76)	—	8.02% (1.07)
A. knobeli	7.11% (1.20)	7.40% (1.16)	6.35% (1.11)	6.35% (1.20)	6.41% (1.12)	6.80% (1.04)	—

than 78% of genetic variation among the geographic areas (F_{ST} = 0.784, p < 0.001; Φ_{ST} = 0.784, p < 0.001, Table 2). All pairwise F_{ST} and Φ_{ST} values among populations were also significant (Table 3), with the highest value (F_{ST} = 0.96; Φ_{ST} = 0.96) between the Cape Peninsula clade and the Limietberg clade. Nucleotide and haplotype diversity was highest for the central CFM clade (Table 4).

Table 2. AMOVA results of the CR.

Source of variation	df	Sum of Squares	Variance Components	Percentage of Variation
Among Populations	3	238.693	5.68762 Va	78.45
Within Populations	94	146.877	1.56252 Vb	21.55
Total	97	385.569	7.25014	

Table 3. F_{ST} estimates (below diagonal) and Φ_{ST} values (above diagonal) among the four *A. atra* clades for the CR. Significance values (p) are given in brackets.

	Northern CFM	Limietberg	Cape Peninsula	Central CFM
Northern CFM	-	0.91 (< 0.001)	0.86 (< 0.001)	0.79 (< 0.001)
Limietberg	0.91 (< 0.001)	-	0.96 (< 0.001)	0.84 (< 0.001)
Cape Peninsula	0.86 (< 0.001)	0.96 (< 0.001)	-	0.45 (< 0.001)
Central CFM	0.79 (< 0.001)	0.84 (< 0.001)	0.46 (< 0.001)	-

Table 4. The molecular diversity indices of the four clades for CR. Standard errors are given in brackets.

Locality	Number of individuals	Number of haplotypes	Molecular diversity indices	
			Haplotype diversity (*h*)	Nucleotide diversity (π)
Northern CFM	13	8	0.910 (0.056)	0.004 (0.003)
Limietberg	5	2	0.400 (0.237)	0.001 (0.001)
Cape Peninsula	7	3	0.714 (0.127)	0.002 (0.002)
Central CFM.	73	23	0.906 (0.017)	0.008 (0.005)

3.2 ND2

The segment of the mitochondrial ND2 fragment (549 bp) in 98 *A. atra* individuals contained 73 variable sites, of which 53 sites were parsimony-informative, with most of the variation observed in third codon positions (44 parsimony-informative characters). The ratios of the A:C:G:T nucleotides were 0.359: 0.249: 0.123: 0.270. The polymorphic sites defined 45 haplotypes ($h = 0.958 \pm 0.009$; $\pi = 0.024 \pm 0.012$). Network analysis of the ND2 dataset (Fig. 5) recovered the same four distinct mtDNA clades associated with geography as were found for CR (Fig. 4).

The ND2 gene showed a slightly higher amount of sequence divergence than the CR among the four clades, with the highest between the northern CFM clade and the central CFM clade ($5.34\% \pm 0.88\%$; Table 1). Within each of these clades, average sequence divergence among haplotypes was once again below 1%.

AMOVA analyses for the ND2 gene indicated that the highest percentage of variance is among the different geographic regions ($F_{ST} = 0.878$, $P < 0.001$; $\Phi_{ST} = 0.890$, $P < 0.001$, Table 5) and not within the geographic regions. The highest pairwise F_{ST} and

Φ_{ST} values were again between the Cape Peninsula clade and the Limietberg clade, plus all pairwise values among all populations were again significant (Table 6). Nucleotide and haplotype diversities were the highest for the central CFM clade (Table 7) as also seen in CR.

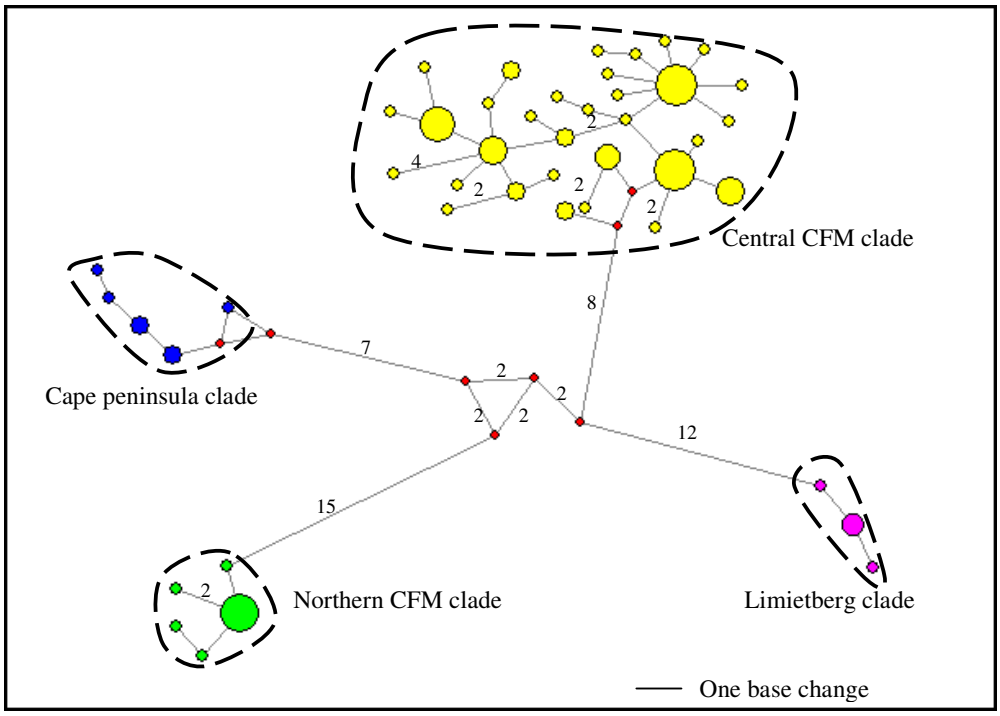


Figure 5. ND2 median-joining network obtained for 45 *A. atra* haplotypes. The four *A. atra* clades, Cape Peninsula clade, northern CFM clade, Limietberg clade and central CFM clade, are indicated by the broken lines. Branch lengths longer than one step are indicated on the branches and red circles indicate intermediate missing haplotypes as suggested by Network.

Table 5. AMOVA results of ND2.

Source of variation	df	Sum of Squares	Variance Components	Percentage of Variation
Among Populations	3	445.470	10.71921 Va	87.84
Within Populations	94	139.469	1.48371 Vb	12.16
Total	97	584.939	12.20292	

Table 6. F_{ST} estimates (below diagonal) and Φ_{ST} values (above diagonal) among the four *A. atra* clades for ND2. Significance values (*p*) are given in brackets.

	Northern CFM	Limietberg	Cape Peninsula	Central CFM
Northern CFM	-	0.967 (< 0.001)	0.957 (< 0.001)	0.897 (< 0.001)
Limietberg	0.964 (< 0.001)	-	0.955 (< 0.001)	0.848 (< 0.001)
Cape Peninsula	0.952 (< 0.001)	0.950 (< 0.001)	-	0.859 (< 0.001)
Central CFM	0.883 (< 0.001)	0.838 (< 0.001)	0.847 (< 0.001)	-

Table 7. The molecular diversity indices of the four clades for ND2. Standard errors are given in brackets.

Locality	Number of individuals	Number of haplotypes	Molecular diversity indices	
			Haplotype diversity (*h*)	Nucleotide diversity (π)
Northern CFM	13	5	0.539 (0.161)	0.001 (0.001)
Limietberg	5	3	0.700 (0.218)	0.001 (0.001)
Cape Peninsula	7	5	0.905 (0.103)	0.003 (0.003)
Central CFM	73	32	0.939 (0.013)	0.007 (0.004)

3.3 Combined mtDNA data set

The two mtDNA data sets revealed the exact same pattern when analysed separately and were combined for a total analysis. A total of 59 mtDNA (988 bp; 439 bp of CR and 549 bp of ND2) haplotypes were identified in 98 *A. atra* samples ($h = 0.981 \pm 0.005$; $\pi = 0.0214 \pm 0.011$). Base frequencies were A:C:G:T = 0.333: 0.242: 0.120: 0.306. The combined dataset revealed 117 variable sites of which 83 were parsimony informative. The haplotype network of the combined analyses (Fig. 6) confirms the individual analyses and clearly indicates the existence of four distinct mtDNA clades that correspond to different geographic locations (Fig. 4). The pairwise F_{ST} and Φ_{ST} values again indicated that the highest percentage of the genetic variation lies among the geographic areas ($F_{ST} = 0.833$, $p < 0.001$; $\Phi_{ST} = 0.852$, $p < 0.001$; Table 8). All pairwise F_{ST} and Φ_{ST} values were significant (Table 9). The highest value was between the Cape Peninsula clade and the Limietberg clade and the central CFM clade showed the highest nucleotide and haplotypic diversity (Table 10). These results were identical to the individual analyses.

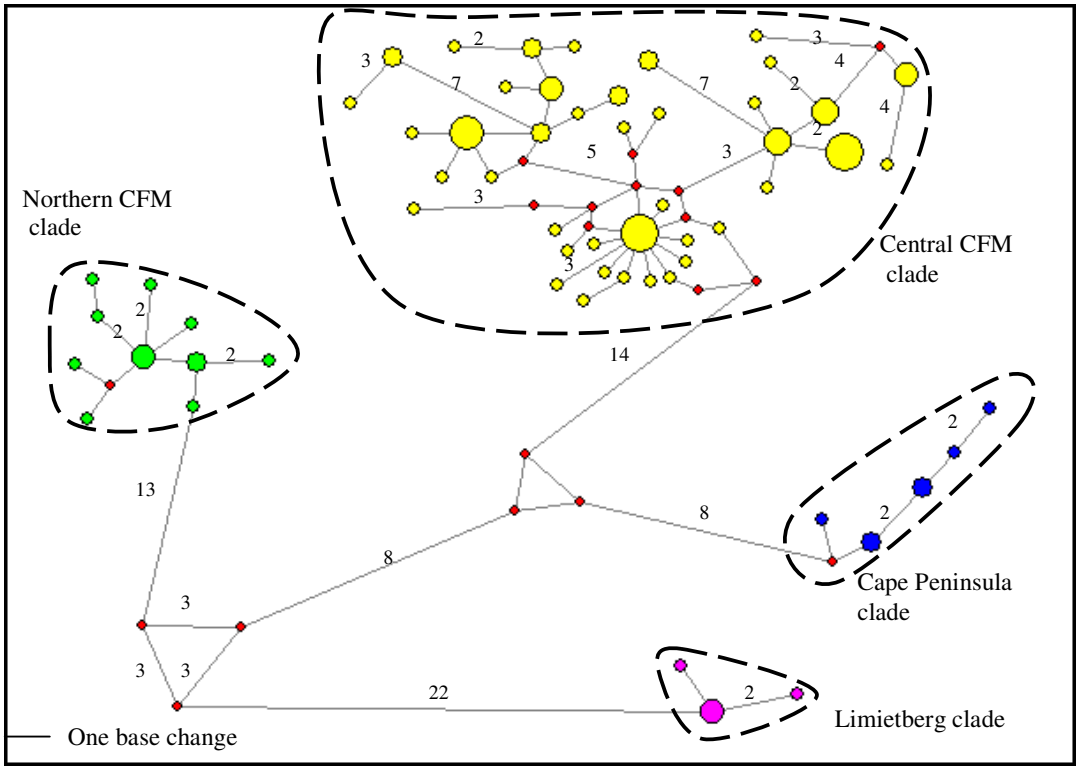


Figure 6. Combined median-joining network obtained for 59 *A. atra* haplotypes. The four *A. atra* clades, Cape Peninsula clade, northern CFM clade, Limietberg clade and central CFM clade, are indicated by the broken lines. Branch lengths longer than one step are indicated on the branches and red circles indicate intermediate missing haplotypes as suggested by Network.

Table 8. AMOVA results of the combined dataset

Source of variation	df	Sum of Squares	Variance Components	Percentage of Variation
Among Populations	3	670.008	16.05038 Va	83.31
Within Populations	94	302.349	3.21648 Vb	16.69
Total	97	972.357	19.26686	

Table 9. F_{ST} estimates (below diagonal) and Φ_{ST} values (above diagonal) among the four *A. atra* clades for the combined data set. Significance values (*p*) are given in brackets.

	Northern CFM	Limietberg	Cape Peninsula	Central CFM
Northern CFM	-	0.945 (< 0.001)	0.930 (< 0.001)	0.857 (< 0.001)
Limietberg	0.942 (< 0.001)	-	0.960 (< 0.001)	0.845 (< 0.001)
Cape Peninsula	0.934 (< 0.001)	0.955 (< 0.001)	-	0.770 (< 0.001)
Central CFM	0.838 (< 0.001)	0.826 (< 0.001)	0.750 (< 0.001)	-

Table 10. The molecular diversity indices of the four clades for the combined dataset. Standard errors are given in brackets.

Locality	Number of individuals (n)	Number of haplotypes (M)	Molecular diversity indices	
			Haplotype diversity (*h*)	Nucleotide diversity (π)
Northern CFM	13	10	0.949 (0.051)	0.003 (0.002)
Limietberg	5	3	0.700 (0.218)	0.001 (0.001)
Cape Peninsula	7	5	0.905 (0.103)	0.003 (0.003)
Central CFM	73	41	0.970 (0.008)	0.008 (0.004)

3.4 SAMOVA analysis and isolation by distance

SAMOVA analysis showed the largest increase in F_{CT} (0.811) when the geographic areas were partitioned into six groups (see Table 11 and Fig. 7). However, beyond these six groups the F_{CT} values still increased, but at a very slow rate. This is probably a response to the decrease in F_{SC} that would continue until all sampling areas are separate (Dupanloup *et al.* 2002). Three of the populations proposed by SAMOVA are consistent with the already identified clades (the Cape Peninsula; northern CFM; Limietberg). Furthermore, SAMOVA suggests that the central CFM clade can be further subdivided into three geographically separate groups (Fig. 7): eastern, middle and western CFM populations. However, there is an anomalous result whereby the most eastern locality (Suurberg) falls within the western CFM population. The northern CFM group is the first single locality to fall out at the larger regional scale, which is consistent with the network and AMOVA results, showing that this region is genetically the most isolated in the CFM (Table 11). The Mantel test revealed no significant relationship (P = 0.399) between the genetic and geographic distances for the comparisons of the six distinct groups identified by the SAMOVA (Fig. 8).

Table 11. Results of SAMOVA analyses. Significance based on 100 simulations, where $*p < 0.05$. The geographic partitioning which shows the largest increase in F_{CT} is indicated in bold.

#groups	Group composition	F_{ST}	F_{SC}	F_{CT}
2	1. Cape Peninsula, Limietberg, Jonkershoek, Saldomsdam, Steenboksberg, Riviersonderendberg, Gordon's Bay, Witteberg, Suurberg, Lady Slipper, Keeromsberg, Baviaanskloof, Port Elizabeth, Tsitsikamma, Kaggakamma, Robertson, Kareedouw, Thomas hut, Waboomsberg, Millwood, Anysberg, Attakwa, Outeniqua, Die Hel, Klein Swartberg, Tradouw Pass, Hartenbos, Cockscomb, Elandsberg, Hudsonvale 2. Northern CFM	0.941	0.847	0.616
3	1. Cape Peninsula, Jonkershoek, Saldomsdam, Steenboksberg, Riviersonderendberg, Gordon's Bay, Witteberg, Suurberg, Lady Slipper, Keeromsberg, Baviaanskloof, Port Elizabeth, Tsitsikamma, Kaggakamma, Robertson, Kareedouw, Thomas hut, Waboomsberg, Millwood, Anysberg, Attakwa, Outeniqua, Die Hel, Klein Swartberg, Tradouw Pass, Hartenbos, Cockscomb, Elandsberg, Hudsonvale 2. Northern CFM 3. Limietberg	0.942	0.801	0.709
4	1. Jonkershoek, Saldomsdam, Steenboksberg, Riviersonderendberg, Gordon's Bay, Witteberg, Suurberg, Lady Slipper, Keeromsberg, Baviaanskloof, Port Elizabeth, Tsitsikamma, Kaggakamma, Robertson, Kareedouw, Thomas hut, Waboomsberg, Millwood, Anysberg, Attakwa, Outeniqua, Die Hel, Klein Swartberg, Tradouw Pass, Hartenbos, Cockscomb, Elandsberg, Hudsonvale 2. Northern CFM 3. Cape Peninsula 4. Limietberg	0.937	0.720	0.776
5	1. Cape Peninsula, Limietberg, Jonkershoek, Saldomsdam, Steenboksberg, Riviersonderendberg, Gordon's Bay, Witteberg, Suurberg, Lady Slipper, Keeromsberg, Baviaanskloof, Port Elizabeth, Tsitsikamma, Kaggakamma, Robertson, Thomas hut, Waboomsberg, Anysberg, Attakwa, Outeniqua, Die Hel, Klein Swartberg, Tradouw Pass, Hartenbos, Cockscomb, Elandsberg, Hudsonvale 2. Limietberg 3. Kareedouw 4. Millwood 5. Northern CFM	0.938	0.803	0.682
6	**1. Jonkershoek, Saldomsdam, Steenboksberg, Riviersonderendberg, Gordon's Bay, Suurberg, Keeromsberg, Kaggakamma, Robertson, Thomas hut** **2. Northern CFM** **3. Limietberg** **4. Lady Slipper, Baviaanskloof, Port Elizabeth, Tsitsikamma, Kareedouw, Millwood, Cockscomb, Elandsberg, Hudsonvale** **5.Witteberg, Waboomsberg, Anysberg, Attakwa, Outeniqua, Die Hel, Klein Swartberg, Tradouw Pass, Hartenbos** **6. Cape Peninsula**	**0.907**	**0.506**	**0.811**
7	1. Jonkershoek 2. Limietberg 3. Lady Slipper, Baviaanskloof, Port Elizabeth, Tsitsikamma, Kareedouw, Millwood, Cockscomb, Elandsberg, Hudsonvale 4. Northern CFM 5. Cape Peninsula 6. Saldomsdam, Steenboksberg, Riviersonderendberg, Gordon's Bay, Suurberg, Keeromsberg, Kaggakamma, Robertson, Thomas hut 7.Witteberg, Waboomsberg, Anysberg, Attakwa, Outeniqua, Die Hel, Klein Swartberg, Tradouws Pas, Hartenbos	0.905	0.465	0.822
8	1. Jonkershoek, Saldomsdam, Steenboksberg, Riviersonderendberg, Gordon's Bay 2. Suurberg 3. Witteberg, Waboomsberg, Anysberg, Attakwa, Outeniqua, Die Hel, Klein Swartberg, Tradouw Pass, Hartenbos 4. Lady Slipper, Baviaanskloof, Port Elizabeth, Tsitsikamma, Kareedouw, Millwood, Cockscomb, Elandsberg, Hudsonvale 5. Cape Peninsula 6. Northern CFM 7. Limietberg 8. Keeromsberg, Kaggakamma, Robertson, Thomas hut	0.904	0.414	0.837
9	1. Keeromsberg, Kaggakamma, Robertson, Thomas hut 2. Witteberg, Anysberg, Attakwa, Outeniqua, Die Hel, Klein Swartberg, Tradouw Pass, Hartenbos 3. Northern CFM 4. Suurberg 5. Waboomsberg 6. Jonkershoek, Saldomsdam, Steenboksberg, Riviersonderendberg, Gordon's Bay 7. Cape Peninsula	0.904	0.372	0.847

	8. Limietberg 9. Lady Slipper, Baviaanskloof, Port Elizabeth, Tsitsikamma, Kareedouw, Millwood, Cockscomb, Elandsberg, Hudsonvale			
10	1. Jonkershoek, Saldomsdam, Steenboksberg, Riviersonderendberg, Gordon's Bay 2. Cape Peninsula 3. Witteberg, Anysberg, Attakwa, Outeniqua, Die Hel, Klein Swartberg, Tradouw Pass, Hartenbos 4. Northern CFM 5. Suurberg 6. Kareedouw 7. Limietberg 8. Waboomsberg 9. Keeromsberg, Kaggakamma, Robertson, Thomas hut 10. Lady Slipper, Baviaanskloof, Port Elizabeth, Tsitsikamma, Millwood, Cockscomb, Elandsberg, Hudsonvale	0.904	0.371	0.847

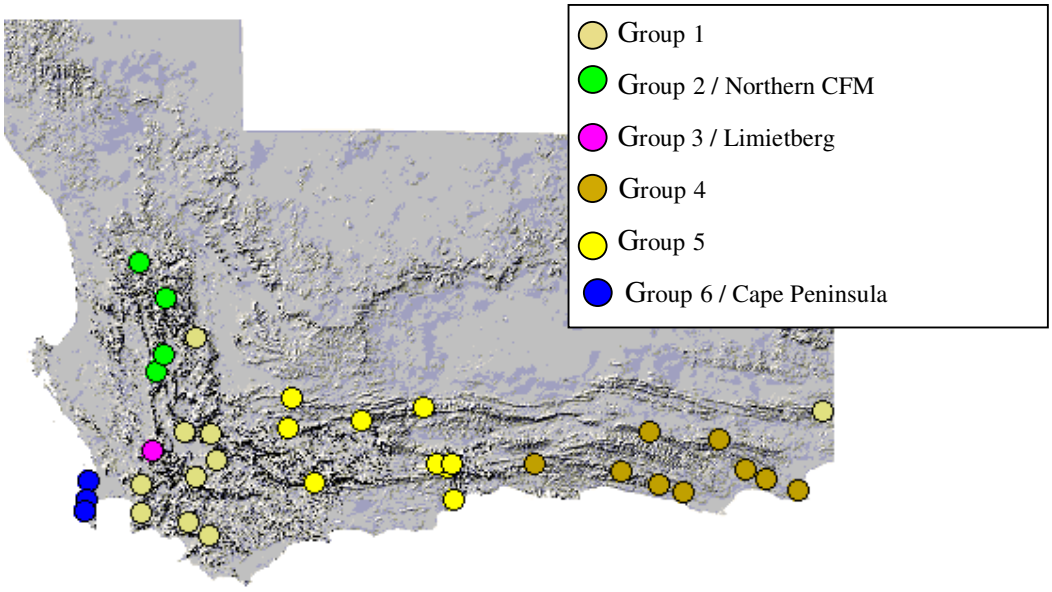


Figure 7. Geographic distribution of the six *A. atra* populations identified by SAMOVA within the CFM.

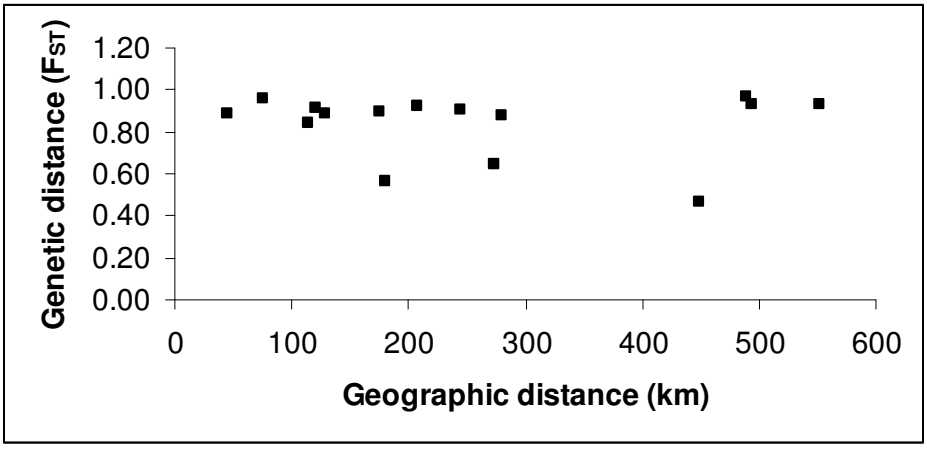


Figure 8. Scatter plot showing the lack of isolation by distance among the CFM *A. atra* sampling sites.

3.5 Nested clade analysis

For the nested clade analyses the four main clades could not be connected with 95% parsimony support so each clade had to be treated as a separate unit. Only the central clade contained enough individuals to carry out a nested clade analysis (Fig. 9). Significant geographical association of clades and sampling locations occur at all six different nesting levels, including the total cladogram (Table 12). The nested clade analysis detected two main groups within the central CFM lineage: 5-1 (middle-eastern group) and 5-2 (western group). Restricted gene flow with isolation by distance was suggested by Templeton's (2004) inference key as the most likely process creating these two clades (Table 12). A mixture of population historical events can be found within these two main clades throughout the hierarchical nested clade structure of haplotypes (Table 12), with restricted gene flow with isolation by distance as the dominant process shaping evolution. When these findings are compared to the SAMOVA analysis not all the localities which form the western group are part of the western group as suggested by SAMOVA.

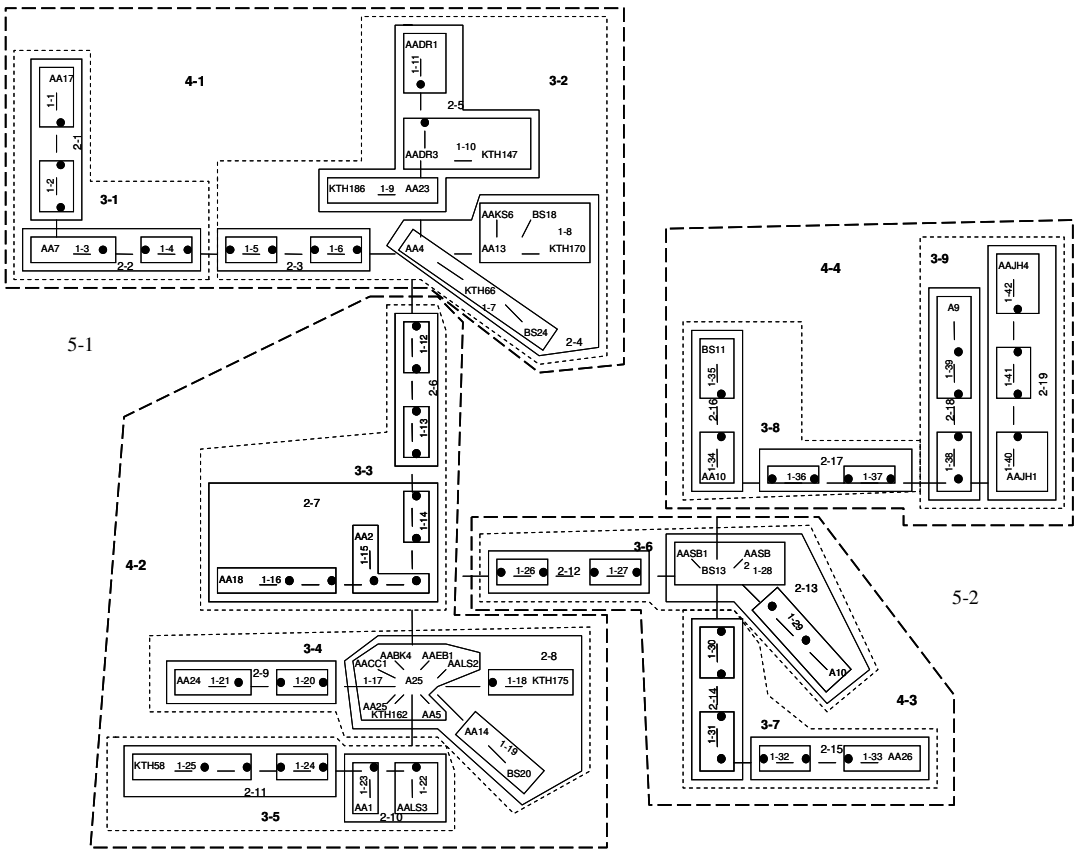


Figure 9. The nesting design inferred only from the central CFM network for *A. atra*. Each line in the network represents one mutational change. Filled circles represent missing haplotypes. The number inside each block indicates the nesting level.

Table 12. Inference chain based on results of geographical dispersion analysis. Only those clades that resulted in a rejection of the null hypothesis are included in this table.

Clade	Chain of inference	Inference
2-4	1-2-3-4-NO	Restricted gene flow with isolation by distance
2-13	1-2-3-4-NO	Restricted gene flow with isolation by distance
3-2	1-2-3-4-9NO	Past fragmentation
4-1	1-2-3-4-NO	Restricted gene flow with isolation by distance
4-3	1-2-3-5-15-NO	Past fragmentation
4-4	1-2-3-5-15-NO	Past fragmentation
5-1	1-2-3-4-NO	Restricted gene flow with isolation by distance
5-2	1-2-3-4-NO	Restricted gene flow with isolation by distance
Total cladogram	1-2-3-4-NO	Restricted gene flow with isolation by distance

3.6 Estimation of divergence times

The Agamid ND2 clock, following the mutational rates used by Macey *et al.* (1998), dates the split of these four *A. atra* groups at 6.5 - 8.5 million years ago (MYA; Table 13). The full range of estimated dates based on the two other calibrated points of 0.6% and 0.4% sequence divergence per million years (Raxworthy *et al.* 2002) is 7 MYA – 13.5 MYA (Table 13). This suggests that the main cladogenesis within the CFM took place at the end of the Miocene and the beginning of the Pliocene.

Table 13. Estimates of divergence times for three different evolutionary rates. The percentage pairwise genetic deference among the four major *A. atra* clades is given in the first column.

	Macey *et al.* 1998 0.65%/my		Raxworthy *et al.* 2002 0.6%/my		Raxworthy *et al.* 2002 0.4%/my	
	Divergence time	Range	Divergence time	Range	Divergence time	Range
Northern CFM vs. Limietberg (4.53%±0.84)	6.97 MYA	5.68-8.26	7.55 MYA	6.15-8.95	11.32 MYA	9.23-13.43
Northern CFM vs. Cape Peninsula (4.55%±0.86)	6.92 MYA	5.68-8.32	7.58 MYA	6.15-9.02	11.38 MYA	9.23-13.53
Northern CFM vs. Central CFM (5.34%±0.88)	8.22 MYA	6.86-9.51	8.90 MYA	7.43-10.36	13.35 MYA	11.15-15.55
Limietberg vs. Cape Peninsula (4.70%±0.83)	7.23 MYA	5.95-8.51	7.83 MYA	6.45-9.22	11.75 MYA	9.68-13.83
Limietberg vs. Central CFM (4.18%±0.08)	6.43 MYA	6.31-6.55	6.90 MYA	6.83-7.1	10.45 MYA	10.25-10.65
Cape Peninsula vs. Central CFM (4.44%±0.79)	7.20 MYA	5.62-8.04	7.40 MYA	6.08-8.72	11.10 MYA	9.13-13.8

3.7 The CFM association with the rest of Southern Africa

The total data set (the 59 CFM haplotypes and 16 samples from localities spread throughout Southern Africa) comprised 988 characters, of which 219 were variable and of these 154 were parsimony informative. Third-position sites of the ND2 region account for over half of the phylogenetically informative sites in the total data set (51%). A similar base-topology was found for all phylogenetic methods with differences restricted to terminal nodes (Figs. 10 - 12). The parsimony analysis recovered 432 equally parsimonious trees of 431steps long (CI = 0.599; RI = 0.861). Modeltest proposed the Hasegawa-Kishino-Yano model (HKY) for both the CR and ND2, however HKY + Γ was proposed for the CR and HKY + Γ + I for ND2. For the combined data set, the HKY plus invariant sites (I = 0.579) plus gamma shape (Γ = 0.678) was proposed by Modeltest, and was subsequently used in the ML analysis. A single tree with a score of -3871.65 was produced by ML analysis (Fig. 12). In the Bayesian inference, identical majority-rule consensus trees were obtained in each of the four runs from the remaining trees after the first 20 000 trees were discarded (Fig. 11; -lnL = 4437.05 - 4451.10).

The trees confirm the monophyly of the three clades (south-eastern clade, north-central clade, *A. knobeli*) as previously described by Matthee and Flemming (2002; Figs. 10-12). The phylogenetic analyses also recover the four main clades within the CFM (with bootstrap support and significant posterior probability values), thus showing congruency with the network analyses presented above. However, high support is confined to the monophyly of clades and little resolution was obtained within the clades.

Sequence divergence ranges from 10 – 11% for ND2 and 6 – 8% for CR among *A. knobeli* and the four CFM clades, while sequence variation is between 7 – 9% for ND2 and 4 – 6% for CR among the northern-central southern African clade and the four CFM clades (Table 1).

The central CFM clade is more closely related to the additional samples from the Matthee and Flemming (2002) study (Nieuwoudtville, Grahamstown, Qwa-Qwa, Bloemfontein, Beaufort West and Transkei) than to any other clade detected in this study. However, the isolated sampling locality of Pretoria seem to be the exception. The sequence divergence between the Pretoria individuals and the central CFM clade (6.63% for ND2 and 4.13% for CR; Table 1) and this sequence divergence value is higher than the sequence variation among the four clades.

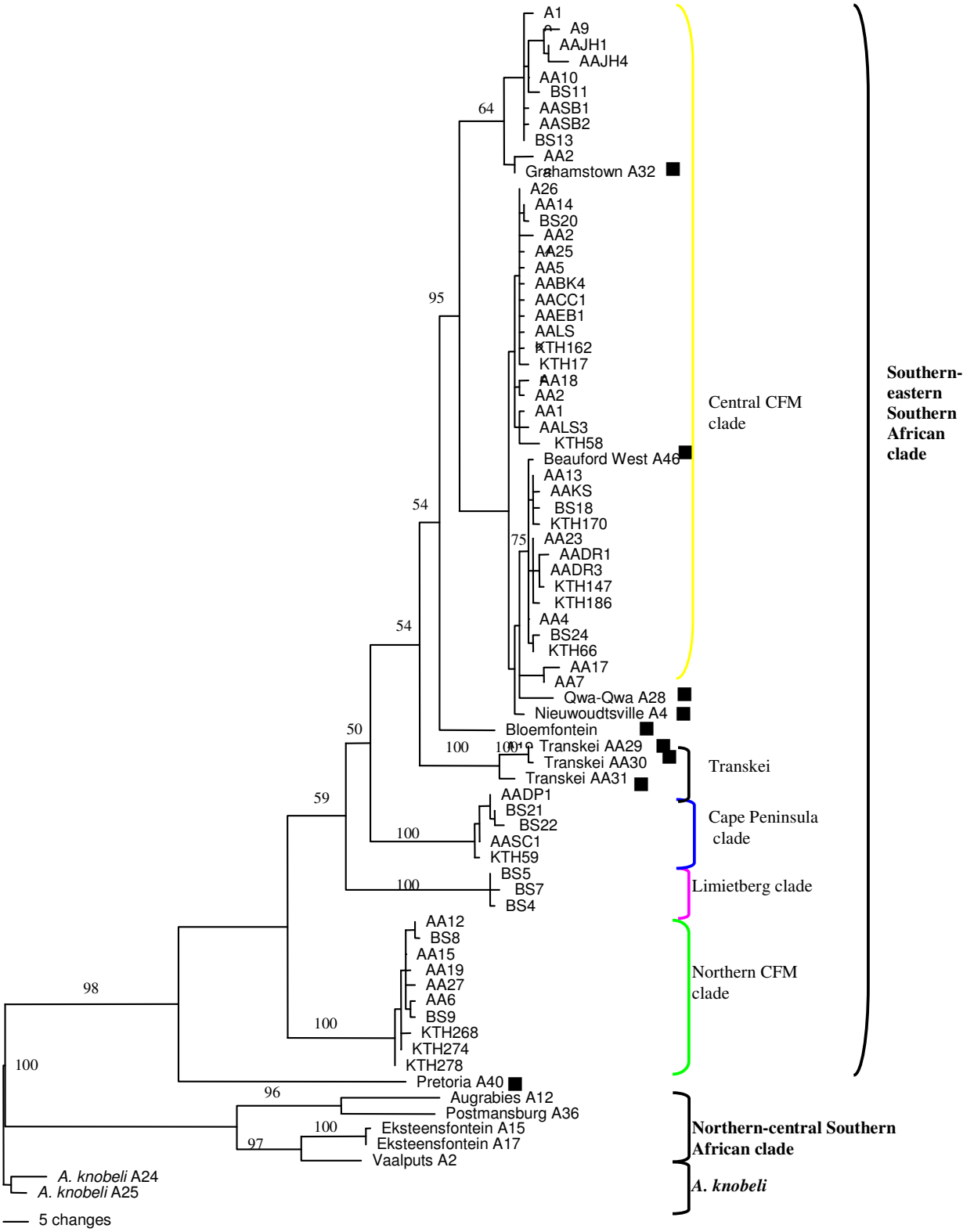


Figure 10. A parsimony phylogram for the 61 *A. atra* haplotypes and the additional samples from the rest of Southern Africa. Individuals outside the CFM and still within the Southern-eastern Southern African clade are indicated by a black square. Bootstrap support values are indicated above nodes.

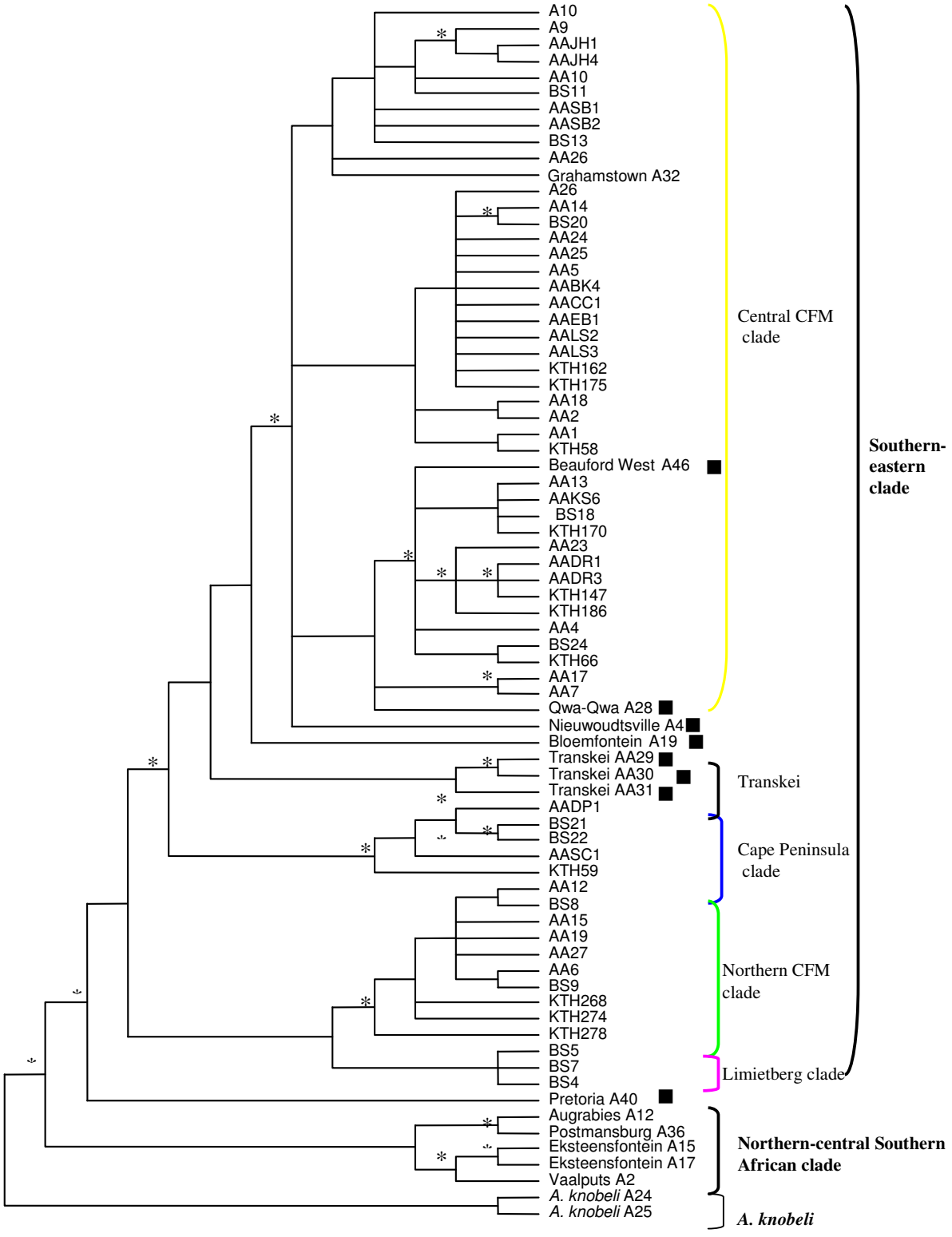


Figure 11. Bayesian topology for Southern Africa *A. atra*. Individuals outside the CFM and still within the Southern-eastern Southern African clade are indicated by a black square. Significantly supported nodes (≥0.95 posterior probability) are indicated by the an asterisk.

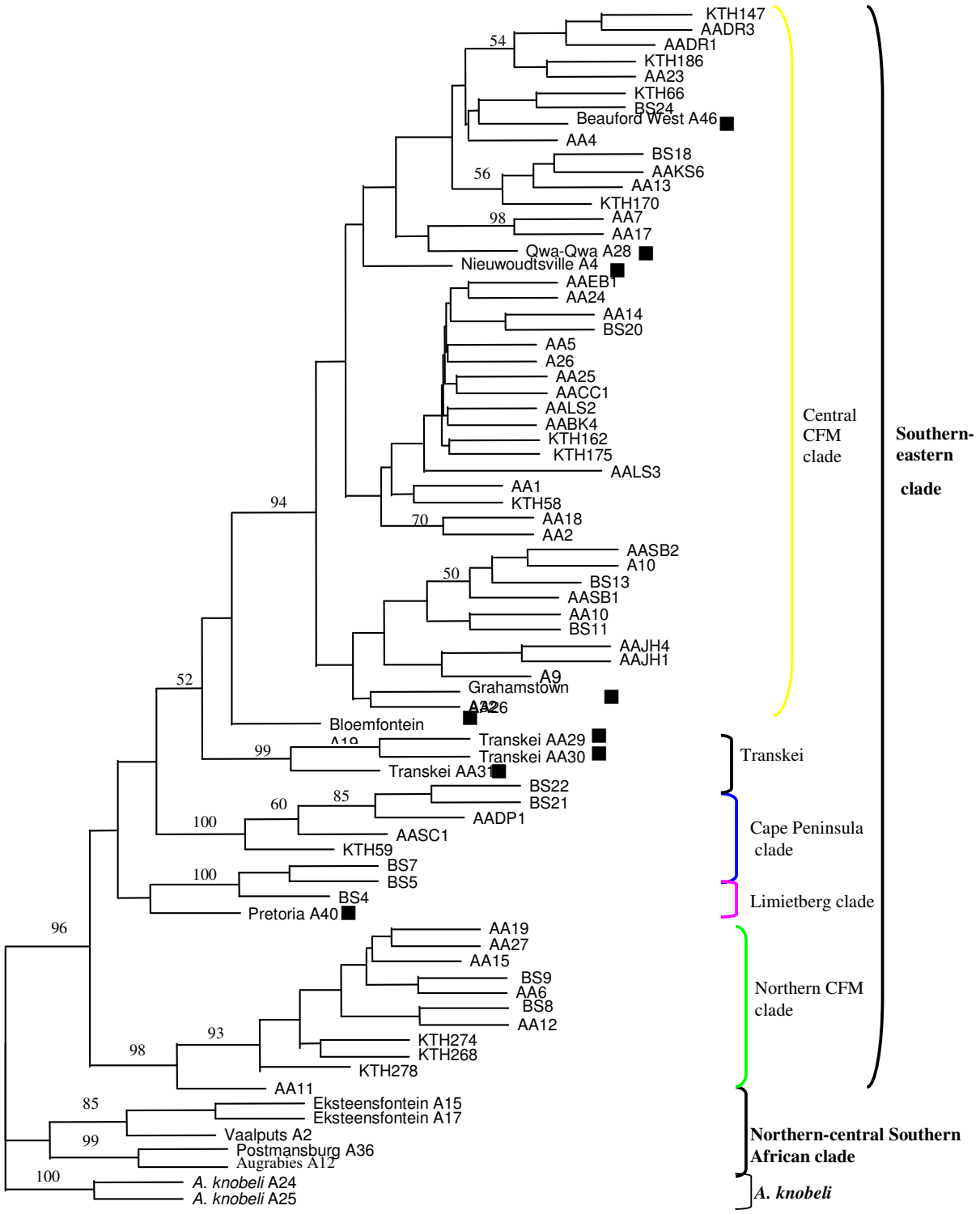


Figure 12. A maximum likelihood phylogram for *A. atra*. Individuals outside the CFM and still within the Southern-eastern Southern African clade are indicated by a black square. The values above the branches indicate bootstrap support higher than 50% for the respective nodes.

Chapter 4. Discussion

4.1 Comparisons of mtDNA regions

Most phylogeographic studies of lizards used the mtDNA genes Cytb, 16S and /or ND2 (*e.g*. Matthee & Flemming 2002; Brown *et al.* 2002; Townsend & Larson 2002). Although CR is often used in other vertebrate studies due to is high variability (*e.g*. Ravaoarimanana *et al.* 2004; Hirota *et al.* 2004; Winney *et al.* 2004), it is seldom used in studies on reptiles and this is one of the first studies to utilise CR in investigating the phylogeographic relationship for a lizard taxon. Given that the CR is a non-protein coding region one would expect that changes in this fragment would occur at least at similar rates to the third position transitions in protein coding genes. Thus, considering all three codon positions, it is expected that the non coding control region will have greater divergence than protein coding mtDNA genes (Hoelzel *et al.* 1994; Sbisa` *et al.* 1997). However, in the present study the average sequence divergences for CR were much lower than ND2 (Table 1) and this pattern has also been previously documented in snakes, *Crotalus viridis* (Ashton & de Queiroz 2001). The authors suggested that the higher sequence divergence found in ND2 relative to the CR is due to the fact that changes occurred more frequently in the ND2 third codon positions than in the CR overall. The present study support this notion as the 3rd codon sites in the ND2 region of *A. atra* once again account for most of the variation as also expressed by the number of phylogenetically informative characters (ND2 = 53; CR = 30). This is also consistent with the functional constraints applied to codon positions within protein-coding genes (Moritz *et al.* 1987). Although third codon positions change faster than first and second codon positions because changes at this position are nearly always synonymous (Koepfli & Wayne 1998).

To visualize the relative rates of evolution of the mtDNA fragments used in the present study, the uncorrected sequence divergence of the CR vs. the overall uncorrected sequence divergence ND2 and separately for each of the codon position of the ND2 gene was plotted (Appendix B). The ND2 gene showed overall greater divergence than CR, which is mainly contributed to the third codon position (Appendix B). The first position shows the same levels of sequence divergence as the CR while the ND2 second position have much lower levels of sequence variation than the CR (Appendix B). This is the same result as observed by Ashton & de Queiroz (2001), suggesting that overall it seems that the ND2 region in reptiles has a high mutation rate than the CR and should thus rather be used when divergences among individuals are expected to be small. The reason for this discrepancy is unknown but is probably due to different functional constraints on the two regions. Protein-coding genes, like ND2 are subject selection acting upon amino acid substitutions (Graur & Li 1991; Yoder *et al.* 1996). In addition, the substitution rates at different codon positions differ due to differences in function. It is also interesting to note that the pattern is different in other vertebrate groups studied thus far (*e.g*. Lloyd 2003).

4.2 *Atra* phylogeography

The present study accentuates the importance of geographic sampling when employing molecular techniques to detect evolutionary lineages within a single species. The previous taxonomic investigation performed by Matthee and Flemming (2002) detected two well-differentiated mtDNA lineages within South Africa (south-eastern clade, north-central clade, Figs. 10 – 12). These lineages were supported with high bootstrap support, significant posterior probabilities and large sequence

divergence values. By employing fine-scale sampling within one of these assemblages (their south-eastern clade), at least three additional distinct *A. atra* mtDNA clades were detected. The extended sampling was mainly confined to the CFM and within this region this study contributed significantly towards resolving the evolution among populations.

In the CFM, four mtDNA clades (Cape Peninsula clade, a northern CFM clade, a Limietberg clade and a central CFM clade; Fig 6) were detected and these were well defined genetically and also geographically distinct. The *A. atra* clades were supported by both mtDNA fragments (ND2 and CR) and isolation by distance does not seem to be an important factor in large-scale phylogeographic patterns in this species (Fig. 8). The uniqueness of the four mtDNA lineages was supported by the median-joining network, phylogenetic analysis, AMOVA results and higher sequence divergence values among, than within, monophyletic groupings. Thus the data show a significant lack of maternal gene flow across the regions, with no haplotype being shared across the four *A. atra* populations.

The analyses suggest that the Cape Peninsula clade is most closely related to the central CFM clade. This is supported by the population analysis, where the pairwise F_{ST} values also revealed that the northern CFM clade and the Limietberg clade are more distant (Tables 2, 4, 6). The support for this is however low (not supported by bootstrap or Bayesian posterior probabilities) and the short internal branches on the phylograms rather tend to suggest that the isolation among these clades was more or less contemporaneous. There is also a discrepancy in distinctness of the Cape peninsula based on ND2 and CR. However, this can be ascribed to the faster rate of

mutation for the ND2 region when compared to the CR Irrespective of the exact relationship to each other, the monophyly of the four main mtDNA clades can be seen as robust, and this is supported by a high number of mutational steps separating them (25 – 49 steps; Fig 6).

The SAMOVA results suggest that six populations can be found in the CFM. Three of these populations correspond to the already identified clades (the Cape Peninsula; northern CFM; Limietberg). However, it also suggests that the central CFM clade can be further divided into three populations (west, middle and east groupings; Fig. 3). This shows that SAMOVA was effective in detecting additional groups within the CFM, making it a useful addition. For example, this analysis allowed the *a posteriori* identification of populations that the networks did not indicate with any sort of statistical confidence.

Nested clade analyses also supported subdivision of the central CFM clade (middle-eastern group and western group) and suggested that this resulted from restricted gene flow with isolation by distance and, in addition, indicated that the western group is also highly diversified. However, not all the localities within the western group found by the nested clade analysis match up with the localities suggested by the SAMOVA analysis. The situation is clearly complex and would require more fine scale sampling in the potential zone of contact. Irrespective, all the analyses showed a high diversification in the western part of the CFR, which is consistent with the patterns reported for the some CFR flora (Linder 2003).

At the larger scale, all *A. atra* populations within the CFM fall within the south-eastern clade previously detected by Matthee and Flemming (2002). Specimens sampled at Nieuwoudtville, Grahamstown, Qwa-Qwa, Bloemfontein, Beaufort West and Transkei fall either within or closer to the central CFM clade than to any other clade detected in this study. The central CFM clade may thus be widely spread over a much greater geographical area. This is supported by the fact that the central CFM clade contains the highest haplotype and nucleotide diversity (Tables 3, 5, 7). From a geographical perspective, most of the south-eastern clade (Matthee & Flemming 2002) populations inhabit the Drakensberg/Great Escarpment mountain range (which is mostly continuous along the southern and eastern coast of Southern Africa), whereas Bloemfontein (basal to the central CFM clade) is more isolated. However, the isolated sampling locality of Pretoria seem to be the exception, which confirm the more isolated nature of the Pretoria population found by Matthee and Flemming (2002). This is particularly evident in the Bayesian analysis, which is more sensitive for short branches (Huelsenbeck & Ronquest 2004). The Pretoria population occupies isolated rocky outcrops and might represent one of the populations at the edge of the distribution of the species.

The four clades found in the CFM are interesting and warrant some further discussion as to the potential factors causing the genetic isolation among these lineages. The CFM is an ancient mountain range, more then 200 MYA old (Gresse *et al.* 1992; Hälbich *et al.* 1983), thus the uplifting of these mountains played no roll in the divergence among *A. atra* clades. Floristically there is a change in species richness and endemism between the western and eastern halves of the CFR, with the western half containing approximately double the number of species and endemism than the

eastern half (Oliver *et al.* 1983; Goldblatt & Manning 2000; Linder 2001; Linder 2003). These differences in the flora species richness and endemism between these western and eastern halves of the CFR are credited to the distinct climate and topography of these two regions (Linder 2003). The eastern region receives summer rain, caused by the south-easterly trade winds from warm Indian Ocean, and winter rains, from the westerly winds bringing rain off the Atlantic. These two systems make the eastern region an area characterized by an all-year rainfall regime. However, the western region falls in the rain-shadow of Caledon and the Riviersonderend Mountains, which effectively cut off the south-easterly trade winds, eliminating summer rainfall in this area. Different climatic histories could have been found in these two regions, because changes (decrease or increase) in the south-easterly trade wind would have a major impact on the eastern CFR, but have little impact on the western part. In contrast, changes in circumpolar circulation would have a greater impact on the western CFR than the eastern areas (Cowling *et al.* 1999; Linder 2003). Some CFR flora showed a high diversification in the west in contrast to the relatively species-poor Southern/Eastern Cape (*Thamnochortus*, Linder & Mann 1998; *Pelargonium*, Bakker *et al.* 1999). This high diversification can be due to colonisation from the south or a climatic change (Linder & Mann 1998; Linder 2003). In the case of the latter, the entire CFR may have originally had the same climate as the Southern Cape has today, with the dry summers in the western parts evolving later, leading to further speciation in the west. These climatic conditions may have also caused the high diversification in *A. atra* in the western parts of the CFM.

Furthermore, in areas that have been repeatedly affected by climatic and vegetation changes, such as the CFM, the observed spatial distribution of genetic variation may

be strongly influenced by extinction/recolonization or expanding/retracting cycles. For instance, when the population contracted, a once continuous population could have been divided into isolated "islands". These "islands" in return would enhance allopatric speciation (Brain 1985; Bauer 1999), especially in species that are habitat specific.

For example, the high level of floral endemism in the Western Cape Province has been associated with habitat fragmentation and range restrictions due to climate variations (Linder *et al.* 1992; Midgley *et al.* 2001; Linder 2003). Furthermore, the Pliocene interglacial and glacial periods are thought to have been a major driving force in floral speciation and differentiation in this region (Richardson *et al.* 2001). Thus, although no obvious physical barrier can be detected between the northern CFM clade, the Limietberg clade and the central CFM clade, these groupings may be the result of climate change or the resulting habitat fragmentation. Southern Africa had experienced a range of extreme aridity and more mesic periods throughout the Pliocene oscillating periods (Van Zinderen Bakke & Mercer 1986; Lancaster 1989). If the molecular clock is correct, the main cladogenesis of *A. atra* within the CFM took place approximately ~6.5 - 9 MYA. Thus, the Pliocene oscillating periods could have played a roll in the evolution of *A. atra*, by fragmenting a once widespread and genetically variable *A. atra* population in different refugia, with re-expansions when the climate became more suitable. This sequence of events would probably have cause a reduced population size, range and genetic variability. Thus, different outcomes of genetic drift in each of the remaining populations may explain the current genetic pattern within the CFM. The Pleistocene has previously been recognised as a period that accounts for the diversity in intraspecific phylogeographic

units (Crochet *et al.* 2004; Hedges 1999). However for some lizard species diversification originated from much older events (*C. oelofseni*, Daniëls et al. 2003).

However, vegetation zones have also responded to the climatic conditions mentioned above. This response was complex given that isolated patches of various vegetation types were regularly created by the climatic fluctuations, several of which may have been isolated from each other for some time (Scott 1995; Midgley *et al.* 2001; Barrable *et al.* 2002). For example, during the Miocene, subtropical forest was the dominant vegetation type in the Cape. However glacial periods in the Pliocene resulted in drier climatic conditions and rainforest zones retracted, exposing novel habitats (Hewitt 2000). In the wetter interglacial periods of the Pliocene the forest zones expanded again. *Agama atra* is absent in dense forests and on rocky outcrops that are surrounded by dense vegetation (Burrage 1974). Thus it is likely that *A. atra* within the CFM has undergone several periods of isolation as a result of forest fluctuations and changes.

The Cape Peninsula clade may be the result of the above-mentioned isolations. It has however also been documented that sea-level changes (due to glacial/interglacial cycles) were responsible for flooding the Cape Flats. The Cape Flats is a coastal plain that separates the Cape Peninsula from the rest of the CFM, and is presently only a few metres above sea level. Sea-level changes have occurred throughout the Cenozoic to the middle Miocene, and the glacial and interglacial cycles of the Pliocene (Deacon 1983; Hendey 1983b). Although the last time the Cape Flats was flooded was in the early Pliocene sea-level changes still occurred throughout the Pleistocene and Quarternary interglacial, but it has risen no more than 6 m (Hendey 1983b). During

these interglacial periods warm, mesic conditions prevailed and the Cape Flats was periodically covered with forest during these wetter periods (Hendey 1983a, b). These periods may have interrupted a more widespread *A. atra* distribution, which occurred during the colder and drier glacial periods. Thus, the *A. atra* population may have become isolated in the Cape Peninsula, where subsequently this group has evolved. What makes this pattern particularly intriguing is the fact that genetic isolation of the Cape Peninsula region has also been detected in other fauna species such as in isopod species of the genus *Mesamphisopus* (Gouws *et al.* 2003) and a freshwater crab species, *Potamonautes brincki* (Daniels *et al.* 2001). The Cape Peninsula is also well known for its high endemism in plant species (Trinder-Smith *et al.* 1996; Linder & Mann 1998; Linder 2003).

4.3 High genetic diversification

Various of studies have also found that high levels of genetic differentiation can occur among geographic populations within a single species (e.g. Gifford *et al.* 2004). High levels of interspecific variation have also been reported in conspecific reptile taxa for the ND2 gene. For example, in the same region, sequence divergence between closely related *Bradypodion* species ranges from 2.3% between *B. ventrale* and *B. taeniabronchum* to 16.5% between *B. setaroi* and *B. pumilum* (ND2, 987 bp; Tolley *et al.* 2004). Variation between two *Laudakia* species (*L. erythrogastra* and *L. caucasia;* Family: Agamadidae) is ± 5% (Macey 1998). At this stage it is not possible to make firm taxonomic conclusions based on the outcome of this study but interestingly, the observed level of ND2 sequence divergence between *A. atra* lineages falls within these interspecific levels when compared to other lizards. Clearly, more intensive

sampling in the contact zones, morphological investigations and nuclear DNA markers are needed for a comprehensive taxonomic revision.

4.4 Conservation implications

In a conservation framework, terms such as management unit (MU) or evolutionary significant unit (ESU) have been proposed to avoid the species definition problem (Ryder 1986; Moritz 1994). MUs are identified as populations that differ considerably in allele frequency distributions and have substantial divergences in mtDNA or nuclear DNA, whereas ESUs are defined by the presence of both. More recently it has been suggested that these populations must also be separated on the basis of morphological or ecological differences. It is not known whether any morphological or ecological differences among the four CFM clades (Cape Peninsula clade, northern CFM clade, Limietberg clade and central CFM clade) exist or whether these clades will be retained if nuclear loci are added to the analysis. Thus, the four clades from the CFM, among which there is probably little or no gene flow, can at this stage be considered as different MUs (Moritz 1994) that deserve separate conservation status.

Conservation genetics entails the use of genetics to preserve species as dynamic entities capable of coping with environmental changes. Studies involving multiple taxa and congruency in their phylogeographical patterns have important conservation implications (Avise 2000; Ditchfield 2000; Sullivan *et al.* 2000; Arbogast & Kenagy 2001; Branch *et al.* 2003). Numerous taxa also have the northern CFM clade spilt, especially for the Cederberg, which is known for its uniqueness and high degree of endemism (e.g. this study; Linder & Mann 1998; Linder 2003; Makokha 2004). In addition, the Cape Peninsula clade can also be seen in a number of different species

(Harrison & Barnard 1972; Daniels *et al.* 2001; Gouws *et al.* 2003) and this region is inhabited by several endemic plant and animal species (Picker & Samways 1996; Trinder-Smith *et al.* 1996; Linder & Mann 1998; Linder 2003). Clearly these areas can be regarded as "hotspots" for speciation. In concert these results suggest that the Cederberg (represented by the northern CFM clade) and the Cape Peninsula are each inhabited by a distinct group of fauna and conservation decisions concerning these areas should be taken with caution in order to preserve as much of the biodiversity as possible.

References

Althoff DM, Pellmyr O (2002) Examining genetic structure in a Bogus Yucca moth: A sequential approach to phylogeography. *Evolution*, **56**, 1632-1643.

Anderson S, Bankier AT, Barrell BG *et al.* (1981) Sequence and organization of the human mitochondrial genome. *Nature*, **290**, 457-465.

Arbogast BS, Kenagy GJ (2001) Comparative phylogeography as an integrative approach to historical biogeography. *Journal of Biogeography*, **28**, 819-825.

Ashton KG, de Queiroz A (2001) Molecular systematic of the Western Rattlesnake, *Crotalus viridis* (Viperidae), with comments on the utility of the D-loop in phylogentic studies of snakes. *Molecular Phylogenetic and Evolution*, **21**, 176-189.

Avise JC (1994) *Molecular Markers, Natural History, and Evolution*. Chapman and Hall. New York.

Avise JC (2000) *Phylogeography: The History and Formation of Species*. Harvard University Press, Cambridge, MA.

Avise JC, Arnold J, Ball RM, Bermingham E, Lamb T, Neigel JE, Reeb CA, Saunders NC (1987) Intraspecific phylogeography: the mitochondrial DNA bridge between population genetics and systematics. *Annual Review of Ecology and Systematics*, **18**, 489-522.

Baard EHW, Branch WR, Channing AC, De Villiers AL, Le Roux A, Mouton PleFN (1999) A review of the amphibians and reptiles of the Cape Floristic Region as indicators of centres of biodiversity, sensitive habitats and sites of special interest.

Western Cape Nature Conservation Board, Stellenbosch. Prepared for WWF-SA. http://www.panda.org.za/megaprojects_cape.htm.

Bakker FT, Culham A, Gibby M (1999) Phylogenetics and diversification in Pelargonium. In *Molecular Systematics and Plant Evolution* (eds. P. M. Hollingsworth, R. M. Bateman and R. J. Gornall), pp. 353-374. Taylor & Francis, London.

Bandelt HJ, Forster P, Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, **16**, 37-48.

Barrable A, Meadows ME, Hewitson BC (2002) Environmental reconstruction and climate modelling of the Late Quaternary in the winter rainfall region of the Western Cape, South Africa. *South African Journal of Science*, **98**, 611-616.

Bauer AM (1999) Evolutionary scenarios in the *Pachydactylus* group geckos of Southern Africa: New Hypotheses. *African Journal of Herpetology*, **48**, 53-62.

Beheregaray LB, Sunnucks P (2001) Fine-scale genetic structure, estuarine colonization and incipient speciation in the marine silverside fish *Odontesthes argentinensis*. *Molecular Ecology*, **10**, 2849-2866.

Branch LC, Clark AM, Moler PE, Bowen BW (2003) Fragmented landscapes, habitat specificity, and conservation three lizards in Florida scrub. *Conservation Genetics*, **4**, 199-212.

Branch WR (1988) *Field Guide to Snakes and Other Reptiles of Southern Africa*. Struik Publishers Ltd., Cape Town.

Branco M, Monnerot M, Ferrand N, Templeton AR (2002) Postglacial dispersal of the European rabbit (*Oryctolagus cuniculus*) on the Iberian Peninsula reconstructed from nested glade and mismatch analyses of mitochondrial DNA genetic variation. *Evolution*, **56**, 792-803.

Brehm A, Harris DJ, Alves C, Jesus J, Thomarat F, Vicente L (2003) Structure and evolution of the mitochondrial DNA complete control region in the lizard *Lacerta dugesii* (Lacertidae, Sauria). *Journal of Molecular Evolution*, **56**, 46-53.

Bromham LD, Rambaut AE, Harvey PH (1996) Determinants of rate variation in DNA sequence evolution of mammals. *Journal of Molecular Evolution*, **43**, 610-621.

Brown RP, Suárez NM, Pestano J (2002) The Atlas mountains as a biographical divide in North-West Africa: evidence from mtDNA evolution in the Agamid Lizard *Agama impalearis. Molecular Phylogenetics and Evolution*, **24**, 324-332.

Brown WM, George M Jr, Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. *Proceedings of the National Academy of Science of the United States of America*, **79**, 1967-1971.

Burland TM, Worthington WJ (2001) Seeing in the dark: molecular approaches to the study of bat populations. *Biological Reviews*, **76**, 389–409.

Burrage BR (1974) Population structure on *Agama atra* and *Cordylus cordylus* in the vicinity of De Kelders, Cape Province. *Annuals of the South African Museum*, **66**, 1-23.

Channing A, Hendricks D, Dawood A (1994) Description of a new moss frog from the south-western Cape (Anura: Ranidae: *Arthroleptella). South African Journal of Zoology*, **29**, 240-243.

Clement M, Posada D, Crandall KA (2000) TCS a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657-1659.

Costello AB, Down TE, Pollard SM, Pacas CJ, Taylor EB (2003) The influence of history and contemporary stream hydrology on the evolution of genetic diversity within species: An examination of microsatellite DNA variation in bull trout, *Salvelinus confluentus* (Pisces: Salmonidae). *Evolution*, **57**, 328-344.

Cowling RM, Cartwright CR, Parkington JE, Allsopp JC (1999) Fossil wood charcoal assemblages from Elands Bay Cave, South Africa: implications for Late Quaternary vegetation and climates in the winter-rainfall fynbos biome. *Journal of Biogeography*, **26**, 367-378.

Cowling RM, Holmes PM (1992) Flora and vegetation. In: *The Ecology of Fynbos, Nutrients, Fire and Diversity* (ed. Cowling RM), pp. 23-61. Oxford University Press, Oxford, UK.

Crochet PA, Chaline O, Surget-Groba Y, Debain C, Cheylan M (2004) Speciation in mountains: phylogeography and phylogeny of the rock lizards genus Iberolacerta (Reptilia: Lacertidae). *Molecular Phylogenetics and Evolution*, **30**, 860-866.

Daniels SR, Mouton PleFN, Du Toit DA (2004) Molecular data suggest that melanistic ectotherms at the south-western tip of Africa are the products of Miocene climatic events: evidence from cordylid lizards. *Journal of Zoology, London*, **263**, 373-383.

Daniels SR, Stewart BA, Burmeister L (2001) Geographic patterns of genetic and morphological divergence amongst populations of a river crab (Decapoda, Potamonautidae) with the description of a new species form mountains streams in the Western Cape, South Africa. *Zoologica Scripta*, **30**, 181-197.

Deacon HJ (1983) An introduction to the fynbos region, time-scale and palaeoenvironments. In Deacon HJ, Hendey OB, Lambrachts JJN (eds.) *Fynbos Palaeoecoalgy: A preliminary synthesis. Southern African National Scientific Programmes Report* 75 CSIR, Pretoria.

Department of Environmental Affairs and Tourism (DEAT) (1997) Environmental potential atlas for South Africa. Pretoria. South Africa: JL van Schalk Publishers.

Ditchfield AD (2000) The comparative phylogeography of neotropical mammals: patterns of intraspecific mitochondrial DNA variation among bats contrasted to nonvolant small mammals. *Molecular Ecology*, **9**, 1307-1318.

Dupanloup I, Schneider S, Excoffier L (2002) A simulated annealing approach to define the genetic structure of populations. *Molecular Ecology*, **11**, 2571-2581.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479-491.

Felsenstein J (1993) PHYLIP (Phylogeny Inference Package) Version 3.5c. Department of Genetics, University of Washington, Seattle.

Fleming AF (1996) An analysis of the *Agama atra* complex (Reptilia: Agamidae) in Namaqualand and Southern Namibia. PhD Thesis, University of Stellenbosch, Stellenbosch.

Gelderblom CM, Bronner GN (1995) Patterns of distribution and protection status of the endemic mammals in South Africa. *South African Journal of Zoology*, **30**, 115-126.

Gifford ME, Powell R, Larson A, Gutberlet RL Jr (2004) Population structure and history of a phenotypically variable teiid lizard (Ameiva chrysolaema) from Hispaniola: the influence of a geologically complex island**.** *Molecular Phylogenetics and Evolution*, **32**, 735-748.

Glor RE, Vitt LJ, Larson A (2001) A molecular phylogenetic analysis of diversification in Amazonian *Anolis* lizards. *Molecular Ecology*, **10**, 2661-2668.

Goldblatt P, Manning J (2000) *Cape Plants. A Conspectus of the Cape Flora of South Africa.* National Botanical Institute, Pretoria.

Gouws G, Stewart BA, Daniels SR (2003) Cryptic species within the freshwater isopod *Mesamphisopus capensis* (Phreatoicidea: Amphisopodidae) in the Western Cape, South Africa: Alozyme and 12S rRNA sequence data and morphometric evidence. *Biological Journal of the Linnean Society*, **81**, 235-253.

Graur D, Li WH (1991) Neatral mutation hypotesis test. *Nature*, **354**, 114-115

Graur D, Martin W (2004) Reading the entrails of chickens: molecular timescales of evolution and the illusion of precision. *Trends in Ecology and Evolution*, **20**, 80-86.

Gresse PG, Theron JN, Fitch FJ, Miller JA (1992) Tectonic inversion and radiometric resetting of the basement in the Cape Fold Belt. In: *Inversion Tectonics of the Cape Fold Belt, Karoo and Cretaceous Basins of Southern Africa* (Ed. by De Wit M, Ransome IGD). Balkema, Rotterdam, 217-228.

Griswold CK, Baker AJ (2002) Time to the most recent common ancestor and divergence times of populations of common chaffinches (*Fringilla coelebs*) in Europe and North Africa: Insights into Pleistocene refugia and current levels of migration. *Evolution*, **56**, 143-153.

Guindon S, Gascuel O (2003) A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology*, **52**, 696-704.

Hälbich IW, Fitch FJ, Miller JA (1983) Dating the Cape Orogeny. In: *Geodynamics of the Cape Fold Belt* (Ed. by Söhnge APG, Hälbich IW). Special publication of the Geological Society of South Africa, 149-164.

Harrison AD, Barnard KH (1972) The stream fauna of an isolated mountain massif, Table Mountain, Cape Town, South Africa. *Transactions of the Royal Society of South Africa*, **40**, 135-153.

Hendey QB (1983a) Palaeontology and palaeoecology of the fynbos region: an introduction. In: Deacon HJ, Hendey QB, Lambrechts NNJ, eds. *Fynbos palaeoecology: a preliminary synthesis*. Pretoria: CSIR, 87-99.

Hendey QB (1983b) Cenozoic geology and palaeogeography of the fynbos region. In: Deacon HJ, Hendey QB, Lambrechts NNJ, eds. *Fynbos palaeoecology: a preliminary synthesis*. Pretoria: CSIR, 35-60.

Hewitt GM (2000) The genetic legacy of the quaternary ice ages. *Nature*, **405**, 907-913.

Hillis D M, Moritz C, Mable BK (1996) *Molecular Systematics*. Sinauer Associates, Inc., Sunderland, Massachusetts U.S.A.

Hirota T, Hirohata T, Mashima H, Satoh T, Obara Y (2004) Population structure of the large Japanese field mouse, *Apodemus speciosus* (Rodentia: Muridae), in suburban landscape, based on mitochondrial D-loop sequences. *Molecular Ecology*, **13**, 3275-3282.

Hoelzel AR, Lopez JV, Dover GA, O'Brien SJ (1994) Rapid evolution of a heteroplasmic repetitive sequence in the mitochondrial DNA control region of carnivores. *Journal of Molecular Evolution*, **39**,191–199.

Huelsenbeck JP, Ronquist F (2001) Mr Bayes: a program for the Bayesian inference of phylogeny.

Huelsenbeck JP, Ronquist F (2004) Bayesian Analysis of Molecular Evolution using MrBayes. http://workshop.molecularevolution.org/software/mrbayes/files/mrbayes_2004.pdf

Hurwood DA, Hughes JM (2001) Nested clade analysis of the freshwater shrimp, *Caridina zebra* (Decapoda: Atyidae), from north-eastern Australia. *Molecular Ecology*, **10**, 113-125.

Hurwood DA, Hughes JM (1998) Phylogeography of the freshwater fish, *Mogurnda adspersa*, in streams of northeastern Queensland, Australia: evidence for altered drainage patterns. *Molecular Ecology*, **7**, 1507-1517.

Jacobsen NHG (1994) The *Platysaurus intermedius* complex (Sauria: Cordylidae) in the Transvaal, South Africa, with descriptions of three new taxa. *South African Journal of Zoology*, **29**, 132-143.

Kerth G, Mayer F, Petit E (2002) Extreme sex-biased dispersal in the communally breeding, nonmigratory Bechstein's bat (*Myotis bechsteinii*). *Molecular Ecology*, **11**, 1491-1498.

Knowles LL, Maddison WP (2002) Statistical phylogeography. *Molecular Ecology*, **11**, 2623-2635.

Knowles LL (2004) The burgeoning field of statistical phylogeography, *Journal of evolutionary biology,* 17, 1-10.

Koepfli KP, Wayne RK (1998) Phylogenetic relationships of otters (Carnivora : Mustelidae) based on mitochondrial cytochrome b sequences. Journal of Zoology, 246, 401-419

Kotlik P, Berrebi P (2002) Genetic subdivision and biogeography of the Danubian rheophilic barb *Barbus petenyi* inferred from phylogenetic analysis of mitochondrial DNA variation. *Molecular Phylogenetics and Evolution*, **24**, 10-18.

Kumar S, Tamura K, Jakobsen IB, Nei M (2001) MEGA version 2.1. Molecular Evolutionary Genetics Analysis software, Arizona State University, Tempe, Arizona, USA.

Lamb T, Bauer AM (2000) Relationship of the Pachydactylus rugosus group of geckos (Reptilia: Squamata: Gekkodidae). *African Zoology*, **35**, 55-67.

Lancaster N (1989) Late Quaternary palaeoenviroments in the southwestern Kalahari. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **70**, 367-376.

Linder HP (2001) On areas of endemism, with an example from the African Restionaceae. *Systematic* Biology, **50**, 892-912.

Linder HP (2003) The radiation of the Cape flora, Southern Africa. *Biological Reviews*, **78**, 597-638.

Linder HP, Mann DM (1998) The phylogeny and biogeography of Thamnochortus (Restionaceae). *Botanical Journal of the Linnean Society*, **128**, 319-357.

Linder HP, Meadows ME, Cowling RM (1992) History of the Cape flora. In: *The Ecology of Fynbos, Nutrients, Fire and Diversity* (ed. Cowling RM), pp. 113-134. Oxford University Press, Oxford, UK.

Lloyd BD (2003) Intraspecific phylogeny of the New Zealand short-tailed bat *Mystacina tuberculata* inferred from multiple mitochondrial gene sequences. *Systematic Biology*, **52**, 460-476.

Lopez JV, Culver M, Stephens JC, Johnson WE, O'Brien SJ (1997) Rates of nuclear and cytoplasmic mitochondrial DNA sequence divergence in mammals. *Molecular Biology and Evolution*, **14**, 277-286.

Macaulay V, Richards M, Sykes B (1999) Mitochondrial DNA recombination - no need to panic. *Proceedings of the Royal Society of London B*, **266**, 2037-2039.

Macey JR, Larson A, Ananjeva NB, Papenfuss TJ (1997a) Evolutionary shifts in three major structural features of the mitochondrial genome among iguanian lizards. *Journal of Molecular Evolution*, **44**, 660-674.

Macey JR, Larson A, Ananjeva NB, Fang ZL, Papenfuss T (1997b) Two novel gene orders and the role of light-strand replication in rearrangement of the vertebrate mitochondrial genome. *Molecular Biology and Evolution*, **14**, 91-104.

Macey JR, Schulte JA, Ananjeva NB, Larson A, Rastegar-Pouyani N, Shammakov SM, Papenfuss TJ (1998) Phylogenetic relationship among agamid lizards of the *Laudakia caucasia* species group: testing hypotheses of biographic fragmentation and an area cladogram for the Iranian Plateau. *Molecular Phylogenetics and Evolution*, **10**, 118-131.

Maddison WP, Maddison DR (1992) MacClade: analysis of phylogeny and character evolution, version 4.0. Sinauer, Sunderland, MA.

Makokha JS (2004) mtDNA phylogeography of Burchell's Sand Lizard (*Pedioplanis burchelli*) in the Cape Fold Mountains. Honours Thesis, University of Stellenbosch, Stellenbosch.

Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research*, **27**, 209-220.

Matthee CA, Flemming AF (2002) Population fragmentation in the southern rock agama, *Agama atra*: more evidence for vicariance in Southern Africa. *Molecular Ecology*, **11**, 465-471.

Matthee CA, Robinson TJ (1996) Mitochondrial DNA differentiation among geographical populations of *Pronolagus rupestris*, Smith's red rock rabbit (Mammalia: Lagomorpha). *Heredity*, **76**, 514-523.

Matthee CA, Robinson TJ (1999) Mitochondrial DNA population structure of roan and sable antelope: implications for the translocation and conservation of the species. *Molecular Ecology*, **8**, 227-238.

Matthee CA, Tilbury CR, Townsend T (2004) A phylogenetic review of the African leaf chameleons: genus *Rhampholeon* (Chamaeleonidae): the role of vicariance

and climate change in speciation. *Proceedings of the Royal Society of London B*, **271**, 1967-1976.

Midgley GF, Hannah L, Roberts R, MacDonald DJ, Allsopp J (2001) Have Pleistocene climatic cycles influenced species richness patterns in the greater Cape Mediterranean Region? *Journal of Mediterranean Ecology*, **2**, 137-144.

Miller-Butterworth CM, Jacobs DS, Harley EH (2003) Strong population substructure is correlated with morphology and ecology in a migratory bat. *Nature*, **424**, 187-191.

Moritz C (1994) Defining 'Evolutionarily Significant Units' for conservation. *Trends in Ecology and Evolution*, **9**, 373-375.

Mouton PLeFN, Herselman YM (1994) Paradoxical reproduction and body size in the rock lizard, *Agama atra atra*, Namaqualand, South Africa. *South African Journal of Zoology*, **29**, 199-203.

Myers N, Mittermeier CG, da Fonseca GAB, Keny J (2000) Biodiversity hotspots for conservation priorities. *Nature*, **403**, 853-858.

Nielson M, Lohman K, Sullivan J (2001) Phylogeography of the tailed frog (*Ascaphius truei*) : implications for the biogeography of the Pacific northwest. *Evolution*, **55**, 147-160.

Newton LR, Nassar JM, Fleming TH (2003) Genetic population structure and mobility of two nectar feeding bats from Venezuelan desert: inferences from mitochondrial DNA. *Molecular Ecology*, **12**, 3191-3198.

Oliver EGH, Linder HP, Rourke JP (1983). Geographical distribution of present-day Cape taxa and their phytogeographical significance. *Bothalia*, **14**, 427-440.

Page R (1996) Treeview: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences*, **12**, 357-358.

Palumbi SR, Kessing BD (1991) Population biology of the trans-arctic exchange: mtDNA sequence similarity between Pacific and Atlantic sea urchins. *Evolution*, **45**, 1790-1805.

Pesole G, Gissi C, De Chirico A, Saccone C (1999) Nucleotide substitution rate of mammalian mitochondrial genomes. *Journal of Molecular Evolution*, **48**, 427-434.

Picker MD, Samways MJ (1996) Faunal diversity and endemicity of the Cape Peninsula, South Africa - a first assessment. *Biodiversity and Conservation*, **5**, 591-606.

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinfomatics*, **14**, 817-818.

Posada D, Crandall KA, Templeton AR (2000) GeoDis: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology*, **9**, 487-488.

Poulakakis N, Lymberakis P, Antoniou A, Chalkia D, Zouros E, Mylonas M, Valakos E (2003) Molecular phylogeny and biogeography of the wall-lizard *Podarcis erhardii* (Squamata: Lacertidae). *Molecular Phylogenetic and Evolution*, **28**, 38-46.

Poynton JC, Broadley DG (1978) The herpetofauna. In: *Biogeography and ecology of Southern Africa* (ed. Werger, MJA), pp. 925-948. Dr. W. Junk, The Hague.

Prinsloo P, Robinson TJ (1992) Geographic mitochondrial DNA variation in the rock hyrax, *Procavia capensis*. *Molecular Biology and Evolution*, **9**, 447-456.

Rassmann K, Tautz D, Rassmann K, Gliddon C (1997) The microevolution of the Galápagos marine iguana *Amblyrhynchus cristatus* assessed by nuclear and mitochondrial genetic analyses. *Molecular Ecology*, **6**, 437-452.

Ravaoarimanana IB, Tiedemann R, Montagnon D, Rumpler Y (2004) Molecular and cytogenetic evidence for cryptic speciation within a rare endemic Malagasy lemur, the Northern Sportive Lemur (Lepilemur septentrionalis). *Molecular Phylogenetics and Evolution*, **31**, 440-448.

Raxworthy CJ, Forstner MRJ, Nussbaum RA (2002) Chameleon radiation by oceanic dispersal. *Nature*, **415**, 784-787.

Richardson JE, Pennington RT, Pennington TD, Hollingsworth PM (2001) Rapid diversification of a species-rich genus of Neotropical rain forest trees. *Science*, **293**, 2242-2245.

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology.* Humana Press, Totowa, NJ, pp 365-386.

Ryder OA (1986) Species conservation and systematics: The dilemma of subspecies. *Trends in Ecology and Evolution*, **1**, 9-10.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual* (2nd Ed). Cold Spring Harbor Laboratory Press, New York.

Sbisa` E, Tanzariello F, Reyes A, Pesole G, Saccone C (1997) Mammalian mitochondrial D-loop region structural analysis: Identification of new conserved sequences and their functional and evolutionary implications. *Gene*, **205**,125-140.

Schluter D (2000) *The Ecology of Adaptive Radiation*. Oxford University. Press, Oxford.

Schneider S, Roessli D, Excoffier L (2000) Arlequin: a software for population genetics data analysis. Version 2.0 Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva.

Scott L (1995) Pollen evidence for vegetational and climatic change in Southern Africa during the Neogene and Quaternary. In: *Paleoclimate and Evolution, with Emphasis on Human Origins* (eds. Vrba ES, Denton GH, Partridge TC, Burckle LH), pp. 65-67. Yale University Press, New Haven, USA.

Slatkin M (1987) Gene flow and the geographic structure of natural populations. *Science*, **236**, 787-792.

Small MP, Stone KD, Cook JA (2003) American marten (*Martes americana*) in the Pacific Northwest: population differentiation across a landscape fragmented in time and space. *Molecular Ecology*, **12**, 89-103.

Sullivan J, Arellano E, Rogers DS (2000) Comparative phylogeography of Mesoamerican highland rodents: Concerted vs. independent response to past climatic fluctuations. *American Naturalist*, **155**, 755-768.

Swofford DL (2002) *PAUP* Phylogeny analysis using parsimony (*and other methods), version 4. 0b10*. Sinauer Association, Inc., Sutherland, MA.

Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, **10**, 512-526.

Templeton AR, Sing CF (1993) A cladistic analysis of phenotypic association with haplotypes inferred from restriction endonuclease mapping. IV. Nested analysis with cladogram uncertainty and recombination. *Genetics*, **134**, 659-669.

Templeton AR, Boerwinkle E, Sing CF (1987) A cladistic analysis of phenotypic association with haplotypes inferred from restriction endonuclease mapping. I. Basic theory and an analysis of alcohol dehydrogenase activity in *Drosophila*. *Genetics*, **117**, 343-351.

Templeton AR (2004) Statistical phylogeography: methods of evaluating and minimizing inference errors. *Molecular Ecology*, **13**, 789-809.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, **24**, 4876-4882.

Tolley KA, Tilbury CR, Branch WR, Matthee CA (2004) Evolutionary history and phylogenetics of the southern African dwarf chameleons, *Bradypodion. Molecular Phylogenetics and Evolution*, **30**, 354-365.

Townsend T, Larson A (2002) Molecular phylogenetics and mitochondrial genomic evolution in the Chamaeleonidae (Reptilia, Squamata). *Molecular Phylogenetics and Evolution*, **23**, 22-26.

Trinder-Smith TH, Cowling RM, Linder HP (1996) Profiling a besieged flora: endemic and threatened plants of the Cape Peninsula, South Africa. *Biodiversity and Conservation*, **5**, 649-669.

Van Zinderen Bakker EM, Mercer JH (1986) Major late Cainozoic climate events and palaeoenviromental changes in Africa viewed in a world wide context. *Palaeogeography, Palaeoclimatology, Palaeoecology*, **56**, 217-235.

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. *Evolution*, **38**, 1358-1370.

Winney BJ, Hammond RL, Macasero W, Flores B, Boug A, Biquand V, Biquand S, Bruford MW (2004) Crossing the Red Sea: phylogeography of the hamadryas baboon, *Papio hamadryas hamadryas. Molecular Ecology*, **13**, 2819-2827.

Willis KJ, Whittaker RL (2000) The refugial debate. *Science*, **287**, 1406-1407.

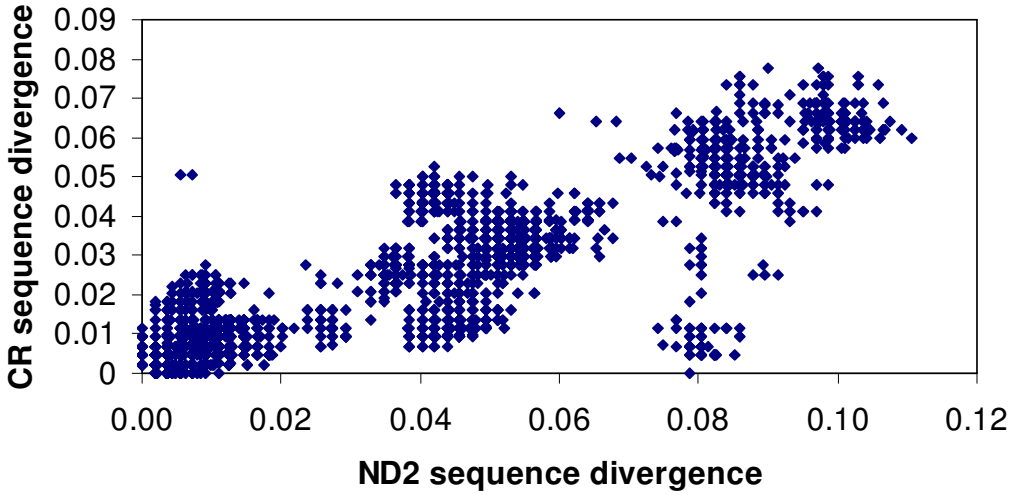
Appendix A. List of the *A. atra* specimens examined, with ID/sample number, sampling locality and GenBank accession numbers for each mtDNA fragment. Sampling locality details are given as decimal degrees for *A. atra* and the locality names correspond to those in Fig. 2.

Sample no.	Locality	ND2	CR	Long East	Lat South
A10	Gordons Bay			18.956	-34.220
A26	Port Elizabeth			25.600	-33.967
A27	Port Elizabeth			25.600	-33.967
A7	Gordons Bay			18.956	-34.220
A8	Gordons Bay			18.956	-34.220
A9	Gordons Bay			18.956	-34.220
MH0710/AA10	Riviersonderendberg			19.520	-33.954
MH0826/AA11	Gordons Bay			18.875	-32.287
MH0828/AA12	Turret Peak, Cederberg			19.216	-32.892
MH0730/AA15	Sneeukop, Cederberg,			19.162	-32.354
MH0836/AA17	Waboomsberg			19.469	-33.258
MH0767/AA18	Kaggakamma			19.536	-32.748
MH0819/AA19	northern Cederberg			19.209	-32.415
MH0195/AA1	Keeromsberg, Hex River Mountains			19.664	-33.565
MH0332/AA20	northern Cederberg			19.095	-32.073
KTH268	northern Cederberg			19.036	-32.161
MH1189/AA23	Attakwa Mountains			21.925	-33.826
MH1115/AA24	Louterwater, Tsitsikamma Mountains			23.673	-33.850
MH1117/AA25	Hudsonvale, Tsitsikamma Mountains			24.191	-33.952
AA26	Suurberg			25.823	-33.289
DDT01/AA27	southern Cederberg			19.317	-32.523
V206/AA2	Thomas Hut, Hex River Mountains			19.410	-33.549
VC024/AA4	Die Hel, Groot Swartberg			21.796	-33.349
V332/AA5	Millwood, Outeniqua Mountains			22.945	-33.816
CF125/AA6	southern Cederberg			19.273	-32.488
MH0689/AA7	Witteberg			20.511	-33.281
MH0690/AA8	Riviersonderendberg			19.491	-33.975
MH0683/AA9	Riviersonderendberg			19.894	-34.049
AABK1	Baviaanskloof Mountains			23.819	-33.614
AABK2	Baviaanskloof Mountains			24.089	-33.523
AABK3	Baviaanskloof Mountains			24.094	-33.524
AABK4	Baviaanskloof Mountains			24.051	-33.520
AACC1	Cockscomb, Groot Winterhoek Mountains			24.792	-33.568
AADP1	Devils Peak, Cape Peninsula			18.449	-33.951
AADP2	Devils Peak, Cape Peninsula			18.440	-33.955
AADR1	Outeniqua Mountains			22.089	-33.824
AADR2	Outeniqua Mountains			22.089	-33.824
AADR3	Outeniqua Mountains			22.089	-33.825
AAEB1	Elandsberg			25.066	-33.817
AAJH1	Jonkershoek			18.994	-34.003
AAJH2	Jonkershoek			18.970	-33.991
AAJH4	Jonkershoek			18.973	-33.993
AAJH5	Jonkershoek			18.976	-33.994
AAKS1	Klein Swartberg			21.208	-33.464
AAKS2	Klein Swartberg			21.207	-33.463
AAKS3	Klein Swartberg			21.200	-33.455
AAKS4	Klein Swartberg			21.200	-33.455
AAKS5	Klein Swartberg			21.392	-33.407
AAKS6	Klein Swartberg			21.392	-33.407
Sample no.	**Locality**	**ND2**	**CR**	**Long East**	**Lat South**

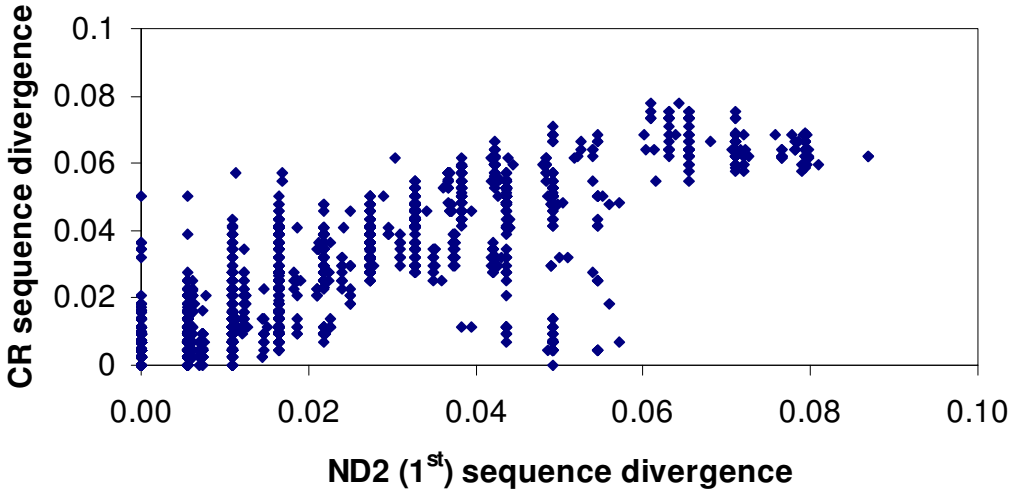
AALS1	Lady's Slipper, Vanstadensberg			25.268	-33.889
AALS2	Lady's Slipper, Vanstadensberg			25.268	-33.889
AALS3	Lady's Slipper, Vanstadensberg			25.261	-33.891
AASB1	Steenboksberg			19.452	-34.316
AASB2	Steenboksberg			19.453	-34.318
AASC1	Scarborough, Cape Peninsula			18.398	-34.208
BS10	Groot Winterhoek Nature Reserve			19.141	-33.033
BS11	Riviersonderend Mountains			19.519	-33.948
BS12	Riviersonderend Mountains			19.523	-33.926
BS13	Riviersonderend Mountains			19.525	-33.928
BS14	Riviersonderend Mountains			19.525	-33.928
BS15	Hartenbos			22.115	-34.133
BS16	Hartenbos			22.115	-34.133
BS18	Robertson Pass, Outeniqua Mountains			22.038	-33.845
BS19	Robertson Pass, Outeniqua Mountains			22.038	-33.845
BS20	Robertson Pass, Outeniqua Mountains			22.038	-33.845
BS21	Silvermine, Cape Peninsula			18.418	-34.103
BS22	Silvermine, Cape Peninsula			18.418	-34.103
BS23	Scarborough, Cape Peninsula			18.398	-34.208
BS24	Tradouw Pass, Langeberg			20.711	-33.990
BS25	Tradouw Pass, Langeberg			20.710	-33.986
BS5	Limietberg			19.101	-33.702
BS6	Limietberg			19.095	-33.681
BS7	Limietberg			19.088	-33.686
BS8	Groot Winterhoek Nature Reserve			19.083	-32.980
BS9	Groot Winterhoek Nature Reserve			19.094	-32.992
G1	Gordons Bay			18.956	-34.220
G2	Gordons Bay			18.956	-34.220
G3	Gordons Bay			18.956	-34.220
KTH147	Attakwa Mountains			21.929	-33.823
KTH148	Tsitsikamma Mountains			23.809	-33.863
KTH162	Tsitsikamma Mountains			24.191	-33.952
KTH170	Attakwa Mountains			21.928	-33.824
KTH175	Kareedouw Mountain			24.429	-34.019
KTH184	Suurberg			25.823	-33.289
KTH186	Attakwa Mountains			21.927	-33.824
KTH274	northern Cederberg			19.030	-32.162
KTH278	northern Cederberg			19.003	-32.138
KTH282	Bainskloof			19.110	-33.603
KTH283	northern Cederberg			19.001	-32.138
KTH40	Salmonsdam, Langeberg			19.656	-34.429
KTH58	Robertson			19.741	-33.777
KTH59	Cape Point, Cape Peninsula			18.474	-33.572
KTH66	Anysberg			20.471	-33.529
KTH9	Salmonsdam, Langeberg			19.656	-34.429
AAKF1	Waboomsberg			19.583	-32.867
KTH358	Bainskloof			19.102	-33.618
A2	Vaalputs			18.527	-30.134
A17	Eksteensfontein			17.254	-28.824
A15	Eksteensfontein			17.254	-28.824
A36	Postmansburg			22.938	-28.390
A12	Augrabies			20.333	-28.583
A40	Pretoria			28.278	-25.616
Sample no.	**Locality**	**ND2**	**CR**	**Long East**	**Lat South**

AA29	Transkei	No information	No information
AA30	Transkei	No information	No information
AA31	Transkei	No information	No information
A19	Bloemfontein	26.158	-29.039
A4	Nieuwoudtsville	19.107	-31.378
A28	Qwa-Qwa	28.667	-28.517
A32	Grahamstown	26.532	-33.302
A46	Beauford West	22.978	-31.798

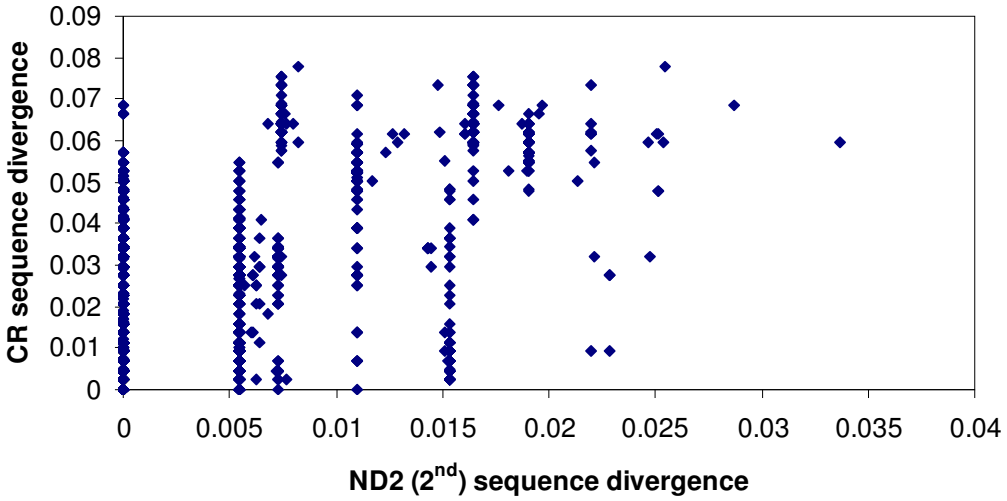
Appendix B: Uncorrected sequence divergence of the CR region vs uncorrected sequence divergences for the ND2 gene.



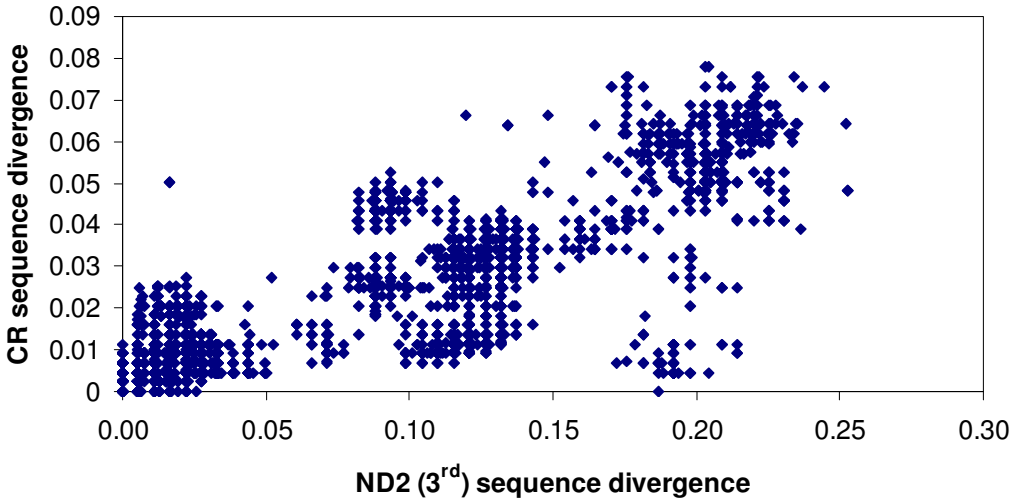
Uncorrected sequence divergence of the CR region vs. the overall uncorrected sequence divergences for the ND2 gene.



Uncorrected sequence divergence of the CR region vs. the uncorrected sequence divergences for the first codon position of the ND2 gene.



Uncorrected sequence divergence of the CR region vs. the uncorrected sequence divergences for the second codon position of the ND2 gene.



Uncorrected sequence divergence of the CR region vs. the uncorrected sequence divergences for the third codon position of the ND2 gene.