Phylogeography of the southern African vlei rat, *Otomys irroratus*, inferred from chromosomal and DNA sequence data

by

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Abstract

This study examines the phylogeography of the southern African vlei rat, *Otomys irroratus* using the mtDNA cyt *b* gene and chromosomal data derived using G-, and C-banding, Ag-NOR staining and fluorescence *in situ* hybridization (FISH using flow sorts of *Myotomys unisulcatus*). A total of $N = 102$ specimens were used from the Western Cape, Eastern Cape, Northern Cape, Free State, Mpumalanga and KwaZulu-Natal provinces of South Africa. Of the $N = 102$, $N = 55$ comprised fresh material while $N = 47$ comprises museum material obtained from the Durban Natural Science Museum of South Africa.

Cytogentic analysis of $N = 55$ specimens collected from seven localities in South Africa revealed intra-specific variation resulting from two rearrangements, namely pericentric inversions and heterochromatin variation. Of the 55 specimens that were analyzed 47% contained inversions or centromeric shifts on four autosomes (OIR1, OIR4, OIR6 and OIR10) which were present singly in specimens (i.e. none of the specimens contained all four inversions concurrently). These inversions were present in both homozygous and heterozygous state over a wide geographic range suggesting that they are floating polymorphisms. Given the potential role of inversions in post-mating isolation (through production of aneuploid gametes), the prevalence of inversions as floating polymorphisms in the vlei rats suggest that they are probably retained in the population through suppression of recombination in the inverted regions of the chromosomes. In addition, differences between populations is due to the presence or absence of heterochromatic arms (and not inversions), which cause variation in the NFa (40 – 49) and supernumerary B chromosomes, resulting in the variation in diploid number ($2n = 28 – 32$). Analysis of $N = 55$ specimens revealed Ag-NORs on 7 autosomal pairs 1, 2, 5, 7, 8 and 9 proximal to the centromere

on the short arm of the chromosome. Pair 8 also displayed Ag-NOR at the distal end of the long arm of the chromosome in individuals from the Free State province. Pair 3 showed two Ag-NORs occurring proximal to the centromere on the short arm and on the terminal end of the long arm, respectively.

I obtained 953bp of mtDNA cyt *b* from fresh material and 400bp from museum material. Using maximum parsimony and Bayesian inference two main clades were retrieved. Clade A specimens occur mainly in the Western and Eastern Cape provinces of South Africa. Clade B specimens occur in the Eastern Cape, Free State, KwaZulu-Natal, Northern Cape and Mpumalanga provinces of South Africa. The mean sequence divergence between the main clades (A and B) is 7.0% and between sub-clades comprising clade B is 4.8%, while within clade A the sequence divergence was 1.91%. Nested clade analysis revealed allopatric fragmentation within *O. irroratus*. Chromosomal characters also support the two evolutionary lineages as clade A has pericentric inversions which occur as floating polymorphisms which are absent in clade B. Clade B in turn is fixed for a complex tandem fusion rearrangement which is absent from clade A. Divergence date estimates indicate that the two clades separated around 1.1 MYA, which coincides with climate changes since the late Pliocene/Pleistocene epochs. Cladogenesis within this species complex could therefore have been influenced by habitat fragmentation. A full taxonomic review of *O. irroratus* is therefore warranted by this study.

Opsomming

Die suider Afrikaanse vlei rot, *Otomys irroratus* word gekenmerk deur fenotipiese konservatisme regoor die spesie se verspreiding en het groot chromosomale variasie met diploied chromosoom getalle wat reeks vanaf 2n = 23 tot 2n = 32. Hierdie variasie binne *O. irroratus* het gelei tot die beskrywing van drie chromosomale groupe naamlik die A sitotipe wat gekenmerk word deur ’n akrosentriese komplement. Die tweede group wat die B sitotipe genoem word besit ten minste agt chromosoom pare met heterokromatiese kort arms, onderwyl die derde group (die C sitotipe) vier chromosoom pare het met heterokromatiese kort arms. Hierdie studie bestudeer die bevolkings genetika struktuur van *O. irroratus* deur 102 monsters te analiseer wat gekollekteer was regoor die spesie se verspreiding binne Suid-Afrika en die mitochondriale merker sitokroom *b* sowel as chromosoom fluoressent hibridisasie te gebruik.

Ek het 55 monsters van sewe lokaliteite binne Suid-Afrika sitogeneties geanaliseer deur gebruik te maak van G- en C-bandbepaling asook die hibridisasie patrone geproduseer deur die vloei-sorteerde chromosoome van *Myotomys unisulcatus.* Die analise het gewys dat 47% van die monsters perisentromeriese inversies besit het, wat slegs aangestref was of die outosome OIR1, OIR4, OIR6 en OIR10. Hierdie inversies was nooit almal teenwoordig binne dieselfde monster nie en was gevind in beide heterosigotiese en homosigotiese vorm. Die inversies kom ook voor oor ’n wye verspreiding wat daarop aandui dat dit swerwende polymorfisme is. Omdat inversies lei tot die produksie van aneuploiede gamete speel hulle belangrike rol in post-parings reproduktiewe isolasie, die verskyning van swerwende inversies binne vlei rotte dui dus daarop dat hulle onderhou word binne populasie verband deur die onderdrukking van rekombinasie in

die gedeeltes van die chromosoom. Verdere verskille tussen populasies behels die voorkoms of afwesigheid van heterochromatiese kort arms wat (nie inversies) wat lei tot die variasies in die Nfa (40 – 49). Die variasie in diploied getal (2n = 28 – 32) is eksklusief as gevolg van B chromosoome. Ag-NOR banding het ook gewys dat daar twee evolusionêre lyne binne *O. irroratus* voorkom.

Verder het filogenetiese analise van al die monsters verkryg deur volgorde-bepaling met behulp van maksimale parsimonie en Bayesian afleiding twee klades geidentifiseer. Klade A diere kom voor in die Wes en Oos-Kaap provinsies van Suid-Afrika terwyl klade B diere voorkom in die Oos-Kaap, Vrystaat, KwaZulu-Natal, Noord-Kaap en Mpumalanga provinsies onderskeidelik van Suid-Afrika. Die gemiddelde volgorde-bepalings verskille beloop 7% tussen die twee hoof klades (A en B) en tussen sub-klades 4.8%, terwyl binne klade A die verskille slegs 1.91% beloop het. Analise van die verwantskap tussen die klades het gewys dat allopatriese fragmentasie heel waarskynlik gelei het tot die populasie genetiese struktuur binne *O. irroratus.* Chromosoom karakters onderskraag die twee evolusionêre lyne waar klade A slegs perisentriese inversies besit wat swerwend wat ontbreek in klade B. Klade B op sy beurt besit ’n komplekse tandemme fusie wat glad nie voorkom in klade A nie. Molekulêre datering het verder gewys dat die twee klades omtrend 1.1 miljoen jaar gelede versprei het, wat ooreenstem met die klimaats veranderinge wat sedert die Peioceen en Pleistoceen plaasgevind het. Klade vorming binne die spesies komples kan daarom as gevolg van habitat fragmentasie plaasgeving het. Hierdie studie dus noodsaak ,n volle taksonomiese ondersoek van *O. irroratus* ten einde vas te stel hoeveel spesies binne die komplex voorkom.

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General Introduction

1. Preamble

The vlei rat, *O. irroratus* is a southern African endemic with a very wide distribution yet shows virtually no phenotypic variation across its range. It is characterised by high karyotypic diversity which has led to several studies aimed at investigating the possibility of multiple intraspecific lineages (Contrafatto *et al.* 1992; Taylor *et al.* 1992; Taylor *et al.* 1994; Contrafatto *et al.* 1997; Taylor *et al.* 2004; Taylor *et al.* 2005). More recently a multi-disciplinary study involving morphometrics, cytogenetics and DNA sequencing uncovered the presence of two unique evolutionary lineages separated by marked sequence divergence (Taylor *et al.* 2009*a*). The lack of adequate sampling, however, prevented insight into the population genetic structure of *O. irroratus*, which subsequently led to the present study investigating the chromosomal variation and phylogeography of *O. irroratus*.

This study focused on the phylogeography of the southern African vlei rat, *Otomys irroratus* (genus *Otomys* Cuvier, 1824). Here a comprehensive sampling strategy was used in conjunction with classical chromosomal banding techniques (G-, C-banding and Ag-NOR staining), fluorescence *in situ* hybridization and DNA sequencing to describe the population genetic structure of this species.

1.1 Evolutionary history of Otomyinae

The Otomyinae (Suborder: Myomorpha: Family: Muridae) is a rodent subfamily endemic to Africa, with most species occurring in southern African (Carlton and Musser 1986). Fossil

data indicate that the family Otomyinae is approximately five million years old (Pocock 1976; Avery 1991; Sénégas and Avery 1998; Sénégas 2001; Denys 2003; Jansa *et al.* 2006). The oldest known fossil of *Otomys* is dated at approximately 3.1 MYA (Sénégas and Avery 1998; Sénégas 2001; Denys 2003; Taylor *et al.* 2004; Matthews *et al.* 2005; Hopley *et al.* 2006). The Otomyinae are distinguished from other murids by the number of lamina which range from one - ten on the third upper molar (M3) and one - seven in the first lower molar (M1) (Carlton and Musser 1986; Skinner and Chimimba 2005). The subfamily is comprised of 20 species which are divided into two genera. *Parotomys* contains two species while *Otomys* contains 18 species. The two genera are distinguished on the size of the bullae which are smaller in *Otomys* (maximum size is 9.5 mm) and larger in *Parotomys* (minimal of 10.8 mm; Meester *et al.* 1986). Of the twenty *Otomys* species, fourteen occur outside southern Africa, while the rest and the *Parotomys* species occur in southern Africa. Phylogenetic results derived from 12S rRNA, 16S rRNA and cyt *b* mtDNA groups the Otomyinae basal to *Aethomys* in the Muridae (Ducroz *et al.* 2001).

In southern Africa the Otomyinae can be subdivided into three groups based on habitat preferences. The two *Parotomys* species (*P. brantsii* and *P. littledalei*), together with *O. unisulcatus*, are xeric-adapted and occupy predominantly the western parts of South Africa. In contrast, *O. irroratus*, *O. laminatus*, *O. angoniensis*, *O. karoensis* and *O. saundersiae* are mesic-adapted and occupy the eastern parts of South Africa. *O. sloggetti* is the only otomyine species that inhabits an Alpine environment, preferring the cold high altitudes of the Drakensberg and Maluti mountain ranges (Lynch and Watson 1992; Skinner and Chimimba 2005).

A considerable amount of work has gone into accurately describing and tracing the evolutionary relationships of the various species within Otomyinae (Taylor *et al.* 1989, Contrafatto *et al.* 1992*a*; *b*, Pillay *et al.* 1994; Contrafatto *et al.* 1997; Taylor *et al.* 2004).

Initially Taylor *et al.* (1989) attempted to resolve the question of whether *Otomys* and *Parotomys* should be considered as two different genera. They made use of protein products for 30 loci detected by starch gel electrophoresis from a sample that contained 18 *O. irroratus*, 13 *O. unisulcatus*, three *P. brantsii* and one *P. littledalei* specimen. The study concluded that although *O. irroratus* is more distantly related to the species studied they could not make an unequivocal distinction between *Parotomys* and *Otomys*. Sperm morphology, which has successfully been used by other workers to draw evolutionary conclusions on muriod rodents (for example Visser & Robinson 1986, Breed, 2005), produced inconclusive results when applied to the Otomyinae (Bernard *et al.*, 1991). The sperm heads of *O. sloggetti* and *P. brantsii* showed the most correspondence (heads of both have a shallow anterior curve and long, slender nuclei), as did *O. unisulcatus* and *O. irroratus* (sharing anteriorly tightly curved sperm heads containing short broad nuclei) (Bernard *et al.* 1991). A cladistic analysis using 45 craniodental and 46 allozyme characters confirmed the monophyly of the Otomyini as a tribe; however poor statistical support prevented a resolved phylogeny (Taylor *et al.* 2004).

Despite several attempts to trace the relationships within the Otomyini, species boundaries within many of the otomyines remain poorly understood. In particular, within the mesic-dwelling *O. irroratus* (the vlei rat) there is extensive geographic chromosomal variation, suggesting the presence of additional evolutionary lineages within this taxon (Contrafatto *et al*. 1992*a*, *b*; Taylor *et al.* 1992; Maree 2002; Taylor *et al.* 2009*a*).

1.2 Life history characteristics of *Otomys irroratus*

Otomys irroratus occurs in areas which are permanently moist and covered with lush vegetation throughout the whole year. The species is widely distributed across large parts of South Africa, Eastern Zimbabwe and Western Mozambique (Meester *et al.* 1986; Skinner and Chimimba 2005). They have stout bodies with short tails which are about 60% of the body length (Skinner and Chimimba 2005). They have bushy coats with long soft hairs; the upper parts of their coats are a grizzled dark or slightly brown. Their sides and under parts are greyer and the tail is dark brown to grey on the upper side and buffy below. The ears are large and rounded and covered with hair (Skinner and Chimimba 2006). Individuals obtain their adult moult around day 84 after birth and display no seasonal variation in moult colour (Davis and Meester 1981).

The feature which separates *O. irroratus* from other species in the Otomyini is a large round petrotympanic foramen; in contrast to the slit seem in its closest relative *O. angoniensis* (Skinner and Chimimba 2005). Other key skull diagnostic characters which define *O. irroratus* are the sharp changeover between the nasal bones. They have three laminations on their first upper molars, two on the second and six on the third upper pair. The first pair on the lower jaw is the largest with four pairs of laminations, while the other two molars have two sets of laminations (Skinner and Chimimba 2005).

Vlei rats are crepuscular and generally solitary or occur in pairs. They make use of saucer – shaped nests constructed of grass or make use of burrows of other species (Bronner 1992a; Skinner and Chimimba 2005). In some instances they do construct their own burrows which have a single nesting chamber made of shredded grass. *Otomys irroratus* is generally antisocial and territorial with aggression shown towards intruders of their own species (Davis 1973). Males

have home ranges of about 1730 m^2 while females have smaller home ranges of 1252 m^2 (Davis 1973).

Otomys irroratus is totally herbivorous and has a digestive tract which is specialized for this lifestyle (Perrin and Curtis 1980). These animals eat nearly all plant species in their habitat, specifically the young parts of the plants, preferring grass to any other type of plant material (Curtis and Perrin 1979). Low metabolic rates and a low thermal conductance rate are bio-energetic and thermoregulatory adaptations to its environment (Perrin 1980a; Haim and Fairall 1987; Skinner and Chimimba 2005). Individuals can survive for three days without food and they practice coprophagy (Willan and Hickman 1986). This habit of eating faeces is an important habit to adults and for the weaning of juveniles as it provides the proper micro flora for food digestion (Ewer 1968; Skinner and Chimimba 2005).

They breed throughout the year, with a dip in breeding occurring during winter, due to limited food availability (Skinner and Chimimba 2005). However, aseasonal breeding cohorts have been noted in the Eastern Cape province of South Africa, and this is argued to be as a result of the all year rainfall regime of this area (Taylor *et al.* 2009*a*). Vlei rats give birth to precocial young which nipple cling for about 14 days. Females can produce nine to twelve young in a season and mature at a weight of 76.0g while males mature at about 96.0g of weight (Skinner and Chimimba 2005). Mature *O. irroratus* males disperse when population densities are high, over distances of about 18.5 m due to habitat constraints (Davies 1973).

1.3 Chromosomal variation in *Otomys irroratus*

Otomys irroratus is characterized by high intraspecific chromosomal variation, which raises the question as to whether this should be regarded as a single species or a species complex.

Over the past decade, thirteen populations of *O. irroratus* have been karyotyped and the diploid number ranges 23 to 32. These populations have been grouped into four parapatric distributed cytotypes A1, A2, B and C (Contrafatto *et al.* 1992*a*, *b*). The main differences between these groups is due to the presence or absence of heterochromatic short arms, a variable number of B chromosomes, a tandem fusion rearrangement, random Robertsonian fusions and pericentric inversions when compared to the standard complement of 2n = 26 plus X and Y (Robinson and Elder 1987; Contrafatto *et al.* 1992*a*; Rambau *et al*. 2001; Engelbrecht *et al.* 2006; Taylor *et al.* 2009*a*).

The type A chromosomal race constitutes an entirely acrocentric karyotype (Contrafatto *et al.* 1992a; Rambau *et al*. 2001). This group can be subdivided into two chromosomal races namely, A1 and A2. The A1 race has been documented in Hogsback, Kamberg and Vergelegen (South Africa), and has a large compound chromosome formed as a result of fusions involving chromosomes 7, 12 and 8 of the standard *O. irroratus* karyotype. This compound chromosome corresponds to the mouse chromosomes MMU17 and 18 (OIR 7), MMU1 and 15 (OIR 8) and MMU12 (OIR12) (Engelbrecht *et al.* 2006). The A2 cytotype is found in KwaZulu-Natal and also has a genome with acrocentric chromosomes but without the tandem fusion composite chromosome. This cytotype has two autosomal pairs which are unique to this cytotype (see Contrafatto *et al.* 1992*a*, *b*).

Cytotype B specimens have at least six to eight pairs of biarmed autosomes and have the widest distribution, also occurring in Zimbabwe (Chingamwe Estates) and in the Eastern Cape Province (Port Elizabeth) (Contrafatto *et al.* 1992*a*, *b*; Taylor *et al.* 1994). Pair six has a pericentric inversion (Robinson and Elder 1987) and pair 4 also has an inversion (Taylor *et al.* 2009*a*). Cytotype C, on the other hand, has four submetacentric chromosomes that are totally C-

positive on their short arms. This chromosomal race has the largest distribution range in South Africa, stretching from the Western Cape in the south to Limpopo province in the north of South Africa (Taylor 2000) (Figure 1.1).

1.4 *Otomys irroratus* population differences

The variation in chromosome number among the populations raises the question as to whether these populations are reproductively isolated. Firstly, laboratory-based breeding experiments between Kamberg (A1, 2n = 24) and Karkloof (A2, 2n = 30) populations were characterized by a pronounced reduction in the number of offspring, which could be the result of a lack of recognition cues (for example: auditory and olfactory) between these populations (Pillay *et al.* 1992). Although copulation events were observed between individuals from the two populations, litter sizes were smaller; young had poor postnatal development and suffered reduced fertility. Furthermore, second generation individuals were completely sterile when breeding attempts were made between individuals from Kamberg and Karkloof, indicating that there may be post-mating isolation between these populations (Pillay *et al.* 1992). Laboratory based experiments also established that behavioural differences may prevent breeding between three adjacent populations of *O. irroratus* (Pillay *et al.* 1995). Higher levels of aggression were observed between Kamberg (A1, 2n = 24), Karkloof (A2, 2n = 30) and Committee's Drift (B, 2n = 28) populations. These results suggest that aggression may potentially operate as a pre-mating isolation barrier (Pillay *et al.* 1995).

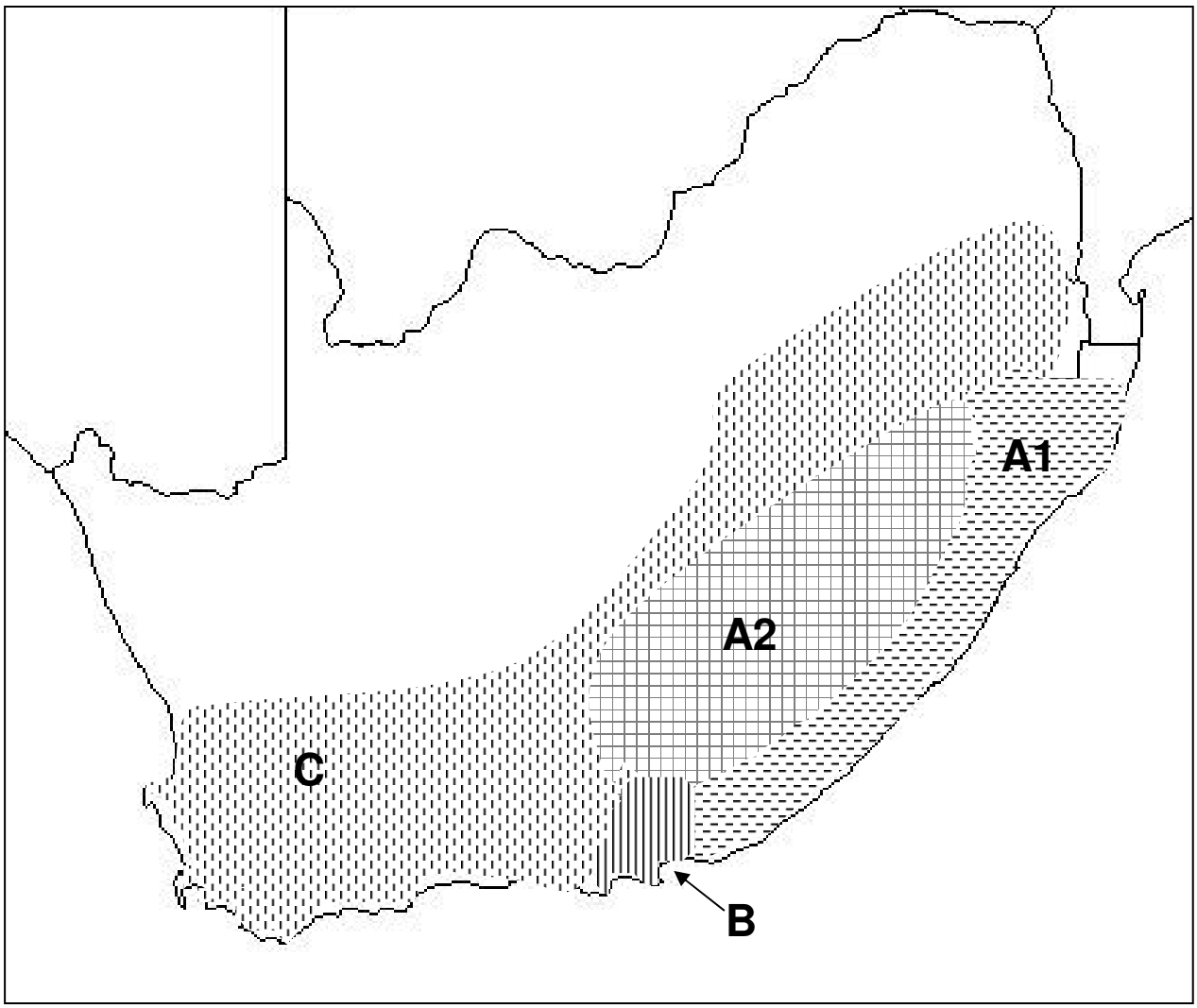


Figure 1.1: The three cytotypes are parapatrically distributed. Cytotype A1 occurs along the East coast and neighbouring inland of South Africa while the A2 cytotype occurs at higher altitudes along the Drakensburg highlands. The B cytotype occurs in a localized region in the Eastern Cape while the C cytotype has the widest distribution across South Africa occurring from the South-West to the North-east of the country. Redrawn from Taylor 2000.

While behavioural studies showed differences between populations, conventional morphometrics failed to show any clear differences among the described cytotypes of *O. irroratus* (Taylor *et al.* 1994). A geometric morphometrics analysis involving 24 dorsal cranium, 22 ventral cranium and 12 mandible landmarks of 67 individuals also failed to detect significant cranial size differences between the various cytotypes. Instead the geometric morphometrics

analysis only revealed cranial shape differences between the South African cytotypes (A1, A2, B and C) and a single allopatric population in Zimbabwe (Taylor *et al.* 2004*a*; 2009*a*).

Allozyme electrophoresis was unable to differentiate the various *O. irroratus* populations (Taylor *et al.* 1992). This study involved 24 protein encoding enzyme loci (of which 12 were polymorphic) obtained from twelve South African populations (Stutterheim, Hogsback, Ngome, Karkloof, Committee's Drift, Kamberg Nature Reserve, Port Elizabeth, Rietvlei, Vergelegen Nature Reserve, Alice, Umgeni Valley and Umkomaas) representing all the described cytotypes. Allozyme analysis revealed a panmictic genetic structure for this species (F_{ST} = 0.37; D = 0.03) (Taylor *et al.* 1992), which suggests high levels of geneflow between populations. Alternatively, it indicates that the marker employed evolves slowly and hence failed to detect population differentiation, especially considering the low levels of heterozygosity ($\bar{H}$ = 0.042 – 0.061). Subsequent analysis using Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS-PAGE) of liver proteins also failed to provide any clear distinction between the various cytotype groups (Contrafatto *et al.* 1997).

In contrast, cyt *b* sequences of the same specimens revealed a close association between the cytotypes B and C (2n = 27 - 32) on the one hand, and A1 and A2, which has the composite chromosome (A1 - between 7, 8 and 12) and the unfused state (A2), respectively (Maree 2002). The chromosomal cytotype A1 showed a sequence divergence of 6.4 – 6.8% from the B cytotype, while a sequence divergence of 6.3 – 6.8% was found between A1 and C. The A2 and B cyto-groups had a sequence divergence of 7.0 – 7.5% separating them while A2 and C had a 6.7 – 6.9% difference between them. Further, the study showed lower genetic distance between B and C (2.0 – 2.1%) while A1 and A2 were shown to be vastly different (4.5 – 4.8%). Maree (2002) argues that A2 could represent a third cytogenetic group. This is congruent with reproductive

data showing that Kamberg (A1) and Karkloof (A2) may be reproductively isolated (Pillay *et al.* 1995). While Maree's (2002) study showed significant differences between the various cytogenetic races, small sample sizes (based on a total of seven sequences) and limited geographic sampling precluded a thorough insight into the population genetic structure between the various cytotypes.

Recently, a multidisciplinary study of *O. irroratus*, using the cyt *b* gene, skull morphometrics and cytogenetics from $N = 30$ specimens retrieved two lineages demarcated by 6.4% sequence divergence (Taylor *et al.* 2009*a*). The two cryptic lineages appear to be distributed parapatricaly spanning the grassland and the Cape Floristic Region (CFR). In the present study I undertook a comprehensive geographic sampling of *O. irroratus* throughout its range in South Africa to resolve its population genetic structure.

1.5 Phylogeography and the utility of mtDNA and nuclear DNA markers

Phylogeography is used to describe the spatial distribution of alleles whose phylogenetic relationships are known or can be approximated (Avise 2000). Historically, allozyme analysis was used for this purpose but allozyme analysis has an intrinsic problem. The alleles on which allozyme analysis is based are often homogenized through balancing selection and as a result of this the phylogenetic history of this molecular marker cannot be deduced with great confidence (Avise 1987). Therefore vicariance events (e.g. the division of a species by a natural event such as the formation of a mountain) will be difficult to trace and consequently results from allozymes may not be a true reflection of a species' phylogeography. This could explain the absence of diagnostic divergence between populations of *O. irroratus* based on allozyme data (Taylor *et al.* 1992; Contrafatto *et al.* 1997).

Mitochondrial DNA sequence data have been used successfully for many years in phylogeographical investigations (Lansman *et al.* 1983; Riddle *et al.* 2000; Carr *et al.* 2008). The general absence of recombination in mitochondria allows for the construction of phylogenies which are unaffected by male dispersal and therefore gene structuring can be visualized across a geological time scale (Avise 1989; Avise 1994). Another advantage of using mtDNA is the high rate of sequence evolution (2% sequence divergence per million years in higher order animals (Brown *et al.* 1979; Brown *et al.* 1982) which makes it perfectly suited for intra-specific variation and phylogeographic investigations. Further, the nucleotide substitution rate of mitochondrial DNA has subsequently been calculated to be at a rate of 1.7 - 3.4 times higher than the fastest evolving nuclear genes (Moriyama and Powell 1997). This means that gene trees of mtDNA reach reciprocal monophyly sooner after speciation than gene trees obtained from nuclear markers (Avise 2000; Sunnucks 2000).

Although mitochondrial DNA has all of the advantages described above, these advantages can also limit population comparisons. For instance, using mtDNA exclusively to uncover population history of a species will be biased as only the maternal evolutionary history will be described (Zhang and Hewitt 2003). The fast pace of mtDNA evolution could lead to a loss of phylogenetic signal and hence oversimplified relationships may be inferred; genetic diversity may be underestimated and evolutionary processes among populations which are separated by great distances may not be accurately uncovered (Zhang and Hewitt 2003). Furthermore, the mtDNA genome has been shown to have pseudo-genes and homoplasic characters which could lead to incorrect evolutionary inferences. By far the biggest limitation of mtDNA is the fact that the genome represents a single locus. This means that a single frame of evolutionary change is investigated for only the maternal side, which could be different from the molecular evolution of other markers for a particular species or population (Zhang and Hewitt 2003).

Given these limitations of mtDNA, the use of combined mtDNA and nuclear DNA markers, is necessary to reveal critical aspects of genealogical affinities. Nuclear DNA, which has less homoplasy in general as a result of its slow rate of evolution, is less informative for taxa which have undergone a recent divergence event (Zhang and Hewitt 2003). The fact that nuclear DNA provides the ability to investigate both sexes is an advantage over mtDNA, but is also a factor which makes it difficult to work with. For instance, nuclear genes may have intra-genomic polymorphisms, be under selection, have variable rates of change and could occur in heterozygous forms within a population. Further, recombined nuclear genes are known to be difficult to sequence as a result of the mosaic form they may take on (Zhang and Hewitt 2003). In this study, chromosomal data (nuclear markers), in conjunction with the mtDNA marker (cyt *b*) is used to investigate the phylogeography of *O. irroratus*.

1.6 The utility of chromosomal data for cladistic analysis

Chromosomes constitute nuclear markers that can be used in a phylogenetic framework and which have been used successfully in mammals to infer evolutionary relationships (Nagamachi *et al.* 1999, Trifonov *et al.* 2002, Pardini *et al.* 2007), especially within the order Rodentia (Guilly *et al*. 1999; Yang *et al.* 2000; Li *et al.* 2004; Romanenko *et al.* 2007; Sitnikova *et al.* 2007). The comparative cytogenetics of rodents has to a large extent been conducted using unbanded chromosomes (e.g., Robbins and Baker 1978) and or classical G and C- and NOR banding protocols (see Robinson 2001 for review). Classical chromosomal banding techniques such as G-banding (Seabright 1971) and C-Banding (Sumner 1972) have provided characters for intra- and inter-species comparisons (see Qumsiyeh 1986) in order to infer evolutionary relationships among taxa. However, classical banding techniques are not effective when taxa are characterised by extensive genome rearrangements, such as in *O. irroratus*. Using banding

patterns alone to delineate complex rearrangements is therefore difficult as these data are subject to convergence (see discussion in Stanyon *et al*. 1999; Robinson 2001 and see Stanyon *et al.* 1995; Dobigny *et al.* 2002; Sanchez *et al.* 1995 for examples).

Fluorescence *in situ* hybridization uses probes (in this case, flow sorted whole chromosomal probes) to identify regions of homology between taxa. It is based on complementary nucleotide base pairing between the sequences of the probe and the target genome, and therefore regions of homology between taxa are established unequivocally at the molecular level. Assessing the degree of homology among genomes of closely related species or distantly related taxa is therefore possible with this approach (Scherthan *et al*. 1994, reviews in Murphy *et al*. 2001, 2005). In other words, differences amongst specimens of a species can be identified with absolute confidence (Scherthan *et al*. 1994; reviews in Murphy *et al*. 2001; 2005).

1.7 Aim of study

The aim of this study is to describe the population genetic structure as well as the phylogeography of the vlei rat, *Otomys irroratus* throughout its distribution range in South Africa using a combination of chromosomal data and DNA sequence data from the mitochondrial gene cyt *b*.

1.7.1 RESEARCH QUESTIONS

(1) **What is the spatial population genetic structure of *O. irroratus* cytotypes?** In the Eastern Cape and KwaZulu Natal Provinces, the contraction and expansion of forests (Lawes *et al.* 2007) may have played a role in the formation of the various cytotypes of *O. irroratus*. A genetic investigation will determine to what extent the cytotypes were affected by these natural geographic barriers, and shed light on the extent of gene flow between the cytotypes.

(2) **Does the level of genetic structuring among populations also coincide with the chromosomal change that has occurred in the populations?** Four main cytogenetic groups are recognized within *O. irroratus*, and therefore it is critical to investigate whether the chromosomal divergence is similarly retrieved at the DNA sequence level. This is critical since previous behavioural data indicated high levels of aggression between populations with the A1 and A2 cytotypes, which would prevent formation of hybrids in nature (Pillay *et al.* 1992). In addition, previous laboratory breeding experiments resulted in reduced reproductive output between the tandem fusion carriers and non-carriers (Pillay *et al.* 1995). These studies suggest both pre-zygotic and post-zygotic isolation may already be in place. Here we will determine to what extent and this may lead to genetic structuring.

*Chapter 2

Chromosomal polymorphisms in the African vlei rats, *Otomys irroratus* (Muridae): inversions, supernumerary chromosomes and heterochomatic additions

*Part of this work formed the bases of a peer reviewed paper accepted for publication: Engelbrecht *et al.* 2010. Entitled: Chromosomal polymorphisms in the African vlei rats, *Otomys irroratus* (Muridae: Otomyini) detected using banding techniques and chromosome painting: inversions, centromeric shifts, and diploid number variation. *Cytogenetic and Genome Research. in press.*

2.1 Introduction

The African vlei rat, *Otomys irroratus* is characterized by widespread intraspecific chromosomal variation and the diploid number ranges from 23 to 32 (Robinson and Elder 1987, Contrafatto *et al.* 1992, Taylor *et al.* 1992, Rambau *et al.* 1997, Rambau *et al.* 2001, Engelbrecht *et al.* 2006). Over the past decade, thirteen populations of *O. irroratus* in South Africa have been karyotyped (Contrafatto *et al.* 1992). Within this variation two chromosomal blue-prints, 2n = 24 and 2n = 28 (Rambau *et al.* 2001), which are reproductively isolated, have been identified (Pillay *et al.* 1992). However, some authors propose the presence of three cytotypes based on the number of heterochromatic short arms present in a specimen's karyotype (Contrafatto *et al.* 1992 *a, c,* Taylor 2000). These cytotypes differ as a result of the presence of a compound chromosome present in the 2n = 24 cytotype that is derived from a tandem fusion of three autosomes (OIR7,

OIR8 and OIR12) in the 2n = 28 cytotype (Rambau *et al.* 2001; Engelbrecht *et al.* 2006). Further, differences among the species are due to heterochromatic additions resulting in variation in chromosomal arms (2n = 23, NFa = 25; 2n = 28, NFa = 37 to 54) and the presence of (1 - 4) supernumerary chromosomes (B - chromosomes).

Recently, Taylor *et al.* (2009*a*) identified the presence of pericentric inversions in Grahamstown specimens involving chromosome 4 in the 2n = 28 cytogroup. While the detection of pericentric inversions is not novel in the vlei rat, the first inversion was detected in chromosome pair 6 by Robinson and Elder (1987); their presence in heterozygous condition suggests that they may be present as floating polymorphisms.

The presence of pericentric inversions in natural populations is important in evolutionary biology because of their ability to produce genetic isolation between conspecific populations within a species (White 1973). Empirical evidence of pericentric inversions leading to species formation has been found in a variety of species including fruit flies, *Drosophila* (*D. persimilus* and *D. pseudoobscura* differ by three pericentric inversions (Wallace 1953; Coluzzi *et al.* 1985); African water rats, *Dasymys cf. incomtus* (Y-chromosome pericentric inversion, Volobouev *et al.* 2000), South American tuco-tucos, *Ctenomys* (Novello and Villar 2006), Japanese talpine moles, *Euroscaptor* and *Mogera* (Kawada *et al.* 2001) and reef fishes, *Centropyge* (Affonso and Galetti Jr. 2005). The genetic isolation between species in these examples is largely due to aneuploid gametes (duplications and deletion of genes that result from the inversion loop that is formed during crossing over during meiosis I) produced by heterozygous carriers (White 1973; King 1993).

Interestingly, pericentric inversions have been widely documented within species suggesting that there may be a mechanism to circumvent the deleterious effects of inversions (Hoffman and Rieseberg 2008). Pericentric inversions have been detected as floating polymorphisms in a variety of taxa including deermice, *Peromyscus maniculatus* (Greenbaum and Reed 1984, Rogers *et al.* 1984), sitka deer mouse *Peromyscus sitkensis* (Hale 1986), cotton rats, *Oligoryzomys nigripes* (Bonvicino *et al.* 2001), multimammate rats, *Mastomys* (Volobouev *et al.* 2001) and white-throated sparrow, *Zonotrichia albicollis* (Thomas *et al.* 2008). Each of these cases suggests that inversions may not be underdominant and that they are retained within populations by suppression of recombination in the inverted regions of the chromosome (Trickett and Butlin 1994; Noor *et al.* 2001; Kirkpatrick and Barton 2006). Since the recombination is suppressed, the chiasma is not formed in the inverted regions of the chromosomes resulting in formation of balanced gametes (Hale 1986). Therefore, suppression of the meiotic mechanism is probably partly responsible for the maintenance of pericentric inversions in natural populations. The recent observation of pericentric inversions in isolated populations of *O. irroratus* (Taylor *et al.* 2009*a*) collected from limited localities raised questions about the prevalence of inversions in the species.

Nucleolar organiser regions (NORs) constitute another chromosome character that can be used as a diagnostic marker (Sanchez *et al.* 1995; Volobouev *et al.* 2002; Arslan *et al.* 2008). Nucleolar organiser regions are chromosomal loci which carry 18S and 28S rRNA genes (Sanchez *et al.* 1995; Trerè 2000; Dobigny *et al.* 2002; Arslan *et al.* 2008). NORs contain argyrophilic proteins which have a high affinity for silver, especially in mammalian chromosomes (Sumner 1990; Gallager *et al.* 1999; Trerè 2000). *Otomys irroratus* have been shown to contain three pairs of NORs on two pairs of autosomal chromosomes, namely pair 3 and pair 7 in a specimen from Tsitsikamma (Robinson and Elder 1987). However, no other

populations cytogenetically investigated thus far have NOR data available (Contrafatto *et al.* 1992 *a*, *b*, *c*; Taylor *et al.* 2009*a*) and therefore their prevalence in *O. irroratus* have never been investigated. Therefore in this study I silver-stained all the collected specimens in order to determine the diagnostic value of NORs (Taylor *et al.* 2009*a*).

The aim of this aspect of the study was:

1. To investigate the chromosomal variation within *O. irroratus* using conventional cytogenetic techniques G-, C-banding and Ag-NOR staining as well as FISH (Fluoresence *in situ* hybridization) using whole chromosome probes derived from the closely related bush karoo rat (*Myotomys unisulcatus*).
2. The variability in the presence of heterochromatin in the short arms of *O. irroratus* has led to the description of three major cytotypes (Contrafatto *et al.* 1992). This was however based on 13 populations; the inclusion of more specimens from different localities allowed a detailed analysis of the cytotype boundaries currently defined.

2.2 Materials and Methods

2.2.1 Specimens and tissue culture

In total fifty five wild caught vlei rats were sampled in the Western Cape, Eastern Cape and Free State provinces of South Africa (Table 2.1). Tail tissue biopsies were taken from specimens, sterilized and thereafter fibroblast cells were established using standard tissue culture techniques. Tissue cultures were maintained in an incubator at 37 ^{o}C and 5% CO_2. Metaphase cells were harvested using 10% Colcemid (in DMEM) to arrest cell division and the hypotonic treatment using 0.075 M KCl, and subsequently fixed in Carnoys fixative (3 parts methanol: 1 part acetic acid). Slides were prepared by dropping approximately 12 µl of the metaphase suspension onto the slide. The quality of the chromosomal harvest was then inspected using a phase contrast microscope. Subsequently, fresh material specimens were identified using their karyotypic data while museum specimens were previously identified using skull and dental morphology. All specimens collected in this study will be deposited in the small mammal museum collection of Durban Natural Science Museum at the termination of this study.

2.2.2 Chromosome banding

Constitutive heterochromatin staining was done following Sumner (1972) with slight modifications. Briefly, slides were treated in 0.2 M HCl solution, followed by 5% $Ba(OH)_2$ and then stained using 5% Giemsa solution in 0.025 M KH_2PO_4 buffer. In order to construct karyotypes, metaphases were G-banded following Seabright (1971). This involved treating metaphase chromosome slides with trypsine (0.025 % in 1 x PBS) for 40 - 50 seconds, followed by a rinse in fetal calf serum buffer (500 µl FCS in 50 ml KH_2PO_4 buffer), and then stained in 10% Giemsa (KH_2PO_4 buffer). Karyotypes were arranged according to size and morphology following the first G-banded karyotype of the species (Robinson and Elder 1987).

2.2.3 Ag-NOR staining

Ag-NOR staining was done following a modified protocol of Goodpasture and Bloom (1975). Freshly prepared slides were firstly stained with DAPI (4', 6 – diamidino – 2 - phenylindole), and the slides were mounted with Vectashield medium. DAPI images were then captured (the coordinates of each captured metaphase spread was noted) and converted to G-band images using the Genus software version 3.7 (Applied Imaging Newcastle, UK). Subsequently, slides were de-stained in a 2x SSC solution for 5 min and air dried. Ag-NOR staining were done by placing 100 µl of 2% gelatine solution (2g gelatine; 98 ml H_2O and 1 ml Formic acid) on the slide followed by 200 µl $AgNO_3$ (50% solution). The slides were then covered with a cover slip and heated for 25 sec at 65 ^oC. After this treatment slides were rinsed with distilled water and stained with a 1% Giemsa solution (KH_2PO_4 buffer). The metaphase spreads were then recaptured in order to determine where NORs are situated on the chromosome.

2.2.4 Fluorescence in situ hybridization (FISH)

A sub-set of *Myotomys unisulcatus* (MUN) flow-sorted chromosomes (MUN2/12, 3/5, 4, 7 and 8) which were flow-sorted for a study investigating phylogenetic relationships within the Otomyini (Engelbrecht *et al.* unpublished) were used to confirm chromosomal rearrangements that were identified using banding techniques. Chromosome flow sorts were amplified with the MW primer (5'CCGACTCGAGNNNNNNATGTGG 3') and labelled with Biotin-16-dUTP (Roche) or Digoxigenin-11-dUTP (Roche) using DOP-PCR (Telenius *et al.* 1992).

Fluorescence *in situ* hybridization was done following Rens *et al.* (2006) with slight modifications, and optimised according to Henegariu *et al.* (2001). Briefly, slides were dehydrated in an ethanol series (70%, 80%, 90% and 100%), air-dried and then denatured for 1 min in 70% formamide / 2x SSC at 65 ^oC. Slides were quenched by placing it in ice cold 70%

ethanol for 3 min and then dehydrated in the ethanol series (70%, 80%, 90% and 100%, 2 min each) and air dried.

A DNA probe mixture consisting of 3 µl DOP-PCR product, 3 µl Salmon sperm and 3 µl mouse-cot DNA were precipitated using Na - Acetate (3M) and 100% ethanol overnight at -80 ^{o}C. Following precipitation the probe mixture was centrifuged, rinsed with 70% ice cold ethanol and pelleted. The dried pellet was eluted in 20 µl hybridization buffer (50% deionized formamide, 10% dextran sulphate, 0.5 M phosphate buffer pH = 7.3, 1× Denhardt's solution) with the use of a vortex. The resuspended DNA probe mixture was denatured at 70 ^{o}C for 10 min and re-annealed for 20 - 30 min at 37 ^{o}C. The re-annealed probe was then applied to the denatured slide and sealed with rubber cement. Following a hybridization period of 24 - 48 hours, slides were washed twice in 50% Formamide / 1 x SSC (5 min in each) followed by two washes in 2 x SSC (5 min in each), rinsed in 4XT (200 ml dH_2O + 50 ml SSC 20X + 125 µl of Tween20) for 10 min. Biotin-labeled probes were detected using Cy3 - avidin (1:500 dilution; Amersham) and Digoxigenin - labeled probes were detected using anti - FITC (1:500 dilution; Amersham) by incubating slides for 20 min at 37 ^{o}C. The slides were then rinsed three times in 4XT (200 ml dH_2O + 50 ml SSC 20X + 125 µl of Tween20) for 5 min each time. Chromosomes were counterstained using DAPI (4', 6 - diamidino - 2 - phenylindole), and the slides were mounted with Vectashield medium.

2.2.5 Image capturing

All images were captured with a CCD camera coupled to an Olympus BX60 microscope set on bright field (for C and G banding). Fluorescence *in situ* hybridization images on the other hand were captured using fluorescent filters and analysed with the Genus software version 3.7 (Applied Imaging Newcastle, UK).

2.3 Results

2.3.1 Karyotype analysis

Of the 55 specimens that were analysed 48 had a diploid number of 2n = 28, and in two populations (Theunissen in the Free State Province and Somerset East in the Eastern Cape Province) diploid numbers ranged from 28 - 32 (*N* = 7) as a result of the presence of supernumerary B chromosomes (1 - 4) (Figure 2.1) (Table 2.1) (Appendix 1). The karyotypes were comprised of bi-armed chromosomes (OIR1 - 9) which had heterochromatic arms and also acrocentrics (OIR10, 11, 12, 13). The X was biarmed and the Y was small and partially heterochromatic (Rambau *et al.* 1997). G-band analysis revealed that 29 specimens had a karyotype similar to that of the standard karyotype (Robinson and Elder 1987). The only deviation from the standard was variation due to heterochromatin additions and deletions on autosomes OIR7 to OIR13. Chromosomal pairs OIR1 to OIR5 always had heterochromatin on their short arms.

Using the number of submetatcentric autosomes containing heterochromatic short arms in a karyotype, following Contrafatto *et al.* (1992*a*), two cytotypes were obtained in this study: (i) The B cytotype which is characterized by six - eight pairs (OIR1, 2, 3, 4, 5, 6, 7, 10) of biarmed autosomes were observed in specimens collected in the Eastern Cape province (Baviaanskloof Nature Reserve (*N* = 5), Somerset East (*N* = 19), Kroomie (*N* = 5) and in the Western Cape, Oudtshoorn, (*N* = 1) (Table 2.1). (ii) The second cytotype, which is defined by four pairs (OIR1, 2, 3, 4) of submetacentrics (i.e. C cytotype) and this was present in specimens sampled in the Western Cape (Porterville, Van Rhynsdorp, Stellenbosch and Beaufort West) and Free State provinces (Theunissen) (Table 2.1). While the distribution of the C cytotype falls within the limits demarcated by Contrafatto *et al.* (1992), this study documents the first description of the B cytotype in the Western Cape.

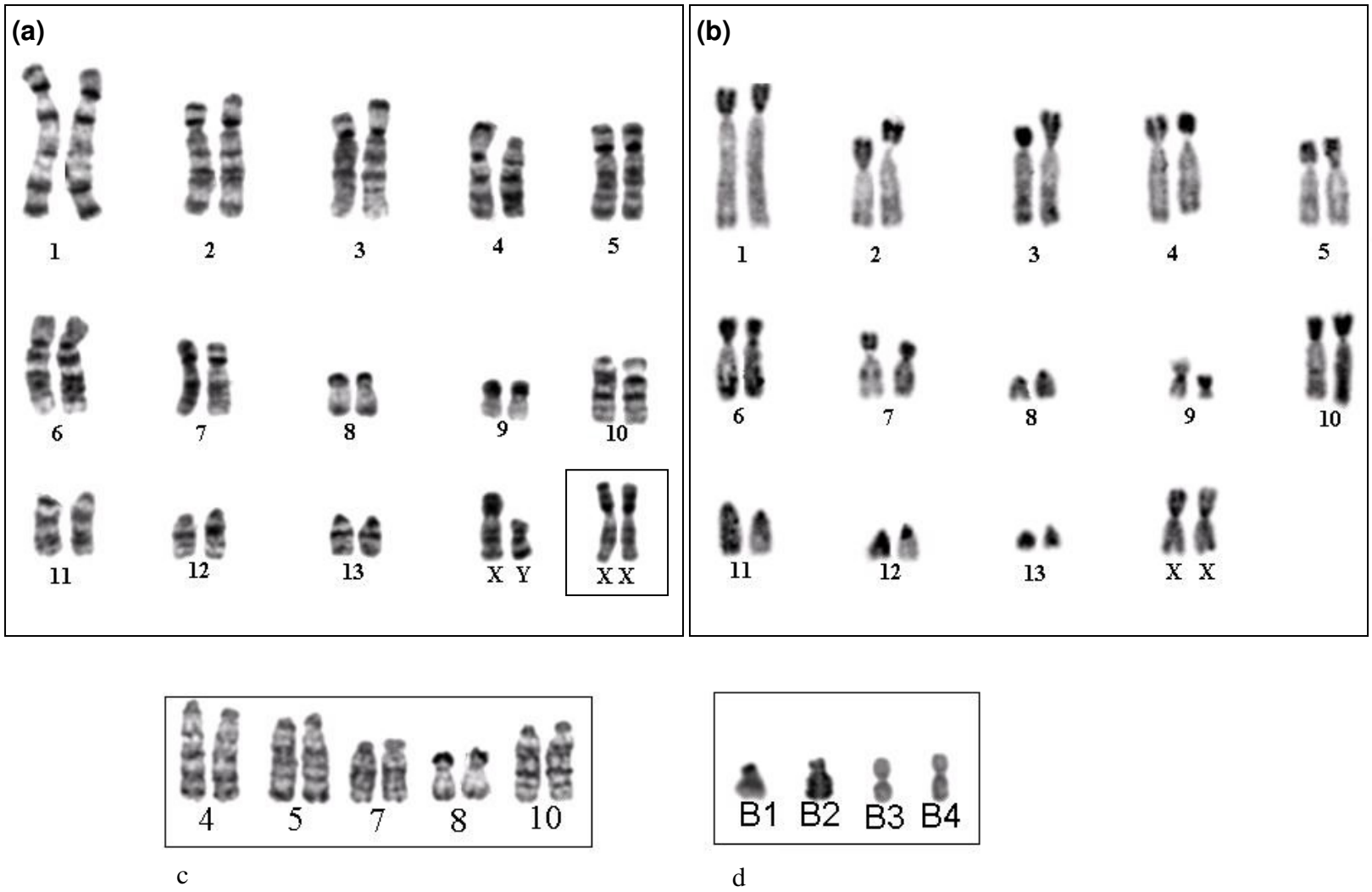


Figure 2.1: (a) The G-banded karyotype from the Oudtshoorn population (insert has two X chromosomes of a female). (b) C- banded karyotype from a Somerset East specimen which is characterised by heterochromatic shortarms. (c) Autosomal pairs OIR4, 5, 7, 8 and 10 which had the loss of heterochromatic shortarms in the Stellenbosch, Porterville, Van Rhynsdorp, Beaufort West (All Western Cape province populations) and Theunissen (Free State province) populations. (d) B chromosomes detected in populations from the north eastern side of South Africa, (B1) Somerset East and Kroomie, Eastern Cape province, (B2) Theunissen, Free State province, (B3 and B4) Somerset East, Eastern Cape province.

Table 2.1: Summary of the chromosomal data from each of the populations that were sampled in the Western Cape, Eastern Cape and Free State provinces of South Africa. The populations displayed variation in diploid number (2n), fundamental number (NFa), and the presence (+) and absence (-) of inversions or centromeric shifts in autosomes OIR1, OIR4, OIR6 and OIR 10, NORs and B chromosomes.

Locality	Coordinates	Sample Size (N)	Diploid number (2n)	NFa	The presence (+) or absence (-) of pericentric inversions				Ag-NORs	B's
Western Cape Province					OIR1	OIR4	OIR6	OIR10		
Porterville	32°, 59', 15" S; 19°, 01', 28" E	8	28	42 – 50	-	+	+	-	6	
Oudtshoorn	33°, 39', 56" S; 22°, 07', 39" E	1	28	50	-	+	-	+	10	
Stellenbosch	33°, 55', 54" S, 18°, 49', 47" E	9	28	40 - 44	-	+	+	+	12	
Van Rhynsdorp	31°, 44', 46" S; 18°, 46', 26" E	1	28	43	-	-	+	-	6	
Beaufort West	32°, 15', 19" S, 22°, 34', 25" E	4	28	44 - 46	-	+	+	-	6	
Eastern Cape Province										
Somerset East	32⁰, 41', 62"S; 25⁰, 37',80"E	20	28;29;30; 32	40 - 52	-	-	+	-	9	1; 2; 4
Baviaankloof	33⁰; 39',38"S; 24⁰, 37", 57"E	5	28	45 – 49	-	-	-	-	7	
Kroomie	27°, 08', 07" S; 20⁰, 32',11"E	5	28; 29	46 - 49	+	-	-	-	9	1
Free State Province										
Theunissen	28°, 30', 6" S; 26°, 48', 7" E	2	28; 30	37 – 38	-	-	-	-	6	2

2.3.2 G-band comparisons and FISH results

Of the 55 specimens that were analyzed in this study, 26 specimens contained pericentric inversions or centromeric shifts involving four autosomal pairs OIR1, 4, 6 and 10 (Table 2.1, and Figure 2.2). With the exception of the pair 6 and 4 inversions which were previously published by Robinson and Elder (1987) and Taylor *et al.* (2009*a*) respectively, the remaining two were new.. These inversions were identified using G-banding and confirmed using five *M. unisulcatus* probes (OUN2/12, 3/5, 4, 7 and 8) that were hybridized on the chromosomal segment spanning the inverted regions (Figure 2.2). The syntenic associations identified by these probes were either disrupted by a centromere (in the inverted homologues) or were retained intact in the un-inverted homologues resulting in the centromere positioned outside the syntenic block (Figure 2.2).

These inversions were present in a heterozygous state (pairs OIR1, OIR4 and OIR10) and as both heterozygous and homozygous states (OIR6; Figure 2.2). The inversion in OIR1 was identified in three specimens sampled in one locality in Kroomie (Eastern Cape province). The rearrangement in OIR4 was detected in three populations in the Western Cape province (Porterville, Oudsthoorn and Beaufort West); OIR6 is present in four localities in the Western Cape province (Porterville, Stellenbosch, Beaufort West and Van Rhynsdorp) and the Eastern Cape (Somerset East and Kroomie). A female from the Somerset East population was heterozygous for the OIR6 inversion and produced two offspring with different morphs of pair 6: one offspring was homozygous (both acrocentrics) and the other was heterozygous for the OIR6 inversion (the babies were discovered when the female was dissected) (See Appendix). The OIR10 inversion was detected in three localities Oudsthoorn, Stellenbosch and Beaufort West (Figure 2.3).

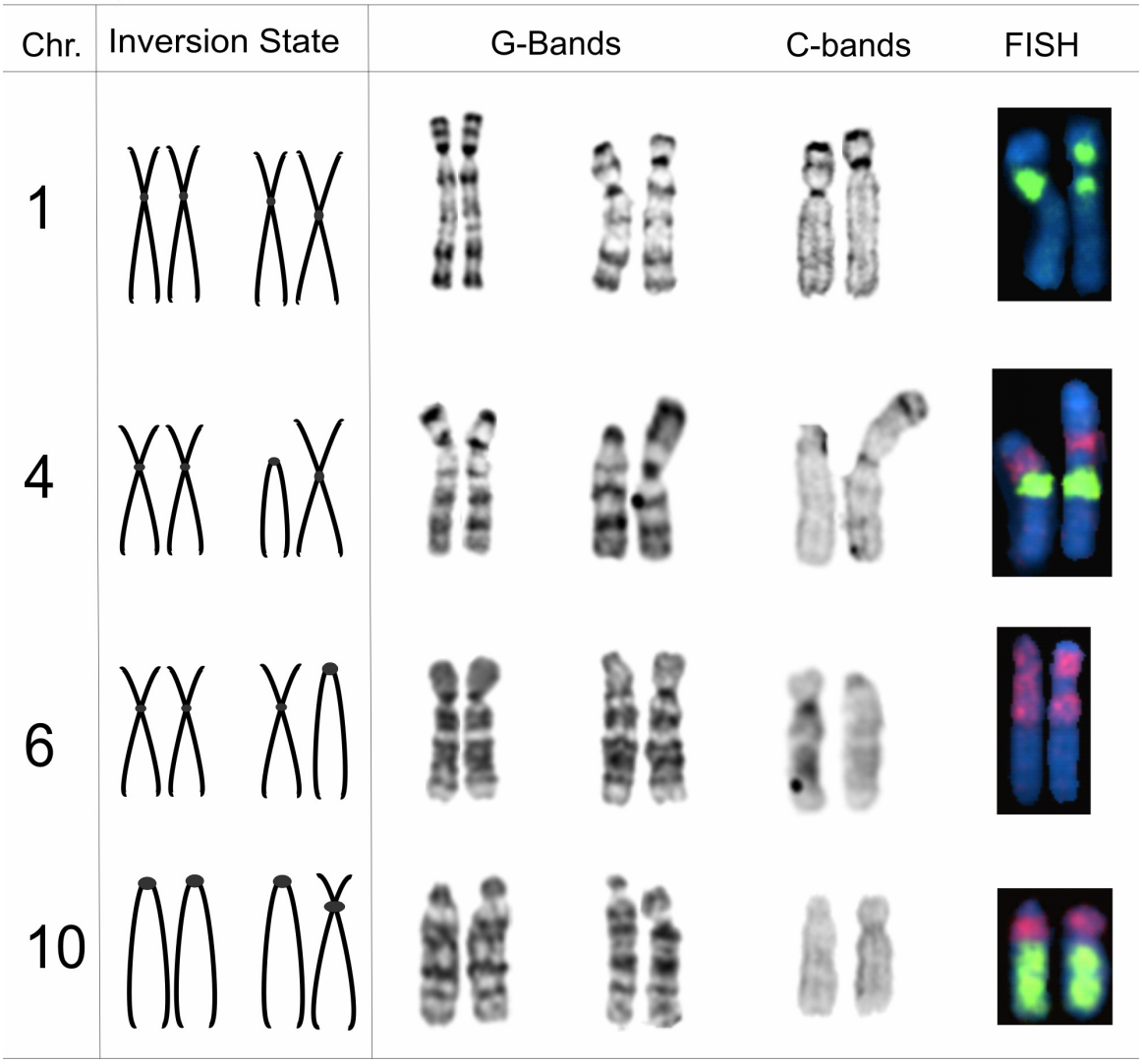


Figure 2.2: The pericentric inversions which were found on pair 1, 4, 6 and 10. The inversions were detected using G-banding, C-banding and FISH with *M. unisulcatus* probes. The pair 1 inversion which occurred in $N = 3$ specimens and was in heterozygous state in all of the samples all was confirmed using MOU7. The inversion on pair 1 was found only in the Kroomie population. The pair 4 inversion was found in two states (heterozygous and homozygous inverted) in $N = 6$ specimens in three population in the Western Cape and was confirmed using MOU 2/12 and 8. The inversion on pair 6 occurred in $N = 14$ specimens and was either heterozygous for the inversion or homozygous this was confirmed with MOU 3/5. The pair 6 inversion was found in 8 populations. The pair 10 inversion occurred in $N = 3$ specimens were present in heterozygous state only and were confirmed with MOU 3/5 and 4. The pair 10 inversions occurred in three populations in the Western Cape. Pericentric inversions were delineated using G-banding, C-banding and FISH (blue indicates DAPI staining, while the red and green indicates Biotin and Dig staining, respectively) in 21 populations of *O. irroratus*.

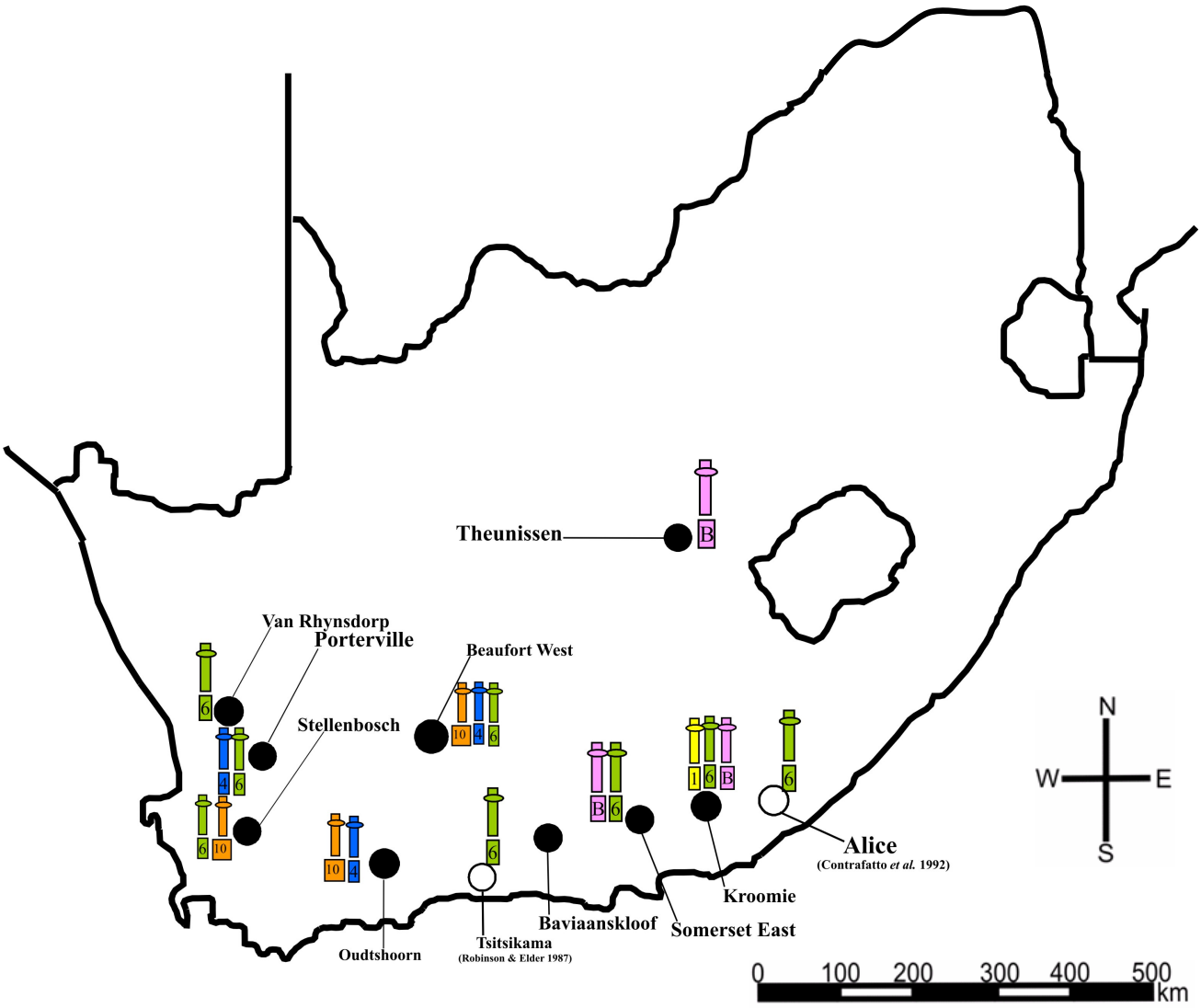


Figure 2.3: The distribution of *O. irroratus* individuals with inversions in South Africa from this study and previous studies. Individuals with B chromosomes occurred only in the Eastern Cape and Free State provinces. The different colours correspond to different chromosomes on which inversions occurred, for instance the pair 6 inversion indicated in green occurred in almost all of the populations that were sampled.

2.3.3 Ag-NOR results

All the specimens in this study which were analysed using Ag-NORs (N = 55) deviated from the results presented in Robinson and Elder (1987) as more than two chromosomal pairs possessed NORs (Figure 2.4; Table 2.1). Specimens from Porterville and Stellenbosch had NORs in autosomal pairs 1, 2, 8 and 9 on the short arm, proximal to the centromere, while pair 3 had two sets of NORs, one proximal to the centromere on the p arm, and the other on the distal end of the q arm thus conformed to the type specimen. The specimen from Oudtshoorn had NORs on autosomal pairs 1, 2, 7 and 9 proximal to the centromere on the p arm and a double signal on one of the homologues of pair 3, and only one signal on the other homologue at the terminal end of the long arm. Specimens from Somerset East, Kroomie, Baviaanskloof (Eastern Cape province) and Beaufort West (Western Cape province) displayed NORs on the same chromosomal pairs (pairs 2; 3 and 5), with the exception of Somerset East which had a NOR on pair 5 proximal to the centromere on the short arm (Figure 2.4).

Theunissen specimens contained NORs on three chromosome pairs: the distal end of OIR3q and OIR8q and proximal to the centromere of OIR9p on the short arm. Analysis of specimens from Hogsback (Eastern Cape) and Dargle (KwaZulu-Natal) revealed NORs on autosomal the terminal end of OIR3q, proximal to the centromere on OIR5q and proximal to the centromere on OIR9q (Figure 2.4).

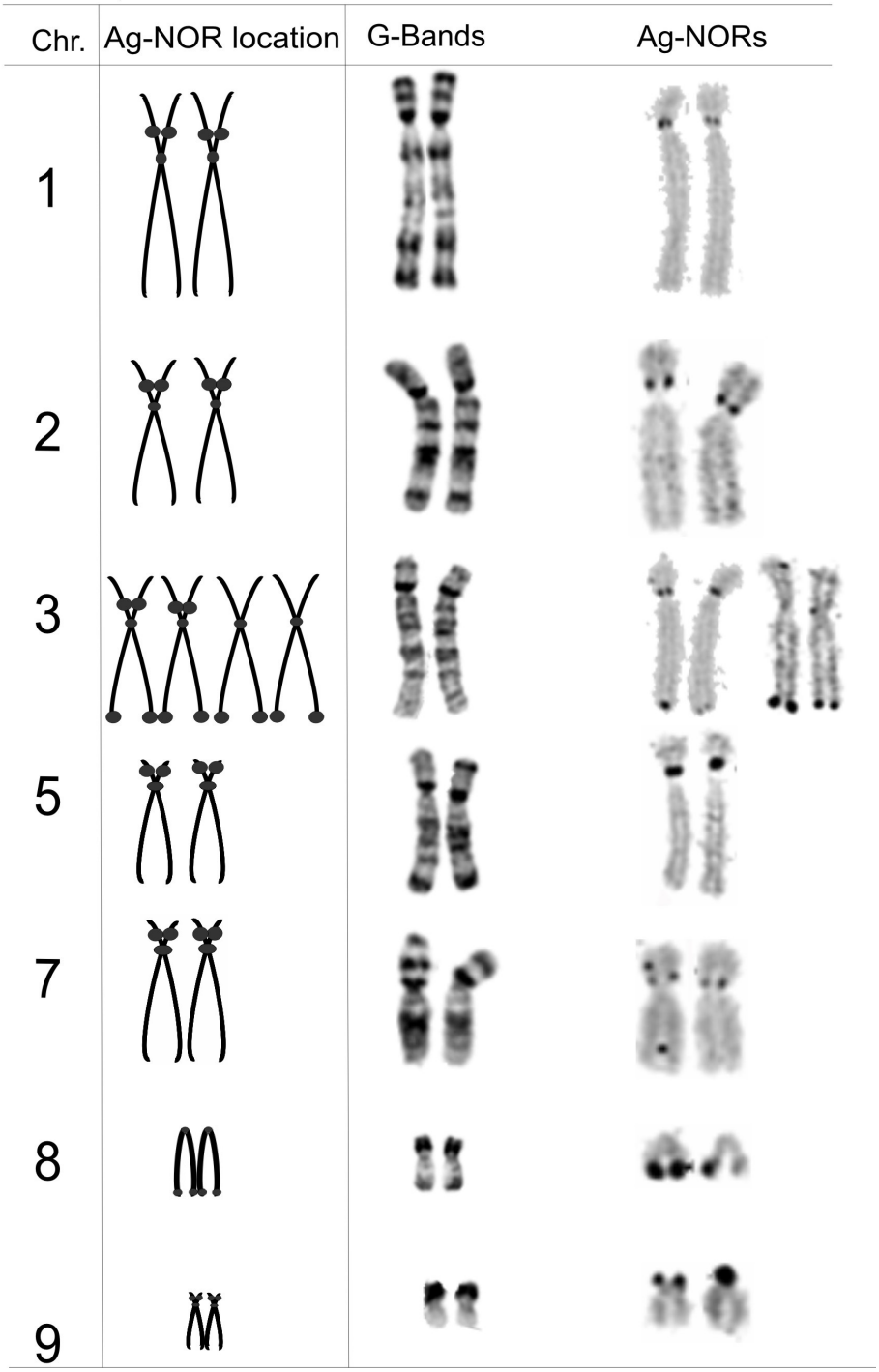


Figure 2.4: The location of Ag-NORs in the karyotype of *O. irroratus*. Analysis of $N = 55$ specimens revealed Ag-NORs on 7 autosomal pairs 1, 2, 5, 7, 8 and 9 proximal to the centromere on the short arm of the chromosome. Pair 8 also displayed Ag-NOR at the distal end of the long arm of the chromosome in individuals from the Free State province. Pair 3 showed two Ag-NORs occurring proximal to the centromere on the short arm and on the terminal end of the long arm, respectively.

2.4 Discussion

The cytogenetic data presented in this investigation provides the first evidence of inversion polymorphisms and confirms the diploid number variation in the widely distributed African endemic, *O. irroratus*. First, of the 55 animals that were analysed, 26 carried inversions (47%) involving either one of the four autosomes: OIR1, OIR4, OIR6 and OIR10. The four autosomal inversions were detected in both heterozygous (biarmed and acrocentric homologue) and homozygous (either two biarmed homologues or two acrocentric homologues) states, clearly indicating that they are floating polymorphisms. Extensive sampling throughout the range of the species in South Africa indicates that these inversions occur in the western and southern parts of South Africa (encompassing the Western Cape and the Eastern Cape provinces). In addition, differences between populations are due to the presence or absence of heterochromatic arms (and not inversions) resulting in variation in the NFa (40 – 49), whereas variation in diploid number (2n = 28 - 32) is due to supernumerary B chromosomes, as previously reported (Contrafatto *et al.* 1992; Rambau *et al.* 1997).

2.4.2 Evolutionary implications of inversions

Naturally occurring chromosomal polymorphisms are very important for understanding the role of chromosomal evolution in speciation. This is particularly important for polymorphisms involving inversions. In the first instance, when two populations differ as a result of inversions this may lead to reproductive isolation partly due to the formation of aneuploid gametes (containing deletions or duplications) when the F1 generation parents carrying the inversions mate. This is the result of illegitimate pairing of homologues at meiosis (Rieseberg 2001, Hoffmann *et al.* 2004). Consequently, pericentric inversions are formidable barriers to gene flow, and may underpin differences between species (particularly sibling species), as found in a variety of taxa including: the African water rats (*Dasymys cf.*

incomtus), which are separated by three inversions (Volobouev *et al.* 2000); south America tuco-tucos (*Ctenomys*), which are differentiated by inversions in the Y-chromosome (Novello and Villar 2006), west African Gerbils (*Taterillus*) which have undergone six pericentric inversions (Dobigny *et al.* 2005). Other examples include the Japanese talpine moles (*Eurocaptor* and *Mogera*), separated by eight inversions (Kawada *et al.* 2001), and reef fishes of the genus *Centropyge* which are separated by 22 inversions (Affonso and Galetti Jr. 2005).

The reproductive isolation in these examples results from a decrease in genes exchanged due to incompatibility, leading to speciation (Navarro and Barton 2003; Hey 2003). Therefore, the occurrence of pericentric inversions in *O. irroratus* in the southern and western parts of South Africa, and their absence in the eastern seaboard, may suggest incipient speciation in this putative species-complex. However, the critical factor in determining the role of a chromosomal rearrangement in speciation is whether or not the rearrangement is underdominant, i.e. if the parental homozygote has greater fitness than their heterozygote offspring (Forsdyke 2004). In such a case the underdominant rearrangement can become fixed in a population through genetic drift in small populations (Templeton 1981; Rieseberg 2001).

While it is well documented that inversions may lead to speciation (Rieseberg 2001; Hoffman and Rieseburg 2008), there is equally compelling evidence from the literature indicating that inversions may occur as floating polymorphisms within populations. These polymorphism have been detected in *Peromyscus* (Greenbaum and Reed 1984; Hale 1986; Tricket and Butlin 1994), in the South American *Oligoryzomys* which carries inversions on four chromosomal pairs (Bonvicino *et al.* 2001), and also in African rodents such as *Mastomys* (Volobouev *et al.* 2001). In these cases inversions are retained within populations

by suppression of recombination in the inverted chromosomal regions during meiosis (see Tricket and Butlin 1994, Noor *et al.* 2001; Rieseberg 2001; Kilpatric and Barton 2006). For instance, in the sitka deer mouse, (*Peromyscus sitkensis*), analysis of synaptonemal complexes using silver staining on heterozygous individuals revealed that asynapsis of the inverted chromosomal regions (and subsequently the absence of inversion loops) and the absence of chiasma within the heterozygous inverted regions resulted in the production of balanced gametes (Hale 1986). Further, the heterozygous homologues did not have duplications, deletions or breakages in the acrocentric and biarmed morphs, clearly indicating that viable gametes were produced by heterozygous carriers. This meiotic mechanism (heterosynapsis) would have allowed the retention of pericentric inversion polymorphisms in the population of the species (Hale 1986).

While the analysis of the *O. irroratus* specimens in this investigation was restricted to G-banding and FISH (as opposed to silver staining of the synaptonemal complexes), it is possible that a similar mechanism may be responsible for the maintenance of the four inversions detected in this study. This is partly supported by the karyotype results that were obtained when a pregnant female *O. irroratus* heterozygous for the OIR6 inversion produced two offspring with different morphs of pair 6: one offspring was homozygous (both acrocentrics) and the other was heterozygous for the OIR6 inversion. While the viability of the two offspring was not determined (they were discovered when the mother was dissected), it is possible that heterosynapsis may have been responsible for the retention of the pair six inversion in the two babies.

The distribution of an inversion throughout the range of a species may be due to several factors (Kirkpatrick and Barton 2006, Hoffman and Rieseberg 2008): reduced

recombination and local selection; epistatic selection that maintains an inversion; or the inversion could be under selection. A fourth explanation is that the inversion is neutral or it could be overdominant or simply underdominant (Kirkpatrick and Barton 2006, Hoffman and Rieseberg 2008).

The presence of inversions within *O. irroratus* throughout a wide geographic area, encompassing the Western Cape and Eastern Cape provinces (> 2000 km), suggests that this rearrangement may not be related to incipient species. Kirkpatrick and Barton (2006) proposed that heterosynapsis and chiasma suppression helps to protect favourable allelic syntenies from recombination, which may lead to evolution of "co-adapted gene complexes" located in the inverted regions (see discussion in Hale 1986 and example in Thomas *et al.* 2008). The polymorphism therefore seems to be localized to the Western Cape and the Eastern Cape provinces in particular.

These two regions differ considerably from the rest of southern Africa in many respects. Firstly, the Western Cape province is a winter rainfall area, and the Eastern Cape has a year-round rainfall regimen, unlike the rest of the country which experiences summer rainfalls and dry winters. Secondly, vegetation types are remarkably different from the rest of the country with the Fynbos biome extending from the Western Cape up to parts of the Eastern Cape where it is replaced by the Albany thicket biome (see Mucina and Rutherford 2006). Given the unique environmental conditions characterizing the Western Cape and Eastern Cape province the possibility that these floating polymorphisms may provide a mechanism to retain and protect region-specific adaptive genes cannot be excluded, although this hypothesis is not tested here (Kirkpatrick and Barton 2006, Hoffman and Rieseberg 2008).

2.4.3 Heterochromatin and B-chromosome variation

Supernumerary B chromosomes (B's) have been reported to occur as floating polymorphisms in the genomes of at least 55 species of mammals (Vujosevic and Blagojevic 2004). They vary in size, shape and molecular composition yet do not seem to contain any functional genes (Camacho *et al.* 2000; Vujosevic and Blagojevic 2004). In *O. irroratus* B chromosomes have also been reported and increase the species' diploid number from 2n = 28 to 2n = 32 (Contrafatto *et al.* 1992 *a, b*). Due to their molecular constitution they are not expected to cause any reproductive impact, therefore their presence or absence is not expected to lead to any barriers to gene flow (King 1993). Chromosomal data from this study show that B chromosomes are more prevalent in populations in the thicket biome of the Eastern Cape and the grassland biome of the Free State (Figure 2.3). They also seem to be completely absent from populations in the Western Cape province which have predominantly fynbos vegetation. Two major clades were found from the sequence data analysis (see Chapter 3), the distribution pattern of B chromosomes in the southern parts of South Africa may be an indication of a lack of gene flow between the two major clades.

In *O. irroratus* the prevalence of heterochromatin on the short arms of chromosomes has led to the description of three major cytotypic groups namely A (divided into A1 and A2), B and C (Contrafatto *et al.* 1992 *a, b*; Taylor *et al.* 1992). Recent molecular data presented in Taylor *et al.* (2009*a*), and molecular data from the cyt *b* gene from N = 98 specimens, for this study (Chapter 3 of thesis), groups individuals with the B and C-cytotype together in one group, with a 1.9 % maximum sequence divergence. I therefore propose that the cytotypic races based on the number of heterochromatic short arms is invalid as no clear distinction can be made between individuals of the B and C-cytotype from the cyt *b* and skull morphometric data (Taylor *et al.* 2009*a*). Furthermore, the number of autosomal arms (NFa) shows no

consistent pattern in the specimens that I analysed from all the populations (i.e. no clinal variation).

2.4.4 Ag-NOR variation and implications

Silver staining of the type specimen by Robinson and Elder (1987) revealed three pairs of NORs on two autosomal pairs 3 and 7. On pair 3 two NORs are present, one on the terminal end of the short arm, while the other is situated proximal to the centromere on the short arm. The study by Robinson and Elder (1987) also revealed that the NOR on pair 7 occurred proximal to the centromere on the short arm. The degree of silver staining is also correlated to the amount of rRNA synthesized, subsequently silver staining have been used frequently, particularly in mammals to quantify the amount of rRNA which is transcribed in a cell (Stanyon *et al.* 1995; Dobigny *et al.* 2002; Teruel *et al.* 2009).

Ag-NOR staining revealed two major groups within *O. irroratus* which contained populations in the south-western parts and the north-eastern parts of South Africa, respectively. The group in the south-western parts of South Africa (Porterville, Stellenbosch; Oudtshoorn and Van Rhynsdorp) is characterised by NORs on autosomal pairs 1, 2, 3 (double signal), 8 and 9. Further the north-eastern group (Somerset East, Kroomie, Baviaanskloof, Beaufort West and Theunissen) is characterised by NORs on 2, 3 (single signal), 5 with 8 and 9 only found in Theunissen (Free State province). These two groups underpin the two major clades retrieved by the molecular investigation based on cyt *b*, which indicates two unique evolutionary lineages separated by ~7% sequence divergence (Chapter 3 of thesis).

2.4.5 Conclusion

This study describes three rearrangements within *O. irroratus*, and shows that chromosomal variation among and within populations of *O. irroratus* is driven by heterochromatin additions and deletions. This in turn affects the number of chromosomal arms which also varied across all of the populations that I sampled. This study also extends the distribution of the B-cytotype (found in Oudtshoorn, Western Cape) which has not previously been recorded in the Western Cape of South Africa. The historically defined cytotypic races however, showed no consistent pattern as to where their geographic borders lie. Similarly, defining the cytotypic races based on the number of heterochromatic short arms, seems invalid, as very low sequence divergences were found among specimens with the B and C cytotype respectively (less than 2% sequence divergence) (see Chapter 3).

Further, the presence of B chromosomes in some populations leads to deviations in the diploid number and generally occurs only in populations in the east of the country (Grassland biome). The presence of B – chromosomes in only one of the two groups (Grassland group) therefore supports the presence of two unique lineages within *O. irroratus*.

The third chromosomal rearrangement which drives chromosomal variation between populations is pericentric inversions, which occur as floating polymorphisms and is probably maintained within the populations through a mechanism similar to the one suggested by (Kirkpatrick and Barton 2006). Overall, this study increases the number of *O. irroratus* populations analysed to date from 13 to 22 populations. Further this study gives support to the hypothesis which suggests that pericentric inversions can occur as floating polymorphisms within populations and highlights the fact that inversions may not always be associated with speciation. The meiotic mechanism which maintains the inversions in the different

populations is however not known at this stage. Future work will thus be directed at studying synaptonemal complexes using silver staining of spermatozoa and also using BAC clones targeting the inverted regions.

Ag-NOR staining in turn provide another nuclear diagnostic character for the recognition of two unique evolutionary lineages within *O. irroratus.* These results however should be treated with caution as Ag-NORs do not always reflect true NORs (Sanchez *et al.* 1995; Dobigny *et al.* 2002). Firstly, silver can bond to regions of the genome in the absence active NORs (Sumner 1990), secondly its been found that Ag-NOR staining can positively single out NORs, yet upon further investigation using FISH and rDNA probes revealed no rDNA signals, conflicting with the widespread believe that Ag-staining is highly reliable (Sanchez *et al.* 1995; Dobigny *et al.* 2002). Therefore although Ag-NOR staining has proved to be a useful diagnostic character for separating the two major clades within *O. irroratus*, these data should be treated with caution. Future studies should therefore be directed at incorporating FISH with rDNA probes in order to affirm the results found here.

Chapter 3

Allopatric fragmentation in the southern African vlei rat, *Otomys irroratus*: phylogeography inferred from mtDNA cyt *b* sequences

3.1 Introduction

Phylogeographical approaches have been used successfully to uncover population demographic structure of numerous small mammal species, including rodents (Avise 2000; Michaux *et al.* 2004; Yuasa *et al.* 2007; Vega *et al.* 2007; Mitsaines *et al.* 2008). In particular, species with wide distribution ranges have often shown multiple evolutionary units (Rambau *et al.* 2003; Michaux *et al.* 2004; Willows-Munro and Matthee 2009). The southern African vlei rat, *Otomys irroratus*, is widely distributed throughout South Africa where it occurs along the coastal belt and adjacent interior into Lesotho, eastern Zimbabwe, Swaziland and western Mozambique (Skinner and Chimimba 2005). Considering the wide distribution of the species, distinct phylogeographical breaks are likely to exist. This factor, coupled to the phenotypic conservatism within *O. irroratus* have led to several studies focused at investigating the presence of evolutionary lineages within the species (Contrafatto *et al.* 1992*a*, *b*, c, 1997; Meester *et al.* 1992; Taylor *et al.* 1992; 2009*b*; Pillay *et al.* 1992; 1995).

In the first instance, *O. irroratus* has a variable chromosome number, 2n = 23 - 32, comprising at least four cytotypes which are defined by the presence or absence of a

compound chromosome, centric fusions, pericentric inversions, B chromosomes, and the addition / deletion of heterochromatic short arms (Contrafatto *et al.* 1992*a*, *c*; Meester *et al.* 1992; Rambau *et al.* 2001; Taylor *et al.* 2009*b*). The observed chromosome polytypy is thought to be a consequence of habitat preference of the species (mesic grasslands) which may create conducive conditions for the fixation of novel chromosomal rearrangements according to the population flush hypothesis (Carson 1975; Robinson and Elder 1987). While the various *O. irroratus* cytotypes appear to be distributed in different bioclimatic regions in South Africa (Taylor *et al.* 1994), allozymes revealed that the four cytotypes (A1, A2, B and C) are genetically invariant (D = 0.03; Taylor *et al.* 1992; Contrafatto *et al.* 1997). However, small mammals such as rodents are known to exhibit low levels of allozyme heterozygosity (Nevo *et al.* 1997). The low genetic variation detected in *O. irroratus* may therefore be due to balancing selection or a slow rate of evolution, which could result in a lack of genetic differentiation among allopatric populations (Avise 1998; Crochet 2000; Piel and Nutt 2000).

The low levels of allozyme variation in *O. irroratus* are in contrast to laboratory breeding experiments where specimens with a diploid number of 2n = 24 (with a complex tandem fusion) were crossbred with specimens with a 2n = 30 (without the tandem fusion), resulting in high levels of antagonism or reduced viability in resultant offspring (Pillay *et al.* 1992, 1995). This suggests that pre- and post-zygotic barriers may preclude gene flow between some populations, and alludes to the presence of at least two evolutionary lineages within the taxon. This was confirmed by a recent analysis of several *O. irroratus* populations, using mtDNA cytochrome *b* (cyt *b*), skull morphometrics and cytogenetics, which retrieved two lineages (Taylor *et al.* 2009*b*). The two evolutionary lineages are demarcated by 6.4% sequence divergence, presence or absence of pericentric inversions and differentiation in cranial morphology differentiation. The two cryptic lineages appear to be distributed

parapatrically, with one lineage occurring in the grassland biome and the other in the Cape Floristic Region (CFR), respectively.

Vlei rats occur in mesic grassland habitats (Skinner and Chimimba 2005); these are prone to fragmentation and therefore could lead to the fixation of new rearrangements in isolated population according to the population flush hypothesis (Carson 1975). Evidence for the fragmentation of *O. irroratus* habitat stems from the fact that forest patches in southern Africa have expanded and contracted since the last glacial maximum (Lawes *et al.* 2007), which may have led to grassland habitat losses and gains respectively, for *O. irroratus*. Similar examples exist in other small mammals of southern Africa, namely *Rhabdomys pumilio* (Rambau *et al.* 2003) and *Myosorex varius* (Willows-Munro and Matthee 2009). In each of these cases, cladogenesis has been linked to climate oscillations since the Pliocene and Pleistocene, which in turn led to shifts in bioclimatic regions. These climatic changes would thus lead to distinct population demographics and clades which correspond to the major biomes in southern Africa.

The last glaciation event on the continent of Africa (~18 000 years ago) led to an estimated 5^{o}C average temperature decrease (Moreau 1962). An increase in the size of the montane biomes also occurred during this time period, according to geological deposits (Moreau 1962; Livingston 1975). In southern Africa, however, during the last glacial maximum temperature trends followed those of the rest of Africa but the mean rainfall across this area was not much different from current rainfall patterns (Moreau 1962). Vegetation types in this sub-region thus did not differ much from what is currently present. Carbon dating of pollen deposits similarly revealed that forest patches in the northern parts of Zimbabwe were isolated from forests along the eastern and southern coasts of South Africa

(Moreau 1962). This data therefore seem to suggest that the forests in the southern parts of southern Africa were more stable than the forests in the northern parts of southern Africa. Grasslands on the other hand, would experience expansions during forest contraction and contractions during forest expansions. The cycle of forest expansions and contractions thus would therefore lead to grassland fragmentation periodically. Genetic structuring is therefore expected between populations living in the northern parts of the species' distribution versus individuals living in the southern parts.

In order to investigate the effect of habitat heterogeneity and the role of natural barriers in shaping the population demographics within *O. irroratus*, a detailed DNA sequence study throughout the range of the species (i.e. phylogeography), together with previous studies on *O. irroratus* (behavioural, allozymes, breeding studies), will give insight into the various factors that may have led to the current population genetic structure of the species.

The aims of this aspect of the project were twofold:

1. To investigate the phylogeography of *O. irroratus* using mitochondrial gene (cyt *b*) sequence data derived from a comprehensive geographic sampling of *O. irroratus* throughout South Africa, in order to determine its population genetic structure, while the geographic coverage was improved by supplementing the data set with museum material.

2. To compare the chromosomal variation with the resulting phylogeny in order to determine the distribution of the recognised cytotypes.

3.2 Materials and Methods

3.2.1: Samples

A total of fifty five specimens of *O. irroratus* were live caught in the Western Cape (12 localities: Stellenbosch, Porterville, Van Rhynsdorp, Beaufort West, De Hoop Nature Reserve, Oudtshoorn, Bainskloof, Tweede Tol, Algeria, Gamkaskloof, Swartberg and Prince Albert), Eastern Cape (11 localities: Baviaanskloof, Somerset East, Kroomie, Hogsback, Thomas Bains Nature Reserve, Alice, Groendal Nature Reserve, Grahamstown, Sam Knott Nature Reserve and Stutterheim), Free State (three localities: Bloemfontein, Theunissen and Ficksburg) and Mpumalanga (one locality: Lydenburg), provinces of South Africa (Figure 3.1; Table 3.1). This data set was supplemented with $N = 75$ museum specimens obtained from the Durban Natural Science Museum (DM) (museum accession numbers provided in Table 3.1), representing the distribution of *O. irroratus*. Sequences from Tygerkloof ($N = 2$) (KwaZulu-Natal), Springs ($N = 1$) (Gauteng) and Zimbabwe ($N = 1$) were obtained from GENBANK and included in the study (see Table 3.1 for GENBANK accession numbers). The utilization of museum material in population genetic investigations is gaining considerable momentum in the literature (e.g. Baker *et al.* 1994; Smith 2007). This material provides the opportunity to greatly increase sample sizes.

3.2.2 DNA extraction PCR and DNA sequencing

Tissue biopsies were taken from wild caught specimens' tails and stored in absolute ethanol, and skin samples were taken from dried museum skins. Total genomic DNA was extracted using a Qiagen DNEasy kit following the protocol of the manufacturer. Extracted DNA was stored at 4^{o}C for later use for PCR (polymerase chain reaction). Cross contamination is commonly associated with museum material (see Stuart *et al*. 2006 for use

of museum material); hence a blank DNA extraction was included each time DNA was extracted from museum material.

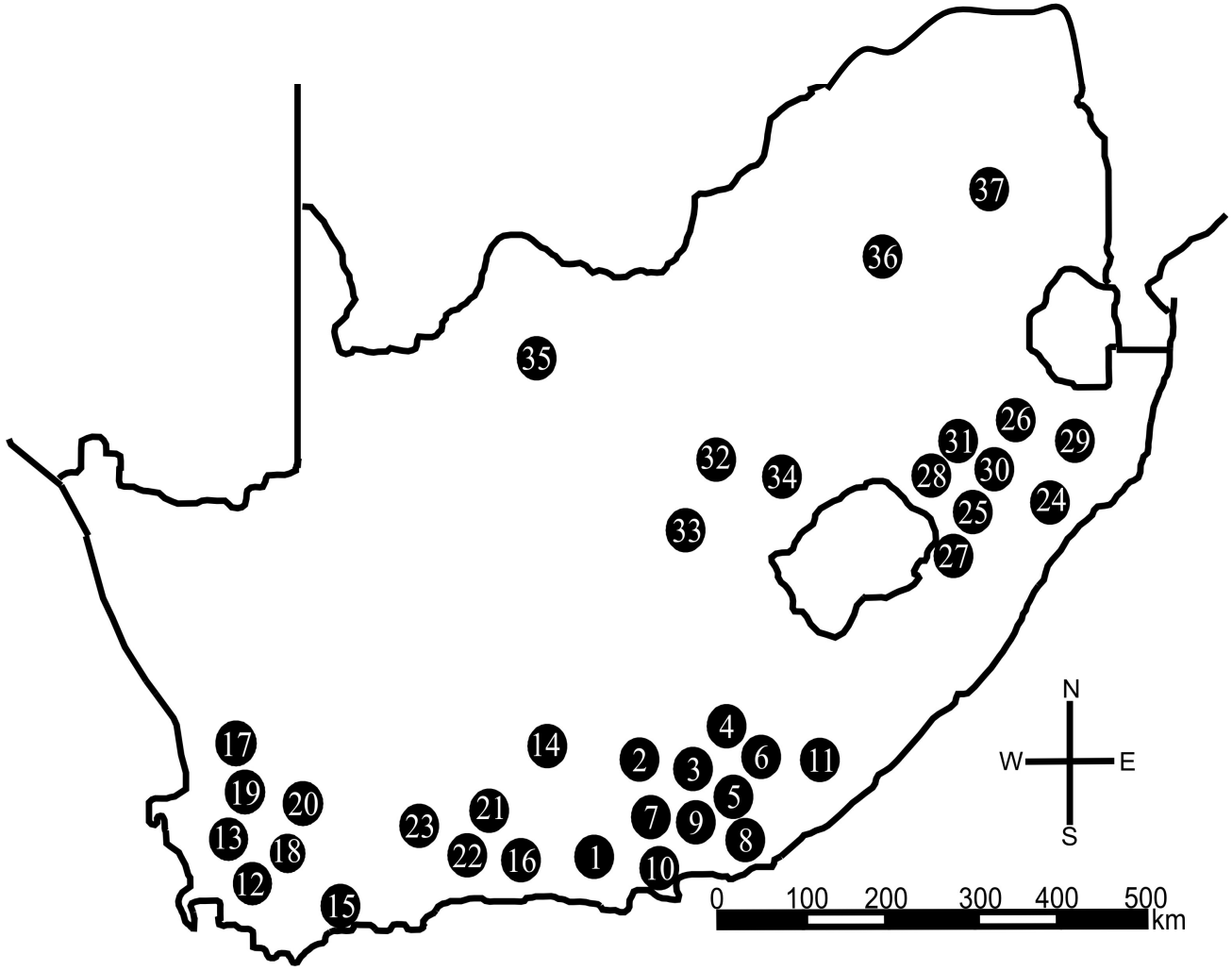


Figure 3.1: The geographic distribution of *O. irroratus* samples used in the current study. The numbers in each circle correspond to the locality in Table 3.1: (1) Baviaanskloof, (2) Somerset East, (3) Kroomie, (4) Hogsback, (5) Thomas Baines Nature Reserve, (6) Alice, (7) Groendal Nature Reserve, (8) Grahamstown, (9) Sam Knott Nature Reserve, (10) Port Elizabeth, (11) Stutterheim, (12) Stellenbosch, (13) Porterville, (14) Beaufort West, (15) De Hoop Nature Reserve, (16) Oudtshoorn, (17) Van Rhynsdorp, (18) Bainskloof, (19) Algeria, (20) Tweede Tol, (21) Gamkaskloof, (22) Swartberg, (23) Prince Albert, (24) Lemonwood, (25) Kamberg, (26) Karkloof, (27) Loteni, (28) Bergville, (29) Umgeni valley, (30) Fort Nottingham, (31) Mgeni, (32) Theunissen, (33) Bloemfontein, (34) Ficksburg, (35) Kuruman, (36) Rietvlei and (37) Lydenburg. The locality of sequences obtained from GENBANK are not indicated here (Tygerkloof; Springs and Zimbabwe).

Table 3.1: List of all the specimens used in this study. For each specimen the locality coordinates are indicated and the number of samples (*N*) that was collected and the number of base pairs (bp) sequenced. NR is nature reserve. For fresh material a 953 bp fragment was sampled, while a 400 bp was amplified from tissue obtained from the Durban Museum of Natural History (DM); museum accession numbers are indicated below. The diploid numbers of karyotyped specimens and GENBANK accession numbers are listed for all the populations.

Locality number and Locations	Province	Locality coordinates	*N*	2n	GENBANK accession numbers/Museum accession numbers
1 Baviaanskloof	Eastern Cape	33° 39' 38" S 24° 37' 57" E	5	28	HM363664-HM363668; Fresh
2 Somerset East	Eastern Cape	32° 41' 62" S 25° 37' 80" E	4	28,29,32	HM363689-HM363692; Fresh
3 Kroomie	Eastern Cape	27° 08' 07" S 20° 32' 11" E	5	28	HM363673-HM363677; Fresh
4 Hogsback	Eastern Cape	32° 36' 19" S 27° 01' 30" E	8	24	HM363657-HM363663; HM363708; Fresh
5 Thomas Bains NR	Eastern Cape	33° 23' 32" S 26° 29' 41" E	1		HM363696
6 Alice	Eastern Cape	32° 47' 00" S 26° 50' 12" E	6	28	HM363698;HM363730; HM363744; HM363746; Fresh
7 Groendal NR	Eastern Cape	33° 42' 07" S 25° 19' 20" E	4		HM363699;HM363700;HM363703HM363720
8 Grahamstown	Eastern Cape	33° 18' 15" S 26° 31' 08" E	7		HM363701;HM363702;HM363714-HM363717;HM363736
9 Sam Knott NR	Eastern Cape	33° 05' 27" S 26° 42' 58" E	3		HM363718;HM363719;HM363728
10 Port Elizabeth	Eastern Cape	33° 57′ 29″ S 25° 36' 00″ E	1		HM363733
11 Stutterheim	Eastern Cape	32° 32' 06" S 27° 22' 57" E	3		HM363747-HM363748;HM363740
12 Stellenbosch	Western Cape	33° 55' 54" S 18° 49' 47" E	5	28	HM363669-HM363672; Fresh
13 Porterville	Western Cape	32° 59' 15" S 19° 01' 28" E	5	28	HM363678-HM363682; Fresh
14 Beaufort West	Western Cape	32° 15' 19" S 22° 34' 25" E	4	28	HM363685-HM363688; Fresh
15 De Hoop NR	Western Cape	34° 26′ 03″ S 20° 32′ 52″ E	1		HM363693; Fresh
16 Oudtshoorn	Western Cape	33° 39' 56" S 22° 07' 39" E	1	28	HM363694; Fresh
17 Van Rhynsdorp	Western Cape	31° 44' 46" S 18° 46' 26" E	1	28	HM363695; Fresh
18 Bainskloof	Western Cape	33° 34' 50" S 19° 09' 13" E	1		HM363697
19 Algeria	Western Cape	32° 26' 19" S 19° 05' 05" E	2		HM363710;HM363712
20 Tweede Tol	Western Cape	33° 34' 28" S 19° 09' 32" E	1		HM363711
21 Gamkaskloof	Western Cape	33° 21' 50" S 22° 03' 41" E	2		HM363713;HM363725
22 Swartberg	Western Cape	33° 21' 36" S 22° 03' 14" E	1		HM363363731
23 Prince Albert	Western Cape	33° 13' 12" S 22° 01' 33" E	1		HM363732
24 Lemonwood	KwaZulu-Natal	29° 28' 03" S 30° 06' 16" E	3		HM363704-HM363706 Fresh
25 Kamberg	KwaZulu-Natal	29° 24' 54" S 29° 40' 21" E	2		HM363707;HM363739
26 Karkloof	KwaZulu-Natal	29° 18' 18" S 30° 13' 30" E	2		HM363734;HM363709
27 Loteni	KwaZulu-Natal	29° 27' 01" S 29° 32' 08" E	2		HM363721;HM363737
28 Bergville	KwaZulu-Natal	28° 44′ 00″ S 29° 22′ 00″ E	1		HM363723
29 Umgeni valley	KwaZulu-Natal	29° 29' 43" S 29° 48' 55" E	2		HM363724;HM363741
30 Fort Nottingham	KwaZulu-Natal	29° 24' 46" S 29° 54' 00" E	4		HM363749;HM363750;HM363726;HM363735
31 Mgeni	KwaZulu-Natal	29° 48′ 36″ S 31° 02′ 08″ E	2		HM363727;HM363738
32 Theunissen	Free State	28° 30' 06" S 26° 48' 07" E	2	28,30	HM363683;HM363684; Fresh
33 Bloemfontein	Free State	29° 07' 02" S 26° 14' 01" E	1	28	HM363656; Fresh
34 Ficksburg	Free State	28° 47' 33" S 27° 53' 35" E	2		HM363742;HM363743; Fresh
35 Kuruman	Northern Cape	27° 27' 12" S 23° 26' 37" E	1		HM363729
36 Rietvlei	Gauteng	25° 53′ 49″ S 28° 17′ 38″ E	1		HM363745
37 Lydenburg	Mpumalanga	25° 05′ 46″ S 30° 26′ 49″ E	1	28	HM363751; Fresh
38 Tygerkloof	KwaZulu-Natal	25° 58′ 05″ S 31° 34′ 01″ E	2		FJ619554.1; FJ619555.1
39 Springs	Gauteng	26° 21′ 07″ S 28° 45′ 00″ E	1		FJ619556.1
40 Zimbabwe	Chingamwe	18° 45′ 00″ S 32° 57′ 00″ E	1		FJ619562.1
Total number of specimens			102		

The complete cyt *b* gene primers, L 14724 (5'-AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA-3') (Kocher *et al.* 1989) and H 15915 (5'–CTG CAG TCA TCT CCG GTT TAC AAG AC-3') (Irwin *et al.* 1991), were used to amplify a 950 bp fragment from the fresh material. Museum samples were amplified using the mitochondrial cyt *b* primers designed specifically to amplify the first 400 base pairs of the gene (tRNA-GluA: 5'- TGA CTT GAA RAA CCA YCG TTG-3' and tRNA-GluJ: 5'- CCC TCA GAA TGA TAT TTG TCC TCA-3') (Palumbi *et al.* 1991). Each PCR reaction contained 14.9 µl of millipore water, 3.5 µl of 25 mM $MgCl_2$, 3 µl of 10 x Mg^{2+} free buffer, 0.5 µl of a 10 mM dNTP solution and 0.5 µl (10 mM) of the respective primer pairs, 0.1 µl of *Taq* polymerase and 2 - 4 µl of template DNA.

All PCR reactions had the following temperature cycle: 94°C for 4 min, 94°C for 30 sec, 48°C for 45 sec and 72°C for 35 sec. The last three steps were repeated for 40 cycles followed by a final extension of 15 min at 72°C. All PCR reactions were carried out and optimized using a GeneAmp® PCR system 2700 thermal cycler (Applied Biosystems). PCR products were separated in 1% agarose gels, and excised with the aid of a sterile surgical blade. An Illustra™, GE Healthcare commercial kit was then used to purify the gene fragments from the agarose gel. An automated sequencer (AB 3100, Applied Biosystems) was used to perform cycle sequencing. In order to authenticate the sequences obtained from the museum material all the sequences were blasted and compared to sequences on GENBANK and aligned with sequences obtained from fresh material.

3.2.3 Phylogenetic analysis

MODELTEST ver. 3.06 was used to estimate the mode of nucleotide substitution for the cyt *b* locus (Posada and Crandall 1998). Using the Akaike information criteria (AIC), which optimize the number of criteria that describes the data, the best-fit maximum likelihood score was chosen (Akaike 1973; Nylander *et al.* 2004). The obtained sequences were edited in Sequence Navigator

ver. 1.0.1 (Applied Biosystems, 1994). Phylogenetic relationships were inferred using maximum parsimony (MP) as implemented in PAUP*4 ver. beta 10 (Swofford 2002). The heuristic search option in PAUP*4 ver. beta 10 was selected with tree bisection and reconnection (TBR) branch swapping using 1000 random taxon stepwise additions and gaps as characters. Confidence nodes on the MP tree were determined with the aid of parametric bootstrapping (Felsenstein 1985). Nodes with bootstrap values above 75% were considered well supported while nodes with bootstrap values below 75% were considered as poorly supported.

Appropriate models were identified by MODELTEST and optimal tree space was investigated with the aid of MrBayes 3.0b4 for ML (Huelsenbeck and Ronquist 2001). For the Bayesian analysis ten Monte Carlo Markov chains were run for five million generations. The chains were sampled randomly for a tree every 1000^{th} generation. A consensus tree was constructed from the generated trees using the 50% majority rule and support for the nodes were estimated by posterior probabilities using the percentage of time a node was recovered. Nodes with posterior probabilities (pP) < 0.95 were considered poorly supported. The two otomyine species *O. laminatus* and *O. karoensis* were used as outgroups as they are sister to *O. irroratus* (Taylor *et al.* 2004; 2009*b*). In order to establish whether the resulting phylogeny and the chromosomal data are correlated, the chromosomal rearrangements identified in Chapter 2 (pericentric inversions, heterochromatin variation and the complex tandem fusion) were plotted on the resulting phylogeny.

3.2.4 Molecular clock

Fossil data indicate that the family Otomyinae is approximately five million years old (Pocock 1976; Avery 1991; Sénégas and Avery 1998; Sénégas 2001; Denys 2003; Jansa *et al.* 2006). The oldest known fossil of *Otomys* is dated at approximately 3.1 MYA (Sénégas and Avery 1998; Sénégas 2001; Denys 2003; Taylor *et al.* 2004; 2009*a*; Matthews *et al.* 2005; Hopley *et al.* 2006). Using the program BEAST (ver. 1.4.8) and three fossil calibration dates (mus / rattus:

~12MYA; Otomyinae: ~5MYA and *Otomys*: ~3.1MYA) the divergence date between the major clades that were retrieved were estimated using the Bayesian approach (with a length of 20 million generations and a burn-in of 200 000) and the nucleotide evolution model HKY+I+G (selected by MODELTEST) sampled every 10 000 iterations (Drummond and Rambaut, 2006*a*). The Bayesian approach was also used to investigate the confidence at the nodes at 95% p*P*. The program BEAUti (ver. 1.4) (Drummond and Rambaut 2006*b*) was used to analyse the output of the BEAST analysis while the program Tracer (ver. 1.3) (Rambaut and Drummond 2005) was used to construct a consensus tree with the divergence dates indicated at the nodes.

3.2.5 Population genetics analysis

A haplotype network was constructed using the method of Templeton *et al.* (1992) at the 95% confidence interval, using the software program TCS ver. 1.13 (Clement *et al.* 2001) and the network was subjected to nested clade analysis (NCA). NCA is a statistical tool which allows for the accurate discrimination between the historical events versus restricted gene flow operating on the current population genetic structure (Templeton *et al.* 1995; Templeton 1998; Templeton 2004). It has been used successfully to unravel the population genetic structure of many taxa including the fresh water crab *Potamonautes perlates* (Daniels *et al.* 2006), the Olive sunbird *Nectrarinia olivacealobscura* (Bowie *et al.* 2004) freshwater mussel *Lampsilis hydiana* (Turner *et al.* 1999) and the greater long-tailed hamster *Cricetulus triton* (Xie and Zhang 2005). Consequently, the haplotype network of the mtDNA gene was nested into a single network and analysed with the software GeoDIS ver. 2.0 (Posada *et al.* 2000).

Arlequin ver. 3.11 was used to determine the population genetic structure using an AMOVA and the clades obtained from the phylogenetic analysis were used for defining groups (Analysis of Molecular Variance) (Excoffier *et al.* 1992). Population subdivision was estimated using F-statistics, which included F_{ST} (fixation index) and F_{IS} (inbreeding coefficient) values respectively

(Excoffier *et al.* 1992; 2005; Frankham *et al.* 2003). Population demographics of *O. irroratus* were also evaluated using mismatch analysis. The mismatch analysis indicates whether or not range expansion or contraction has occurred in the historic past (Rogers and Harpending 1992). Mismatch distributions of pairwise nucleotide differences of each observed group was calculated and compared with expected values for an expanding / contracting population (Rogers and Harpending 1992). Tests for goodness-of-fit statistics based on the sum of square deviations (SSD) for a model of sudden expansion was determined in Arlequin version 3.11 (Excoffier *et al.* 2005). After range expansion or contraction is established, Fu's F*s* test was done to test whether the populations are in equilibrium (Fu 1997).

Neutrality tests were then done to ensure that the pattern of sequence polymorphism observed in the populations conformed to the neutral model. Tajima's D value was also calculated in order to evaluate whether the populations are in stasis or expanding using Arlequin ver. 3.11 (Excoffier *et al.* 2005). After an episode of population growth, coalescence theory predicts that external branches are elongated and there is an excess of low frequency mutations compared to the neutral model (Petit *et al.* 2000). Tests for goodness-of-fit and Fu's F_S were generated in Arlequin ver. 3.11 using parametric bootstrapping with 10 000 replicates (Felsenstein 1985).

3.3 Results

3.3.1 Gene and Phylogenetic analysis

Of the *N* = 75 museum samples extracted only *N* = 43 were successfully sequenced. A total of 98 samples thus from 38 localities were successfully amplified and sequenced; 950bp was obtained from the fresh material (*N* = 55) and 400bp from the museum material (*N* = 43) (Table 3.1). Further an additional four sequences were obtained from GENBANK, resulting in the total number of sequences analysed being *N* = 102. The missing base pairs for the museum material were coded as absent characters in the data set. In order to determine whether pseudo-genes were amplified or not, sequences were checked for stop codons using EMBROSS/Transec using the vertebrate mitochondrial setting and checking the six possible reading frames (http://www.ebi.ac.uk/Tools/emboss/transeq/index.html).

The partial fragments, of the cyt *b* gene and the complete fragment were firstly analysed as separate data sets. This was done in order to check whether the two data sets were comparable. Preliminary analysis retrieved identical patterns hence the sequences were combined into a single data matrix. All the sequences were deposited in GENBANK (Accession numbers: HM363654-HM363751). MODELTEST selected the HKY+I+G as the model of substitution using the AIC criteria (-lnL = 3405.58; AIC = 6823.16) (Hasegawa *et al.* 1985). The proportion of invariable sites (I) was 0.56 and the gamma distribution shape parameter (G) was 0.68 for the among site variation. The substitution model's rate matrix was R(a) [A-C] = R(c) [A-T] = R(d) [C-G] = R(f) [G-T] = 1.00 while R(b) [A-G] = 10.93 and R(e) [C-T] = 13.32, similar values were retrieved for both data sets (the 400bp and 953bp fragment) hence only the combined data set is discussed. The base frequencies are A = 31.43 %, C = 30.00 %, G = 11.50 % and T = 27.04 %. No significant variation in base composition was evident between sequences as shown by a chi squared test (x^2 = 15.95, df = 1, p = 0.01), as previously found in cyt *b* of other rodents (Michaux *et al.* 2004; Mitsaines *et al.* 2008).

The tree topologies retrieved for NJ, MP and BI were nearly identical with regard to the major clades retrieved hence only the MP tree topology is discussed. The MP tree was constructed from 157 parsimony informative characters; analysis of these characters yielded 100 trees with a length of 363 steps. The consistency index (CI) was 0.60 while the retention index (RI) was 0.92. Both MP and BI trees retrieve *O. irroratus* as monophyletic (100% / 0.99 p*P*) (Figure 3.2). Two major clades with good statistical support were retrieved among ingroup *O. irroratus* samples (100% / 1.00 p*P*), with an average 7.0% sequence divergence between the two. Clade A occurs in the south western parts of South Africa (Western and Eastern Cape provinces) (96% / 0.96 p*P*) while Clade B occurs in the north eastern parts of South Africa (Eastern Cape, Free State, KwaZulu-Natal, Mpumalanga, Gauteng and Northern Cape provinces) (79% / 0.96 p*P*) (Figure 3.2).

Two groups are evident within clade A, the first is comprised of 13 specimens which are exclusive to the Eastern Cape provinces (100% / 0.99 p*P*), the second group has 39 specimens which occur in the Western and Eastern Cape (Figure 3.2). Clade A is characterized by shallow sequence divergences between specimens (1.91%). Clade B also has two sub-clades with one sub-clade comprised of ten individuals derived from a wide geographic range in the northern part of South Africa (Mpumalanga, Northern Cape, KwaZulu-Natal and Free State provinces) (77% / 1.00 p*P*). The larger group has 33 specimens predominantly occurring in the south eastern half of South Africa (Eastern Cape, KwaZulu-Natal) (100% / 0.96 p*P*). Further specimens from Alice ($N = 6$) originating in the Eastern Cape province grouped in both clades A and B indicating an area of sympatry. Specimens from Hogsback in the Eastern Cape grouped in two sub groups comprising clade B, similarly specimens for Fort Nottingham (KwaZulu-Natal) were present in the two sub groups of clade B, and these are separated by a 4.8% sequence divergence (Figure 3.2). In turn, divergence time estimates date the split between the two major clades at 1.1 MYA, suggesting a late Pliocene/Pleistocene cladogenesis.

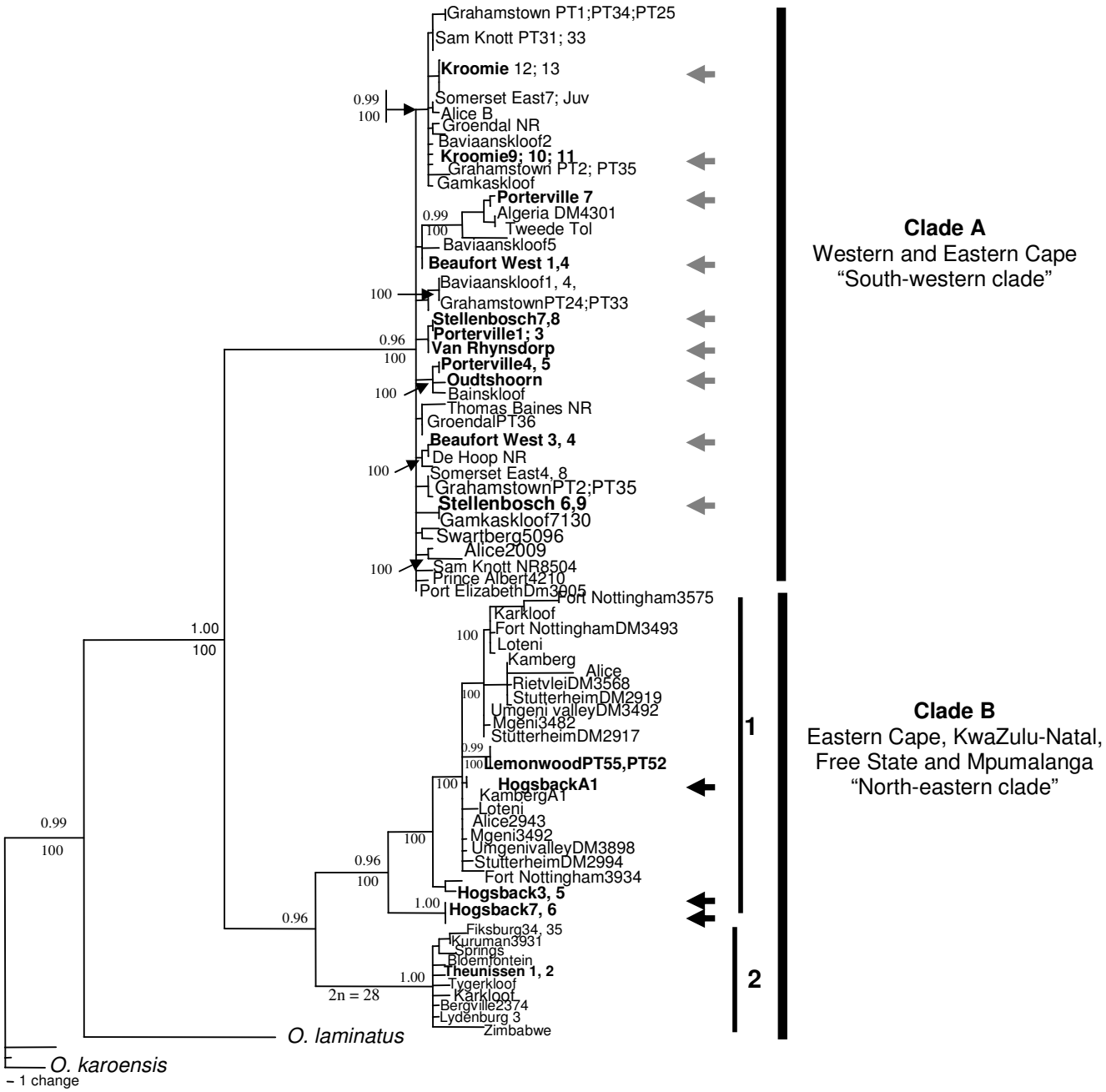


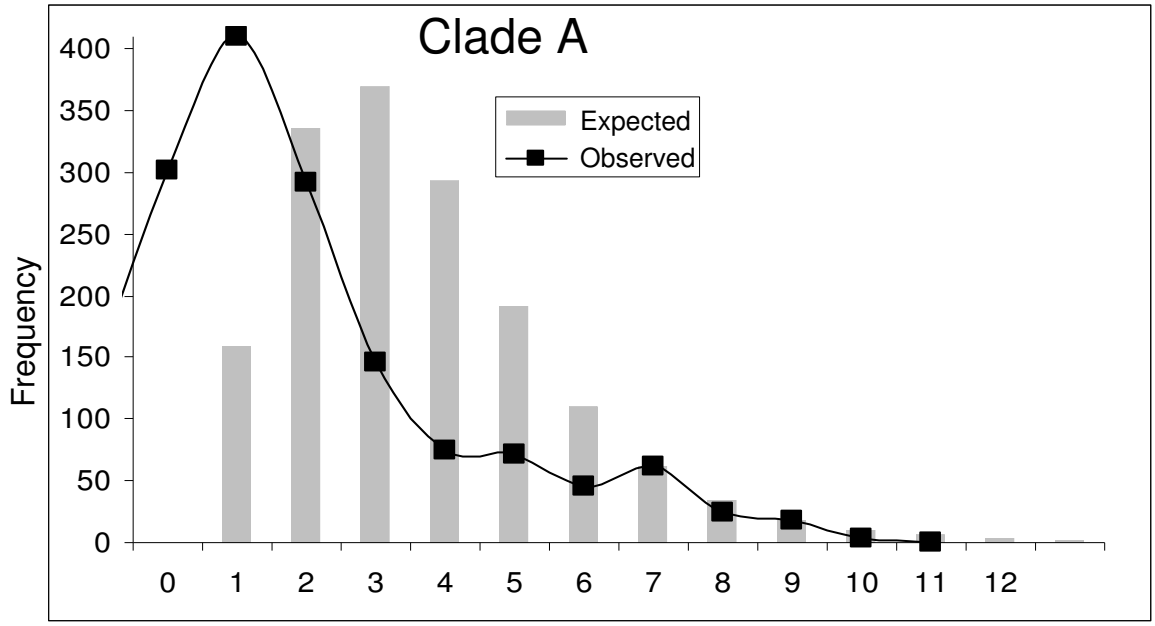
Figure 3.2: The maximum parsimony phylogram for *O. irroratus* for the cyt *b* gene (*N* = 102) based on 69 haplotypes. Statistically well supported clades have posterior probabilities which are > 0.95 p*P* (above nodes), while bootstrap values above 95% is indicated below supported nodes. The two major clades A and B that were retrieved are indicated by the vertical lines. Specimens that were karyotyped are indicated in bold, and specimens with pericentric inversions are indicated by grey arrows, while specimens with the complex tandem fusion are indicted by the black arrows. NR indicates Nature Reserves.

3.3.2 Chromosomal analysis

When the chromosomal rearrangements were plotted onto the phylogenetic tree (Figure 3.2), fixed chromosomal rearrangements separated the two major clades. Clade A contained specimens with pericentric inversions (N = 26; grey arrows; Chapter 2) which were completely absent in clade B, while a complex tandem fusion (N = 10; black arrows; Chapter 2) occurred in clade B which were absent from clade A specimens (indicated in Figure 3.2 Chapter 2). Also, specimens from the B and C cytotype co-occurred in clade A with shallow sequence divergences separating them (1.91% maximum sequence divergence). Specimens in clade B, which is sub divided into two clades, had specimens with the A1, A2, B and C cytotypes. Specimens with the A1 and A2 arrangement grouped (group 1) together (100% / 0.96 pP), while specimens with the B and C cytotype formed a group (group 2) (75% / 1.00 pP), the two sub-clades were separated by an average 4.8% sequence divergence (Figure 3.2).

3.3.3 Population genetics

An AMOVA revealed that Φ_{ST} was 77.10% (Va = 77.10%, df = 36, sum of squares 408.71, variance component = 1.38). There was highly significant pairwise F_{ST} values between the two major clades F_{ST} = 0.87, ($p < 0.01$) (Table 3.2). Within the two clades some genetic substructure was found with F_{ST} values ranging from 0.40 ($p < 0.01$) in clade A to 0.44 ($p < 0.01$) in clade B. The Tajima's D value over all of the sampled localities was 0.52 and the positive value therefore suggests balancing selection or a decrease in the population size (Tajima 1983), while a p-value of 0.83 was reported by Fu's F_S test for all the sampled localities, indicating that the populations are in stasis. A bell shaped (unimodal) distribution of the frequency of the pairwise differences was found in clade A which is indicative of a recently expanded population (Figure 3.3a). In contrast clade B has a multimodal pairwise difference distribution of a stable population (Rogers and Harpending 1992) (Figure 3.3b).



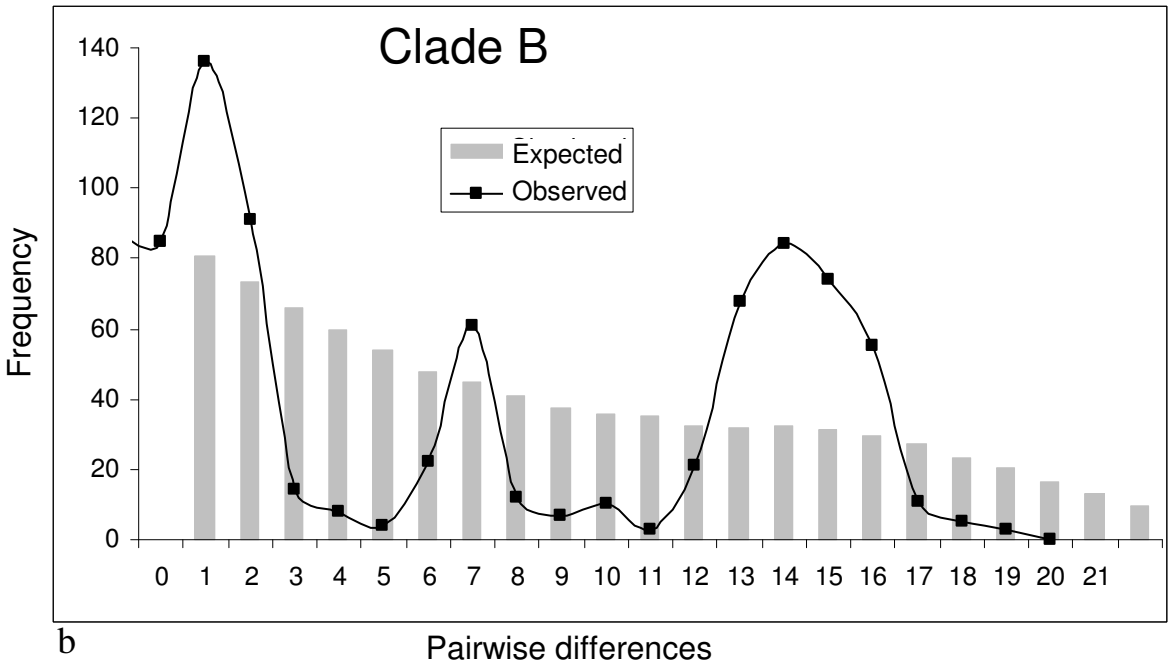


Figure 3.3: Mismatch distribution for the cyt *b* gene of the two *O. irroratus* clades. Each vertical bar represents 1000 simulations of the 102 mtDNA sequences. The expected population growth-decline model was determined with Arlequin ver. 3.00 and is indicated by the vertical bars (3.3a, b). The observed frequency is indicated by the solid line. (3.3a, b) Clade A shows a bell shaped distribution for the observed frequency (grew from $\theta = 0.1$ to 12 at $\tau = 1$ unit of mutational time past) indicating a recent expanded population while, (3.3b) Clade B shows three peaks for the observed frequency indicating a stable population ($\theta = 0.1$).

Table 3.2: Analysis of molecular variance for $N = 38$ *O. irroratus* populations. F_{CT} represents genetic differentiation between groups; F_{SC} shows genetic variation among populations within groups; F_{ST} shows the overall genetic variation among populations. All the values showed significant ($P < 0.05$) genetic differentiation.

Source of variation	d.f	sum of squares	Variance component	Percentage of variation
Among groups	1	408.71	8.33 Va	77.1
Among populations within groups	36	149.3	1.08 Vb	10.07
Within populations	61	84.66	1.38 Vc	12.84
Total	98	642.68	10.811	
F_{SC}				0.44
F_{CT}				0.77
F_{ST}				0.87

3.3.4 Nested clade analysis

Sixty nine haplotypes were retrieved for the 102 cyt *b* sequences (Table 3.2). A haplotype network was constructed using the program TCS which grouped all the specimens into a single network at the 95% confidence level (Figure 3.4). The two major clades which were revealed in the phylogeny were also evident from the haplotype network. The number of haplotypes (*Nh*) per locality ranged from one to four and most of the haplotypes were separated from each other by one to three mutational steps. Two genetically distinct groups are apparent from this network, indicated by the high number of mutational steps (18 in total) separating them. The two sub groups in clade B are separated by eight mutational steps (Figure 3.4).

Forty-five one-step clades, 16 two-step clades, six three-step clades and two four-step clades were evident from nested clade analysis. GeoDis (ver. 2.0) also retrieved two divergent groups which correspond to the two lineages obtained using phylogenetic analysis (clades 4-1 and 4-2). Clade A occurs in the south western parts of South Africa (4-2), while Clade B occurs in the north eastern parts of South Africa. The analysis indicated that eight of the clades had a statistically significant relationship between nested clades and geography (Table 3.4). Clade 1-15 was the only clade on the first nesting level to have a statistically significant *p*–value. Inadequate geographic sampling, however, prevented any conclusive interpretation. Three clades on the second nesting level showed statistically significant *p*-values (2-6, 2-8 and 2-15) (Table 3.4). For clade 2-6 however, inadequate sampling precluded any robust conclusion and isolation by distance as a result of short distance dispersal vs. long distance dispersal could not be confirmed. For clade 2-8 restricted gene flow with isolation by distance led to the current genetic structure. This haplotype occurs in the Oudtshoorn region which occurs in the little Karoo of the Western Cape province. The

analysis retrieved an inconclusive outcome for the clade 2-15. On the third nesting level two clades had statistical support (3-2 and 3-5). Clade 3-2 showed restricted gene flow with some long distance dispersal; clade 3-2 includes most of the Western Cape province samples (clade A). The clade 3-5 has haplotypes which originates from Hogsback in the Eastern Cape province and restricted gene flow with isolation by distance is implicated as the driving force leading to the contemporary genetic structure in this clade. Only one clade on the fourth level had sufficient statistical support (4-1); however an inconclusive outcome was retrieved from the inference key for this clade. The total cladogram showed that allopatric fragmentation led to the current genetic structure within *O. irroratus.*

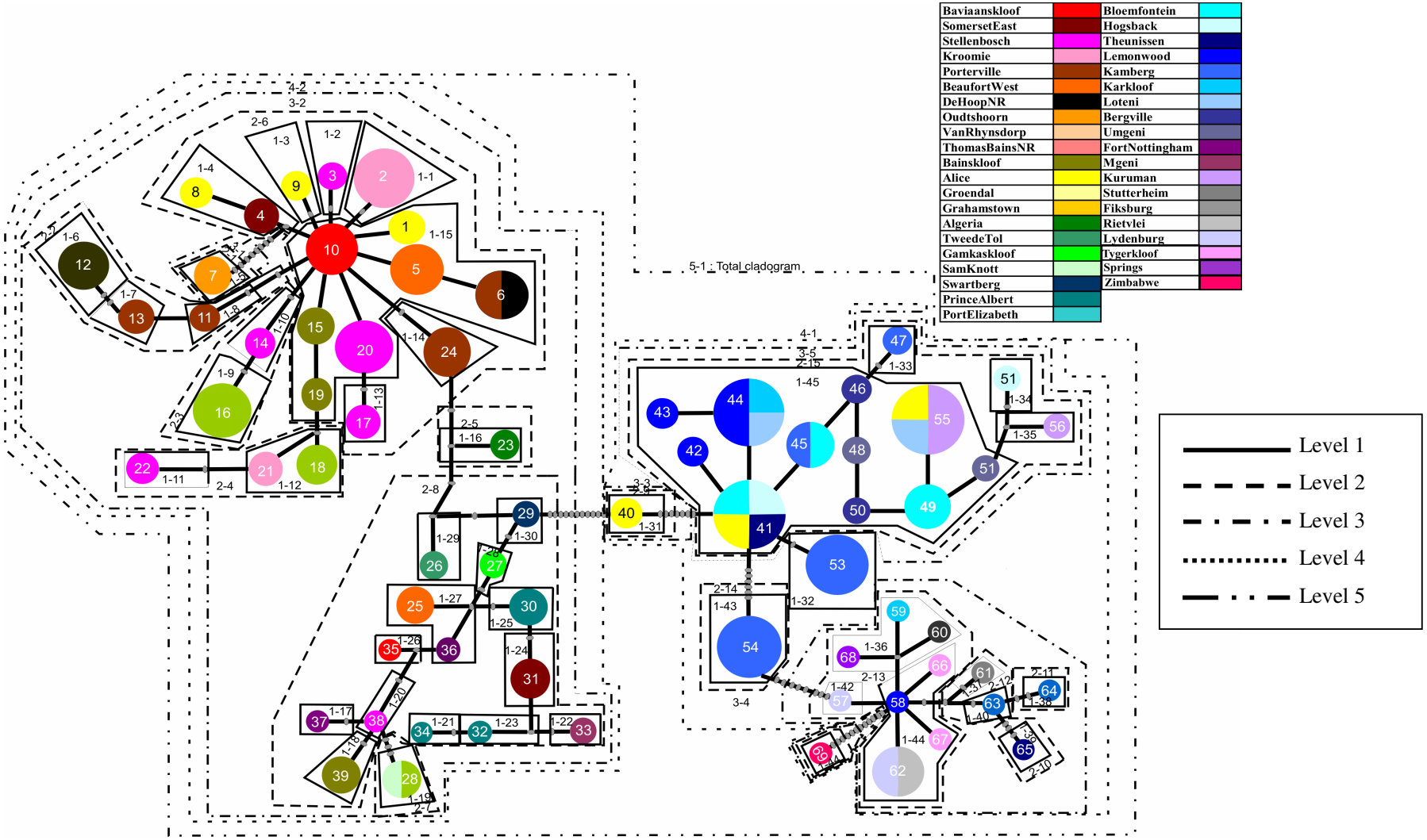


Figure 3.4: Redrawn minimum spanning haplotype network of 69 haplotypes retrieved from 102 sequences at the 95% confidence interval. Each colour indicates a sampling locality and the numbers inside the circle corresponds to the haplotype found at that location. The size of the circles is relative to the number of different haplotypes which occurred at that particular location. The numbers correspond to the haplotype name and the grey circles between haplotypes correspond to the mutational steps (unsampled haplotypes) between the observed haplotypes. The clades on the different nesting levels are demarcated by different border lines.

Table 3.3: The frequency of haplotypes *N* = 102 specimens obtained from 40 localities.

		Haplotypes																																	
Population	***N***	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Baviaanskloof	5																3		1										1								
Somerset East	4															1				1																
Stellenbosch	4					2																				2										
Kroomie	5		4																			1														
Bloemfontein	1																																			
Hogsback	8																																			
Porterville	5						1					1		1											2												
Theunissen	2																																			
Beaufort West	4																														2		1		1		
De Hoop NR	1																																	1			
Oudtshoorn	1																										1									
Van Rhynsdorp	1						1																													
Thomas Baines NR	1																											1								
Bainskloof	1																							1												
Alice	6	1							1	1																										
Groendal NR	4				1																											3				
Grahamstown	7			1											1			2			1		1													
Lemonwood	3																																			
Kamberg	2																																			
Karkloof	2																																			
Algeria	2												2																							
Tweede Tol	1							1																												
Gamkaskloof	2										1																								1	
Sam Knott NR	3																				2															
Loteni	2																																			
Bergville	1																																			
Umgeni valley	2																																			
Fort Nottingham	6																																			
Mgeni	2																																			
Kuruman	1																																			
Swartberg	1																																			1
Prince Albert	1																													1						
Port Elizabeth	1																												1							
Stutterheim	3																																			
Fiksburg	2																																			
Rietvlei	1																																			
Lydenburg	1																																			
Tygerkloof	2																																			
Springs	1																																			
Zombabwe	1																																			

Table 3.3: (Continued):

Population	*N*	Haplotypes																																
		37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69
Baviaanskloof	5																																	
Somerset East	4			2																														
Stellenbosch	4																																	
Kroomie	5																																	
Bloemfontein	1																										1							
Hogsback	8									1		1						3	3															
Porterville	5																																	
Theunissen	2																					1					1							
Beaufort West	4																																	
De Hoop NR	1																																	
Oudtshoorn	1																																	
Van Rhynsdorp	1																																	
Thomas Baines NR	1																																	
Bainskloof	1																																	
Alice	6				1	1														1														
Groendal NR	4																																	
Grahamstown	7		1																															
Lemonwood	3																			2	1													
Kamberg	2					1											1																	
Karkloof	2								1															1										
Algeria	2																																	
Tweede Tol	1																																	
Gamkaskloof	2																																	
Sam Knott NR	3	1																																
Loteni	2								1											1														
Bergville	1																								1									
Umgeni valley	2										1				1																			
Fort Nottingham	6						1	1	2					1									1											
Mgeni	2												1			1																		
Kuruman	1																									1								
Swartberg	1																																	
Prince Albert	1																																	
Port Elizabeth	1																																	
Stutterheim	3					1				1				1																				
Fiksburg	2																											1	1					
Rietvlei	1					1																												
Lydenburg	1																													1				
Tygerkloof	2																														1	1		
Springs	1																																1	
Zombabwe	1																																	1

Table 3.4: Nested clade analyses results for 41 populations of *Otomys irroratus* collected throughout South Africa. Nesting results are based on 10 000 replicates in GEODIS (Posada *et al.* 2000). Templeton's inference key was used (2004). An asterisk indicates a significant $p < 0.05$ value.

Clade	x^2	Probability	Inference chain	Inferred pattern
1 - 15	55.00	0.04	1-19-20-No	Inadequate geographic sampling
2 - 6	74.22	0.032*	1-2-3-5-6-7-8-No	Sampling inadequate to discriminate between IBD (SDD vs. LDD)
2 - 8	0.00	<0.001*	1-2-3-4-No	RGF with IBD
2-15	30.52	0.76	1-2-11-17-No	Inconclusive outcome
3 - 2	167.63	0.011*	1-2-3-5-6-7-Yes	RGF dispersal but with some LDD
3 - 5	9.93	0.37	1-2-3-4-No	RGF with IBD
4 - 1	0.00	0.00	1-2-11-17-No	Inconclusive outcome
Total	102.0	0.00	1-2-3-4-9-No	Allopatric Fragmentation

IBD, isolation by distance; SDD, short distance dispersal; LDD, long distance dispersal; RGB, Restricted gene flow

3.4 Discussion

3.4.1 Main findings

The topology inferred from the cyt *b* mtDNA sequence data clearly demonstrates the presence of two lineages each containing two sub-clades within *Otomys irroratus*. These two major clades are characterised by elevated sequence divergence values and separated by fixed chromosomal differences (data not shown). Clade A includes the distribution of the type locality of *O. irroratus* (Tsitsikamma, Western Cape; Robinson & Elder 1987) while clade B represents a novel lineage, referred to as *O. auratus* by Taylor *et al.* (2009) (the species still require formal description). The two clades A and B are sympatric at Alice in the Eastern Cape, and are genetically distinct, as evident from the general absence of shared haplotypes (excluding the Alice specimens). Molecular data of the sequences indicates that cladogenesis in *O. irroratus* occurred in the Pliocene/Pleistocene. Further, mismatch distributions indicate that clade A underwent a recent expansion while clade B has a mismatch distribution indicative of a stable population suggesting that the two groups are on different evolutionary trajectories. Allopatric fragmentation was inferred from NCA, suggesting that historic events lead to the current genetic structure within *O. irroratus*.

3.4.2 Population demography, biogeography and NCA

The results from this study corroborate the findings of Taylor *et al.* (2009*b*), which suggested that cladogenesis within *O. irroratus* was probably the result of climate oscillations since the Pliocene epoch. The estimated divergence times within *O. irroratus* is 1.1 MYA which is in line with the climate changes which took place since the late Pliocene 2.9 – 0.8 MYA (Ellery *et al.* 1991; de Menocal 2004; Lawes *et al.* 2007). The divergence between the two clades is dated at 1.1MYA while the two clades are aged at 0.62 MYA (clade A) and 0.66 MYA (clade B) in this study. These changes were possibly brought about by a two degrees

Celsius drop in temperature and the addition of a 15% lower precipitation (Ellery *et al.* 1991) led to the contraction of grassland and fynbos biomes, thus fragmenting *O. irroratus* populations (Taylor *et al.* 2009*b*).

Taylor *et al.* (2009*b*) further argue that species such as *O. irroratus* moved to the highland regions of the Drakensberg and Cape Fold Mountains (where the climate would have been suitable) during this period. With the onset of climate amelioration again to more favourable conditions the species extended its range to lower elevations again. This argument is in line with Robinson and Elder (1987) who proposed that due to *O. irroratus*' specific habitat requirements it is prone to habitat fragmentation leading to the fixation of novel chromosomal rearrangements. Support for this is evident in the mismatch distribution which shows a recent expansion for clade A. This recent expansion could have been due to favourable climatic conditions which prevailed during the Pleistocene, which enabled the expansion of Fynbos, northwards up the west coast of South Africa, thus allowing for *O. irroratus* to expand in this direction (Moreau 1962). This clade also contains pericentric inversions which are completely absent in clade B.

Interestingly, haplotypes are shared between sampling localities on either side of the Cape Fold Mountains (CFM) (Porterville; Van Rhynsdorp; Stellenbosch and Algeria on the western side and Oudtshoorn; De Hoop NR; Swartberg and Beaufort West on the eastern side of the CFM), which is a known phylogeographic barrier (Tolley *et al.* 2008; 2009; Swart *et al.* 2009). This indicates that the Cape Fold Mountain range may not be a barrier to dispersal for the species as some long distance dispersal has been implicated from the nested clade analysis on (Table 3.4). The hypothesis advanced by Taylor *et al.* (2009*b*), renders a plausible explanation. The latter authors argue that with the onset of dry conditions *O. irroratus* would

have sought refuge in highland areas where conditions would be suitable, and later expanded their ranges when conditions were suitable again. Apart from the dry conditions, the grassland habitat of *O. irroratus* may have been able to expand during higher temperature cycles as woodlands would have contracted during this time (Ellery *et al.* 1991; Lawes *et al.* 2007). The lack of genetic structure within clade A could therefore be a result of these processes.

The area of parapatry found at Alice between clades A and B in the Eastern Cape province has also been found in other taxa, namely the fourstriped fieldmouse *Rhabdomys pumilio* (Rambau *et al.* 2003) and the forest shrew *Myosorex varius* (Willows-Munro and Matthee 2009). This area is a known crossover zone between the fynbos and grassland biomes in southern Africa as well as the two forest types Afrotemperate forest and Scarp forests (Mucina and Rutherford 2006). The distribution of the two main clades thus closely follows the geographic extent of these two biomes in the southern parts of South Africa. It also mirrors the contraction and expansion of the forests which occurred since the Pliocene/Pleistocene epochs (Lawes et al. 2007). Given that cladogenesis and forest contractions / expansions occurred during the same time period, it is conceivable these events may be linked.

3.4.3 Species boundaries

A plethora of species concepts exist (Mayden 1997; Bond and Sierwald 2002; Sites and Marshall 2003; Monaghan *et al.* 2009). Of these the most widely used is the biological species concept (Mayr 1963). One alternative to the biological species concept, the phylogenetic species concept, has been developed employing DNA sequence data in species distinction criteria (Wiley 1981; Cracraft 1989; 1992). Apart from this, other concepts such

as the mtDNA phylogroups concept (Avise and Walker 1999), amongst others also exist (see Mayden 1997; de Queiroz 2007).

Many authors suggest speciation has occurred when sequence divergences above 7.0% are evident between species (Bradley and Baker 2001; Rambau *et al.* 2003; Baker and Bradley 2006). Bradley and Baker (2001) suggest the genetic species concept as follows "A species is a group of genetically compatible interbreeding natural populations that are genetically isolated from other such groups". They also propose that elevated sequence divergence within rodents (usually more than 5% for cyt *b*) indicates the presence of novel species. The biological and the phylogenetic species concepts, however, does have their disadvantages (Bradley and Baker 2001; de Queiroz 2007). However, since speciation processes are complex, it is advisable to use an integrated approach where multiple diagnostic characters (including genetic characters, morphometric data and chromosomal data) is advocated as proposed by Crandall *et al.* (2000) and Bond and Stockman (2008).

In the case of *O. irroratus*, Templeton's test of cohesion (TTC) is applied by means of nested clade analysis and inferences about phylogeography are based on a phylogenetic tree (Templeton 2001; Templeton *et al.* 1995). Templeton's test of cohesion applies two hypotheses for species distinction criteria; the first suggests that evolutionary lineages are exclusive while the second suggests that lineages are exchangeable both genetically and ecologically. If both hypotheses are rejected at similar levels of confidence, the presence of novel lineages is inferred (Templeton 2001; reviewed in Sites and Marshall 2003). In the case of *O. irroratus* my cytochrome *b* data supports pre- and post zygotic isolation experiments (Pillay *et al.* 1992; 1995). Further, the resolved phylogeny retrieved using multiple

phylogenetic analyses (NJ, MP, Bayesian inference) also suggest that the phylogenetic species concept can be applied in *O. irroratus*.

Several problems are, however, associated with mtDNA sequence data when applied in species distinction criteria (Moritz and Cicero 2004; Monaghan *et al.* 2009). Although the utilization of mtDNA has proven to be a huge benefit to population genetics, phylogeography and biogeographical investigations across all known taxa, it has limitations in designating new species due to its rapid evolution and the presence of pseudo-genes in some instances (Avise 2000; Zhang and Hewitt 2003; Michaux *et al.* 2004; Yuasa *et al.* 2007; Mitsaines *et al.* 2009 and Hickerson *et al.* 2010 for review). By far the most limiting factor of mtDNA is the fact that it only reflects the matrilineal evolutionary history (Zhang and Hewitt 2003). Considering these limitations, it is necessary to use nuclear DNA markers in conjunction with mtDNA. To this end, many studies have incorporated nuclear DNA sequence data when making investigations into phylogeography, and when dealing with species boundaries (for example Cho *et al.* 1995; Bagley *et al.* 1998; Hare 2001). In this study chromosomal data (nuclear markers), in conjunction with the mtDNA marker as well as the presence of behavioural differences (pre-and post-zygotic isolation) supports the presence of two cryptic lineages within *O. irroratus* (Pillay *et al.* 1992; 1995). When taken together with other data sets the two lineages may represent two species and thus support Taylor *et al.* (2009*b*). Clade A occurs in the CFR and Albany thicket biome and is characterised by pericentric inversions, while the clade B occurs in the northern grassland biome and is fixed for a complex tandem fusion in some populations. They synonymysed clade A in this study to *O. irroratus* as it originates from the type locality (Tsitsikamma, Western Cape; Robinson & Elder 1987) while clade B was referred to as *O. auratus* (Taylor *et al.* 2009*b*).

3.4.4 Conclusions

This study described the population genetic structure of *O. irroratus* and found the presence of two unique evolutionary lineages. Firstly the two clades seem to correspond to the two major biomes along the south coast of South Africa (CFR and Grassland biomes), and show contrasting population genetic structures. One of the clades (clade A) seems to have expanded recently as indicated by shallow sequence divergence and the presence of floating chromosomal polymorphisms, while clade B is fixed for a chromosomal rearrangement and shows pronounced sequence divergence. Full taxonomic reviews is therefore warranted by this study as the two evolutionary lineages within *O. irroratus* show elevated sequence divergence, present contrasting population genetic structures and are separated by chromosomal rearrangements. *Otomys irroratus* presents another example of southern African taxa where the presence of cryptic species is found (Denys and Jaeger 1986; Britton-Davidian *et al.* 1995; Taylor *et al.* 1995; Rambau *et al.* 2003; Mullin *et al.* 2004 Willows-Munro and Matthee 2009). However to confirm these cryptic taxa cranial morphological analysis needs to be performed and a diagnostic key needs to be developed to distinguish the species. Considering the results presented here, biodiversity indices for southern Africa may currently underestimate the number of taxa present as many widespread taxa characterized by morphological conservatism may have diverged at the molecular level. Thus, highlighting the importance of molecular studies aimed at investigating population demographics within rodents and other taxa which often do not present morphological diagnostic characters.

Chapter 4

General Conclusions

This study increases the number of *O. irroratus* populations cytogenetically analysed to date, from 13 to 22 populations. My data confirm and extend previous work by confirming the presence of B chromosomes, fluctuating heterochromatin and the presence of pericentric inversions which occur as floating polymorphisms. Here the first evidence of inversion polymorphisms is found in *O. irroratus*; forty seven percent of the specimens analysed had inversions involving either one of the following autosomes: OIR1, OIR4, OIR6 and OIR10. These inversions were detected in either the heterozygous or homozygous form, clearly indicating that they are floating polymorphisms. Extensive sampling throughout the range of the species in South Africa indicates that these inversions occur in the south-western parts of South Africa (encompassing the Western Cape and the Eastern Cape provinces). Inversions are renowned speciation barriers and lead to reproductive isolation due to the formation of aneuploid gametes (containing deletions or duplications).

In the case of *O. irroratus*, these inversions are retained in the populations studied across more than 2000 km, suggesting that a mechanism such as heterosynapsis may maintain these polymorphisms within the species. Alternatively, these polymorphisms are probably maintained within *O. irroratus* in order to retain favorable alleles (see Kirkpatrick and Barton 2006 for review), as these rearrangements occur in a unique bioclimatic region in South Africa (Chapter 2). This study therefore supports the hypothesis which suggests that pericentric inversions can occur as floating polymorphisms within populations.

The presence of supernumerary B chromosomes is also found within *O. irroratus*. B chromosomes occur as floating polymorphisms and have been reported in many mammalian taxa (N = 55) and do not have any reproductive impact (Camacho *et al.* 2000; Vujosevic and Blagojevic 2004). Chromosomal data from this study show that B chromosomes are more prevalent in populations in the Eastern Cape and Free State provinces (Figure 2.3) within the Grassland biome. They also seem to be completely absent from populations in the Western Cape province which have predominantly fynbos vegetation. This study therefore confirms and extends previously published data as deviations in the diploid number between populations from 2n = 28 – 32 were found and generally B chromosomes occurred only in populations east of the country (Grassland biome) (Chapter 2). Since B chromosomes do no contain functional genes, their restricted distribution could be coincidental.

The variation of heterochromatic locations in the genome of *O. irroratus* has led to the description of four major cytotypes (Contrafatto *et al.* 1992). Variation in the NFa (38 - 52) is therefore not as a result of inversions. Only the pair 6 inversion caused a reduction in the fundamental arm number. Recent molecular investigations (this study and Taylor *et al.* 2009*b*) placed specimens with the B and C cytotype in one clade, with shallow sequence divergences separating them (1.91 % maximum sequence divergence). I therefore propose that the cytotypic groups, based on the number of heterochromatic short arms are invalid, as specimens clustered together based on their geographic location rather than cytotype (Chapter 2 and Chapter 3).

Ag-NOR staining is often used to quantify the amount of rRNA which is transcribed in a cell and has also been utilized as a phylogenetic marker (Stanyon *et al.* 1995; Dobigny *et al.* 2002; Teruel *et al.* 2009). Two major groups were defined by Ag-NOR staining within *O.*

irroratus which contained populations in the south-western parts and the north-eastern parts of South Africa, respectively. The two groups defined by Ag-NORs coincide with the two lineages (clade A and B defined by the mtDNA study here in Chapter 3) within *O. irroratus*. Ag-NOR staining is not always reliable and future work should therefore incorporate FISH with rDNA probes (see Chapter 2). Similarly, the meiotic mechanism which maintains the inversions in the different populations is not known at this stage and future work will be directed at studying synaptonemal complexes using silver staining and also using BAC clones flanking the inverted regions (Chapter 2).

Results from the mtDNA sequence data of the cyt *b* gene supported the presence of two genetically distinct lineages which were also found by Taylor *et al.* (2009*b*). Apart from the high sequence divergence separating the two clades, this study has identified an area of sympatry between the two lineages (in Alice in the Eastern Cape province). This conclusion is drawn from the lack of shared haplotypes between the two clades suggesting they are also reproductively isolated. This contact zone has been similarly found in several studies investigating South African taxa.

Population genetic analysis also revealed that clade A (which occurs in south-eastern parts of South Africa) have undergone a recent expansion, while clade B (which occurs in the north-eastern parts of South Africa) shows a mismatch distribution of a stable population, therefore indicating two separately evolving lineages. Nested clade analysis also revealed that historic events most probably led to the current genetic structure within *O. irroratus* due to allopatric fragmentation.

In support of the sequence data the chromosomal rearrangements present within *O. irroratus* such as the pericentric inversions which occur exclusively in clade A and the tandem fusion rearrangements, exclusive to clade B, underpin the two unique lineages found in this study. Further, the estimated divergence time between the two clades is dated to 1.1 MYA while, divergence within the two clades is dated at 0.62 MYA (0.86 p*P*) (clade A) and 0.66 MYA (0.89 p*P*) (clade B), which is in line with climate oscillations which took place since the Pliocene/Pleistocene epochs 2.9 – 0.8 MYA (see Chapter 3). However, Bayesian inferences are unable to show prior posterior distributions, especially when limited or no fossil calibration points are available. In order to obtain consistent results, the analysis needs to be re-run several times (Drummond and Rambaut 2007), here however several fossil calibration points were available and consistent were obtained from the analysis.

Otomys irroratus presents another example in southern African rodent taxa where the presence of cryptic speciation is found (Denys and Jaeger 1986; Britton-Davidian *et al.* 1995; Taylor *et al.* 1995; Mullin *et al.* 2004). Considering the results presented here, biodiversity estimates for southern Africa may currently underestimate the number of taxa present as many widespread taxa characterized by morphological conservatism may have diverged at the molecular level. This highlights the importance of molecular studies aimed at investigating population genetics within rodents and other taxa which are often characterised by morphological conservatism. In particular the utilization of museum material in population genetic investigations is gaining considerable momentum in the literature (e.g. Baker *et al.* 1994; Smith *et al.* 2007). Museum material thus, provides the opportunity to greatly increase sample sizes, certainly in the case of *O. irroratus*. The sequences obtained from museum material however, requires a cautionary approach as they need to be checked carefully in

order to avoid errors resulting from cross contamination (see Stuart *et al*. 2006 for use of museum material). Not only does the practice of using museum material motivate the existence of museum collections but do they also present the opportunity to accurately identify type specimens held in collections (Goodman *et al.* 2007; Smith *et al.* 2007).

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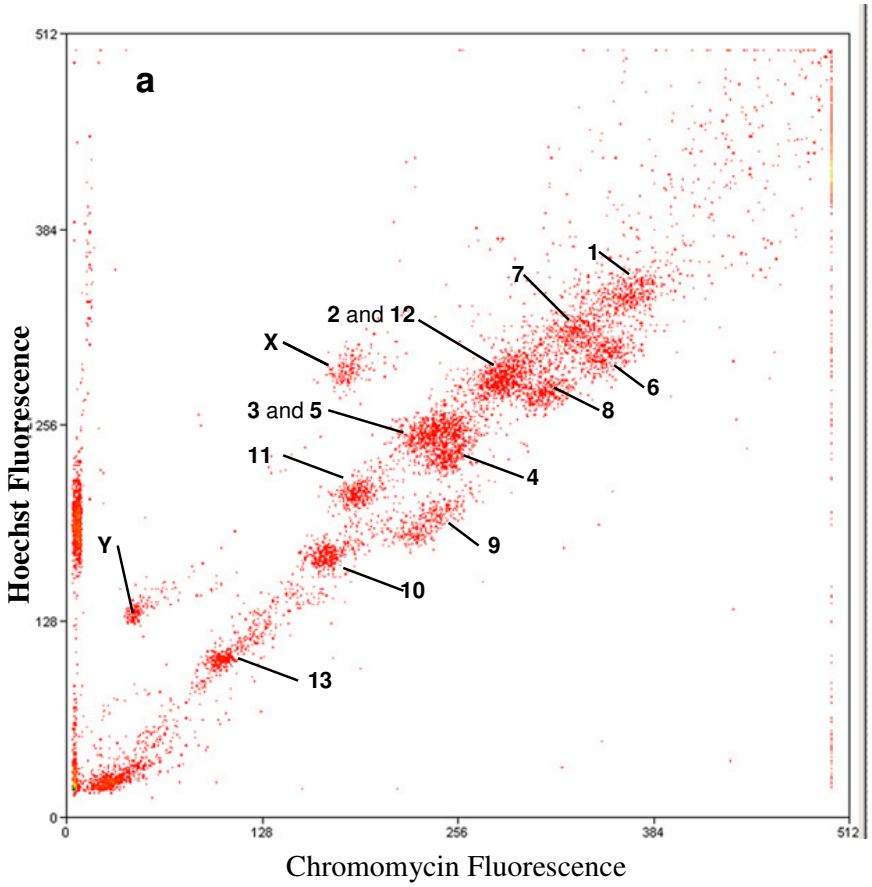
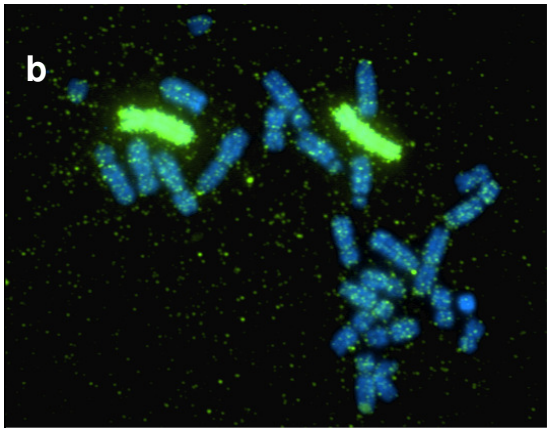
Appendix 1





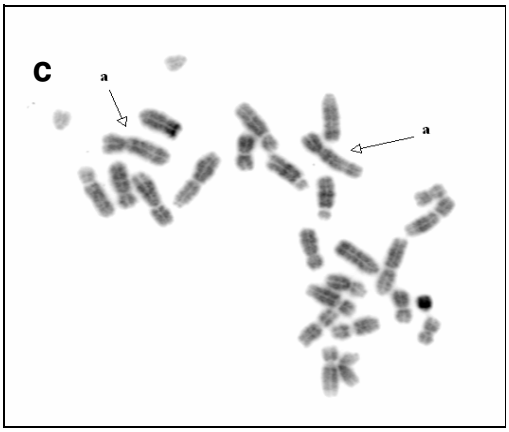


Figure 1: (a) Bivariate flow karyotype of *Myotomys unisulcatus* with chromosomal assignments. The chromosomes of *M. unisulcatus* (2n = 28) separated into 13 units. The chromosomal pairs 2, 12 and 3, 5 have similar sizes and base ratios and sorted together. Eleven peaks each contained a unique chromosome (1, 4, 6, 7, 8, 9, 10, 11, 13, X and Y), and two peaks hybridized to two chromosomes (2 and 12, 3 and 5). Each unit was identified by hybridizing a particular unit onto metaphase chromosomes of *M. unisulcatus* (for example here in (b) the chromosome painting of the digoxigenin labelled probe OUN 1 to metaphase chromosomes of *M. unisulcatus* is shown). The DAPI-inverted band image (c) of (b) was then used to identify the chromosomes corresponding to the flow sorted probe.

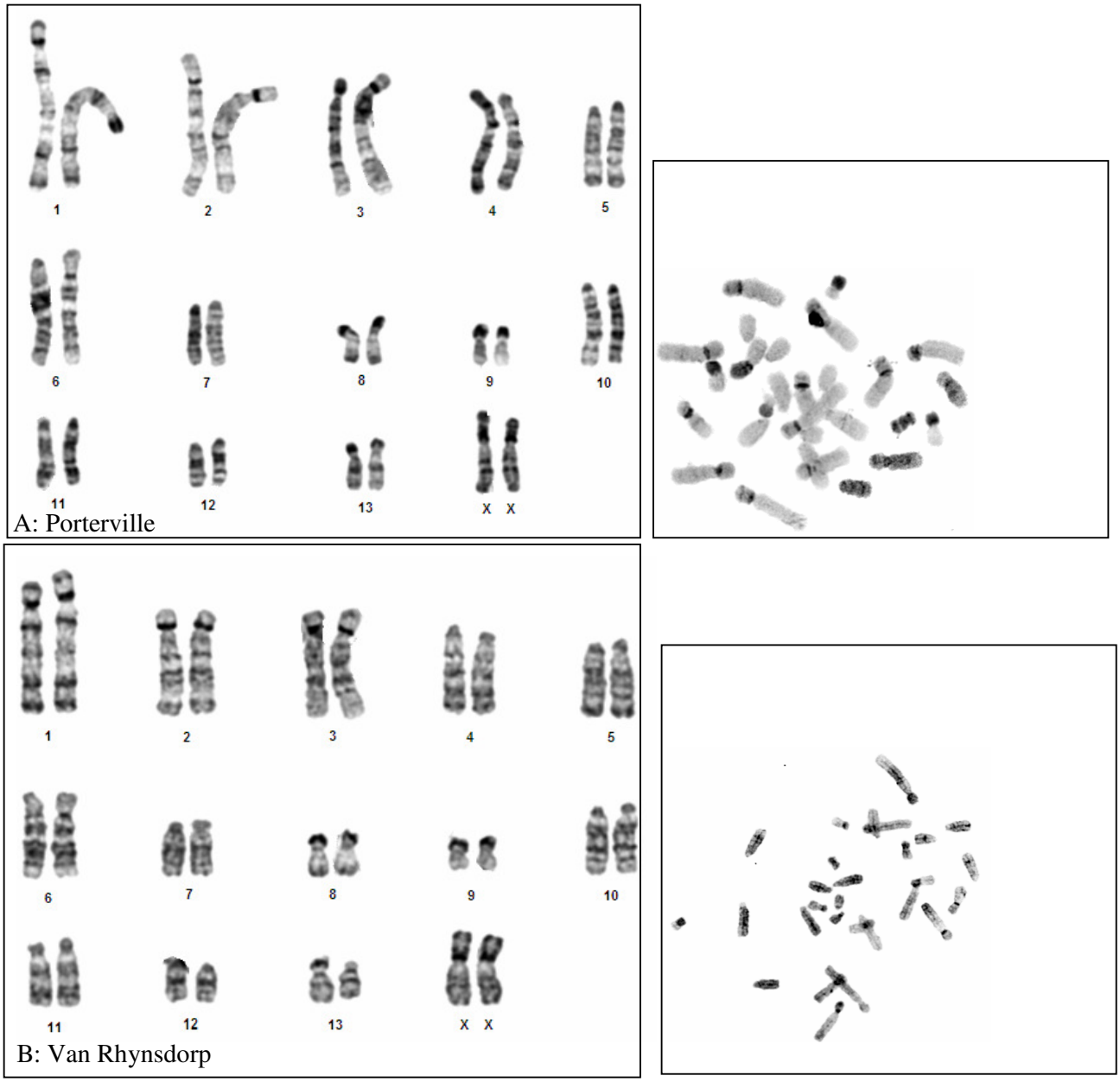
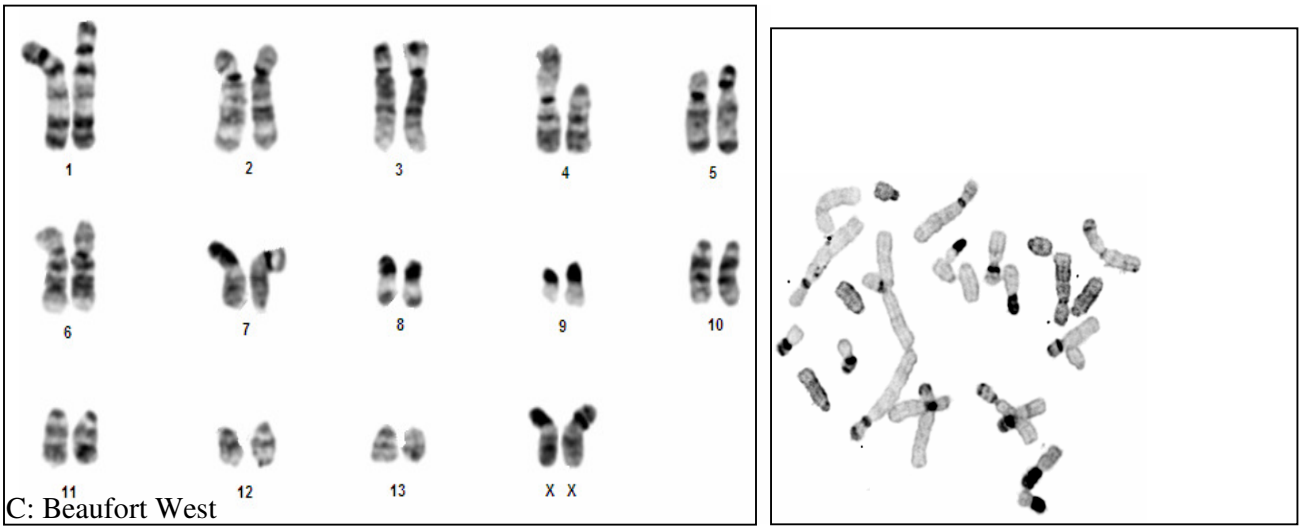
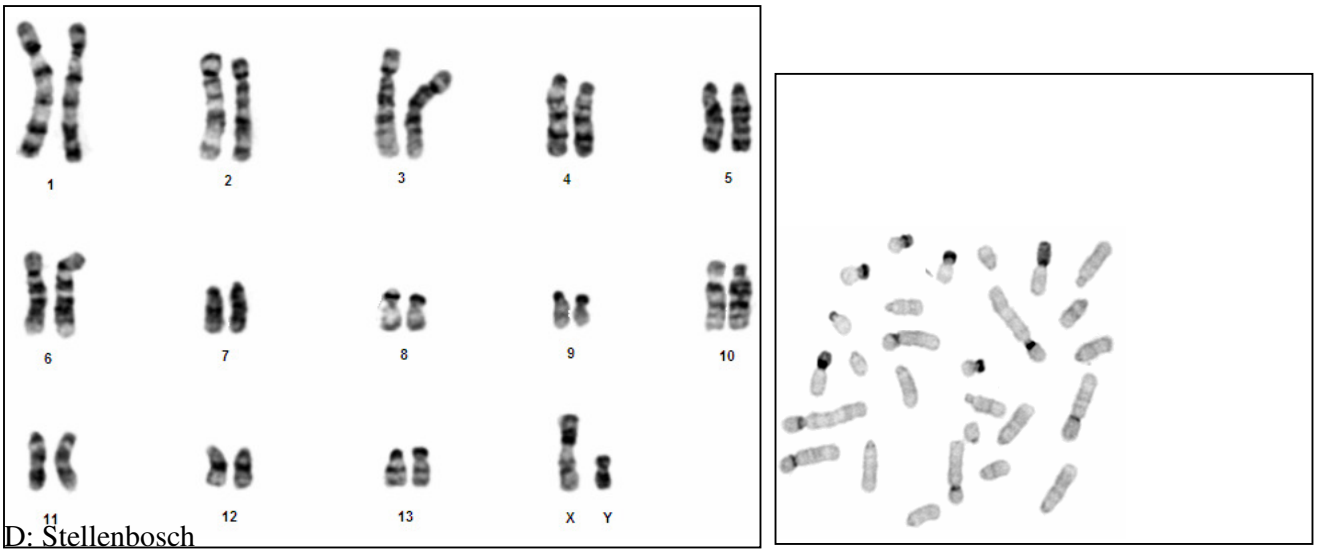


Figure 2: (A) All the specimens (*N* = 8) caught at Porterville in the Western Cape had the C-cytotype. Four males and four females were analysed and the diploid number were 2n = 28 in all the specimens. The pair 4 inversion were noted in four specimens all with a heterozygous configuration, the specimen here had a loss of heterochromatin in pair 4, with the corresponding C-bands in the adjacent box. **(B)** The single specimen collected in Van Rhynsdorp in the Western Cape had the C-cytotype configuration, the C-bands revealed four pairs with heterochromatic shortarms. The female had a karyotype identical to the type specimen, with a pericentric inversion on pair 6.

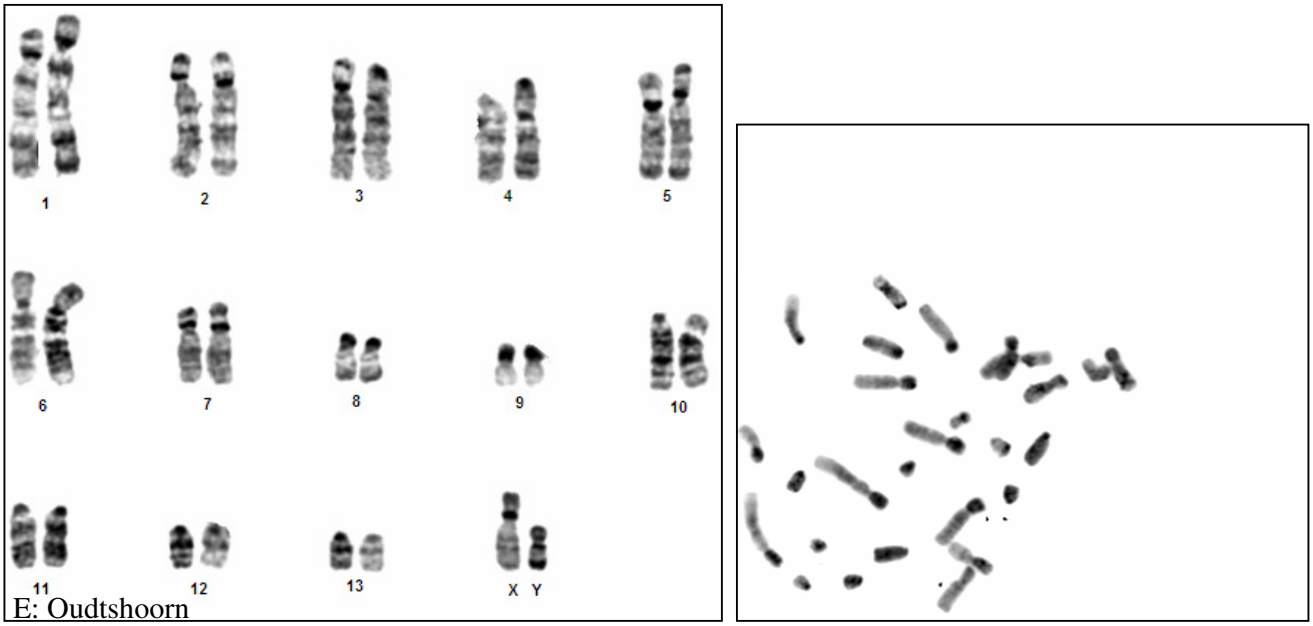


C: Beaufort West

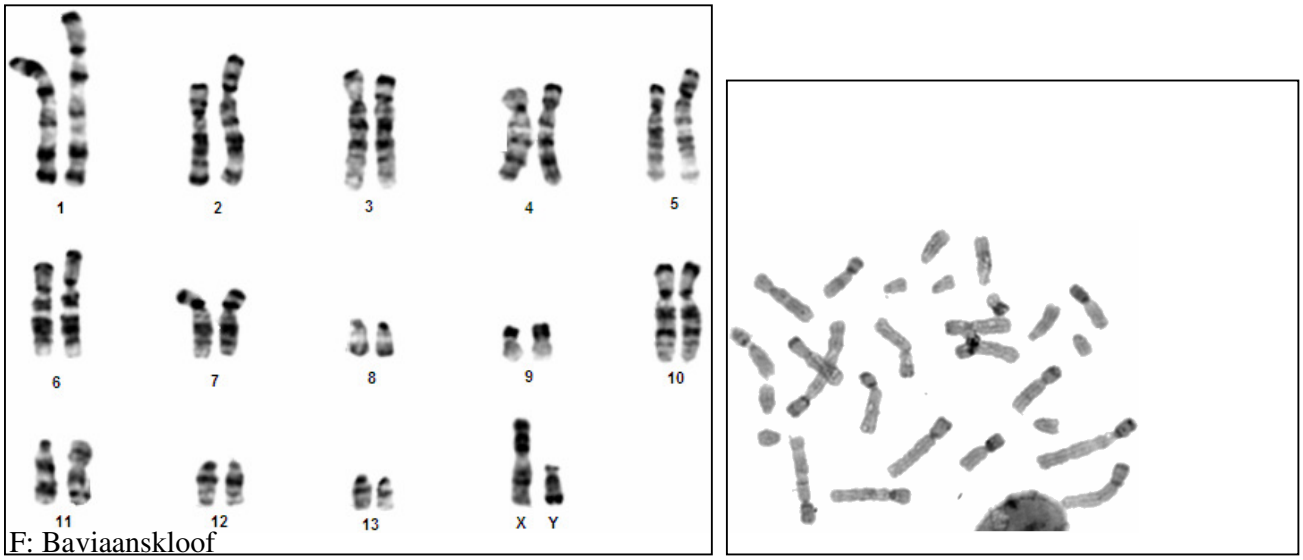


D: Stellenbosch

Figure 3: (C) Three specimens were collected in Beaufort West (Western Cape), all with the C-cytotype karyotype arrangement. All three specimens had the pair 4 pericentric inversion in heterozygous fashion, while two specimens had the pair 6 inversion also in heterozygous condition. One specimen had the pair 4, pair 6 and pair 10 pericentric inversions. **(D)** Eight specimens were analysed from the Stellenbosch population in the Western Cape. All the specimens had the C-cytotype arrangement; one specimen had the pair 6 pericentric inversion, while another had the pair ten inversion. All the other specimens showed no difference from the standard karyotype. The C-bands of both populations are presented next to the G-bands above.

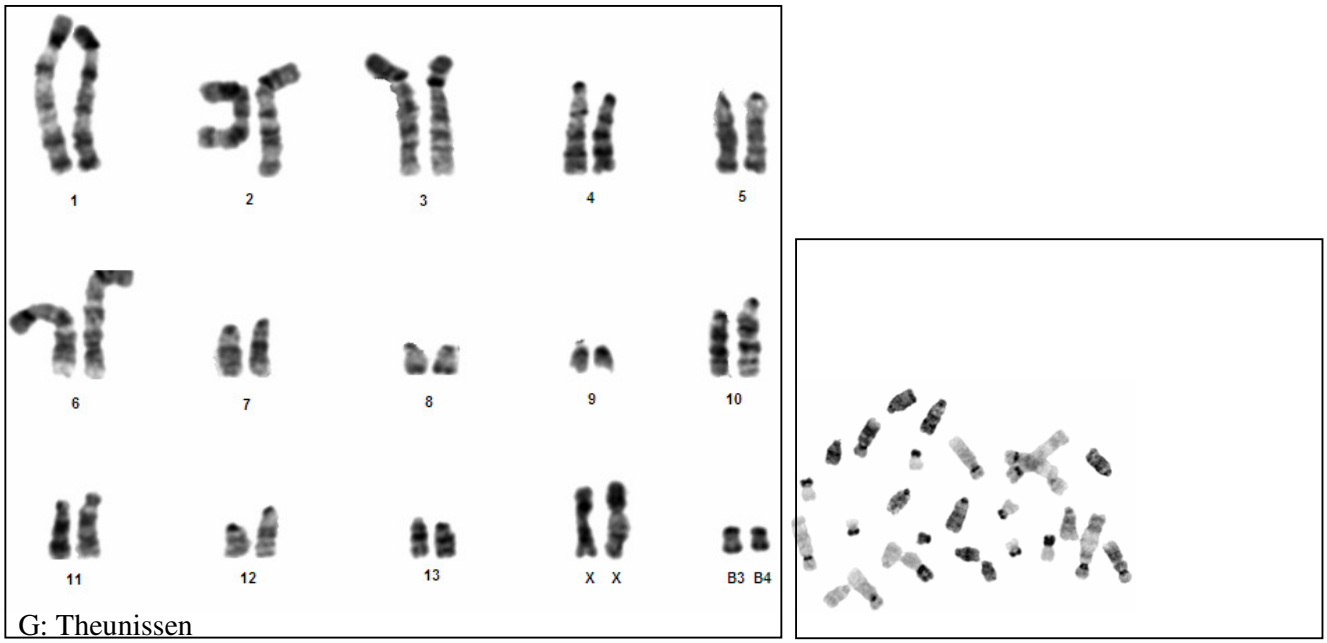


E: Oudtshoorn

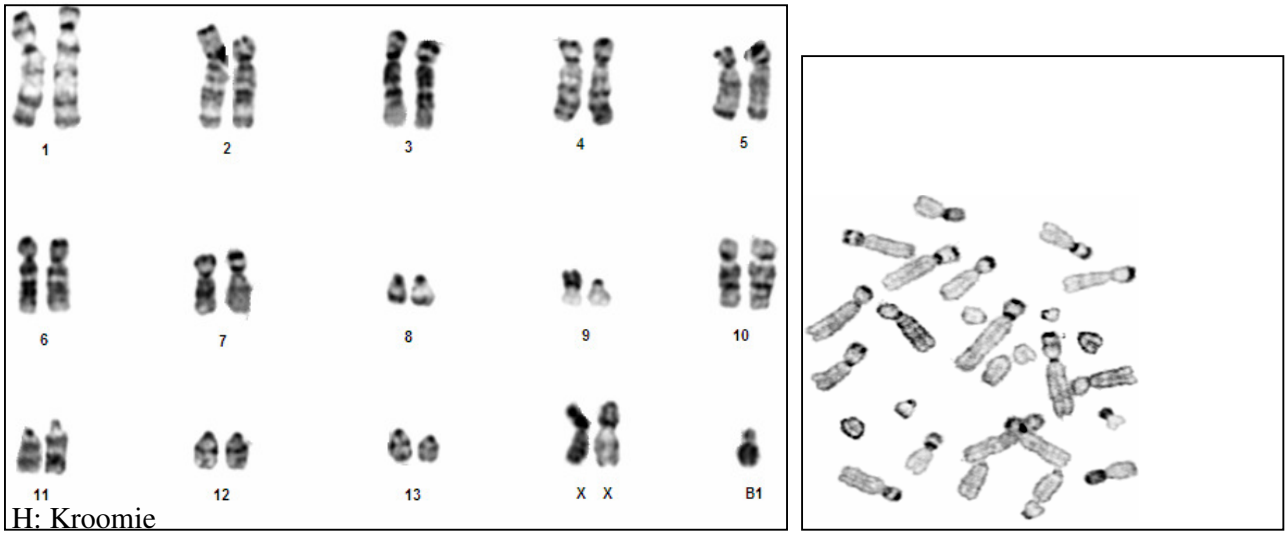


F: Baviaanskloof

Figure 4: (E) One specimen was collected from Oudtshoorn in the Western Cape. It had the B-cytotype and possessed the pair 4 and pair 10 pericentric inversions. None of the other chromosomes differed from the standard karyotype. **(F)** Five specimens were collected at Baviaanskloof (Eastern Cape); all the specimens had the B-cytotype. Representatives of the C-bands of both populations are presented next to their respective G-bands above.

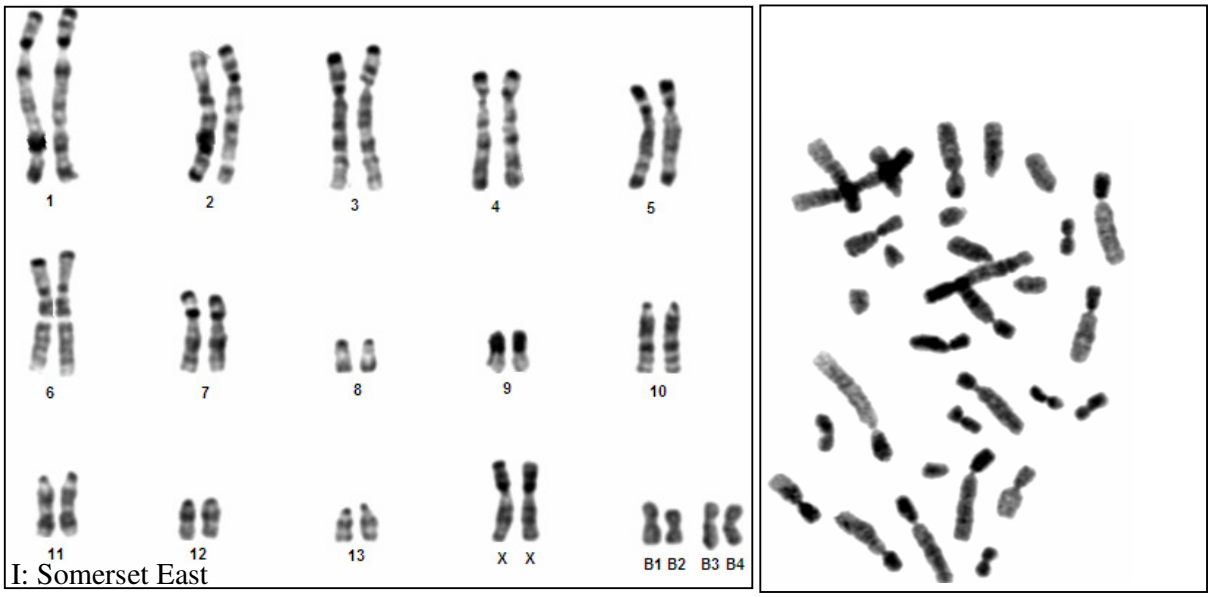


G: Theunissen

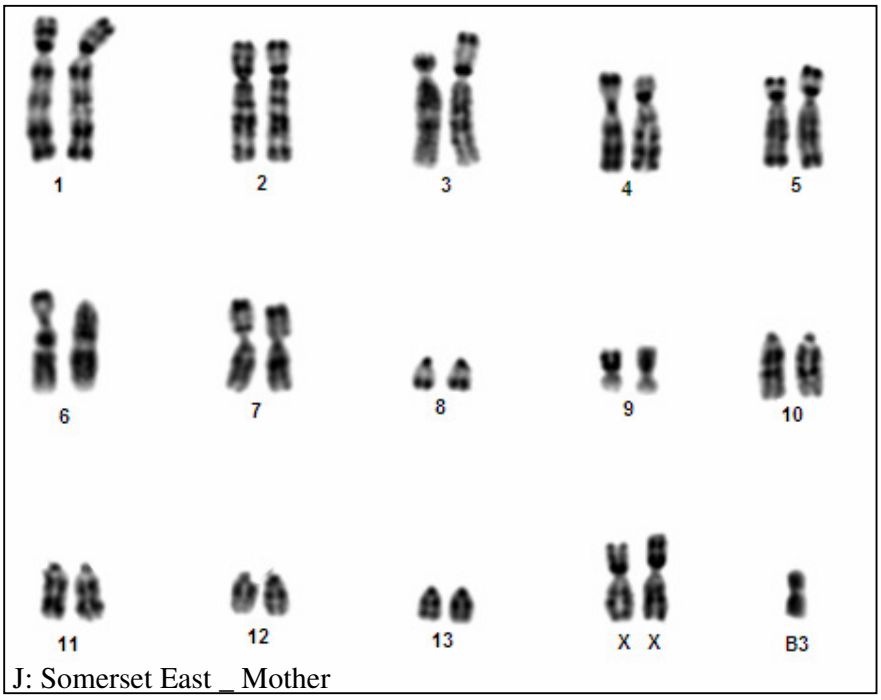


H: Kroomie

Figure 5 (G) Two specimens were collected in Theunissen in the Free State province. Both had the C-cytotype (2n = 28 / 30) and did not contain any inversions. One specimen had two B-chromosomes increasing the diploid number to 2n = 30. **(H)** Five specimens were collected at Kroomie (Eastern Cape) all the specimens had the B-cytotype. Three specimens had the pair 1 inversion which was site specific for this sampling locality. Representatives of the C-bands of both populations are presented next to their respective G-bands above.

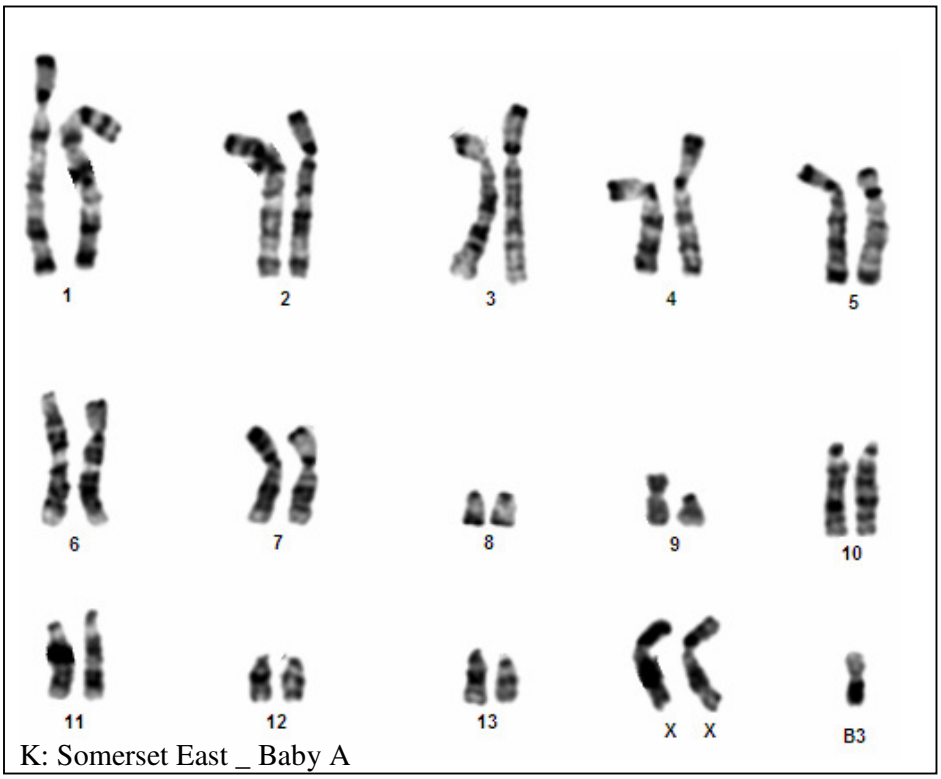


I: Somerset East

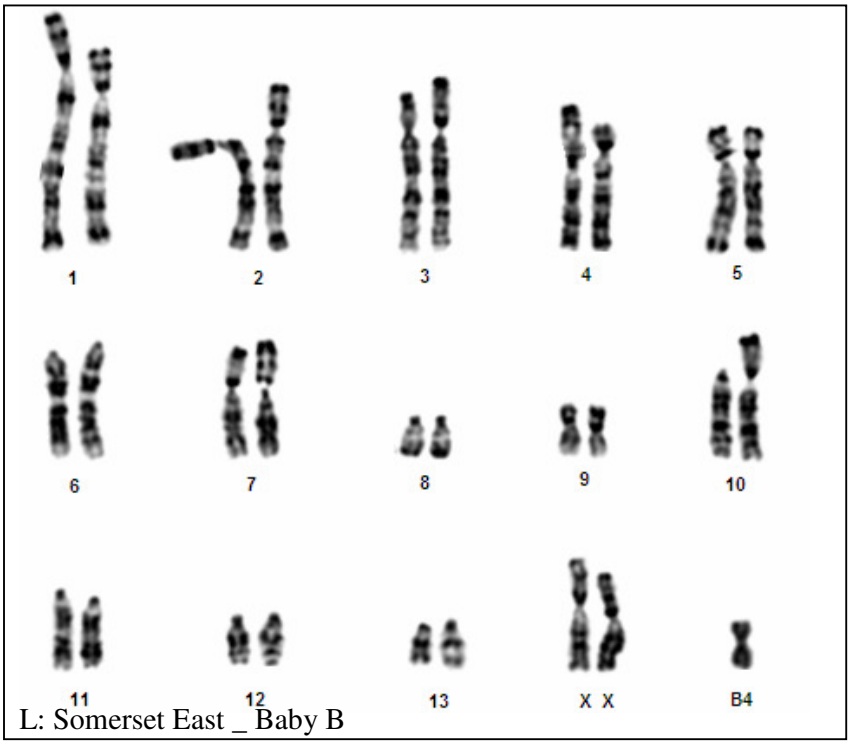


J: Somerset East _ Mother

Figure 6 (I) Nineteen specimens were analysed from Somerset East in the Eastern Cape. All the specimens had the B-cytotype, with 7 specimens had the pair 6 inversion, either in heterozygous or homozygous fashion. Five specimens contained supernumerary B-chromosomes leading to diploid number variation (2n = 28, 29 and 30). A Representative of the C-bands of the Somerset East population is presented next to the G-bands of (I) above. . **(J)** The mother of the two babies who were caught in Somerset East's karyotype is shown here. The specimen had the B cytotype and had the pair 6 inversion in heterozygous condition and one B-chromosome.



K: Somerset East _ Baby A



L: Somerset East _ Baby B

Figure 7 (K, L) The karyotypes of the two babies were both of the B-cytotype. One baby had the pair 6 inversion in heterozygous condition (K), while the other had it as homozygous inverted (L). Baby (K) had no heterochromatin on pair 10, while baby (L) had heterochromatin on one homolog of pair 10. Both had supernumerary B chromosomes which the babies must have inherited from the mother or father.

Figure 3.5: F_{ST} values (below diagonal) between populations, numbers 1 to 38 corresponds to population numbers indicated in Table 3.1. Above the Diagonal are the average pairwise differences between populations.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	_	2.4	1.9	3.0	25.0	20.3	3.6	25.0	2.6	2.6	2.6	1.6	1.6	2.6	3.2	17.7	2.0	3.0	21.4	20.7	22.7	7.6	9.6	2.9	2.3	21.4	26.0	21.4	22.0	21.4	25.0
2	0.7	_	1.0	1.8	25.0	20.9	2.8	25.0	2.0	2.0	2.0	1.0	1.0	2.0	2.0	18.3	1.3	1.9	22.0	21.0	23.0	7.0	9.0	2.0	1.3	22.0	26.0	22.0	22.5	22.0	25.0
3	0.6	0.0	_	1.3	24.5	20.4	2.3	24.5	1.5	1.5	1.5	0.5	0.5	1.5	1.5	17.8	0.8	1.5	21.5	20.5	22.5	6.5	8.5	1.5	0.8	21.5	25.5	21.5	22.0	21.5	24.5
4	1.8	0.9	0.8	_	24.8	21.7	3.2	24.8	2.8	2.8	2.8	1.8	1.8	2.8	1.8	19.1	1.8	2.1	22.8	21.3	23.3	7.8	9.8	2.3	1.5	22.8	25.8	22.8	23.1	22.8	24.8
5	24.0	24.3	24.2	24.6	_	15.4	24.0	1.0	24.5	24.0	24.0	25.0	25.0	26.0	25.0	16.7	25.0	25.0	16.0	14.5	7.5	23.0	26.0	25.5	24.7	15.0	1.0	15.0	12.3	15.0	0.0
6	16.9	17.9	17.7	19.1	13.0	_	20.5	14.6	19.7	18.9	18.9	19.9	19.9	20.9	21.9	6.5	20.4	21.3	3.9	4.9	9.4	19.9	21.0	21.4	20.9	4.1	16.4	4.1	6.2	4.1	15.4
7	0.9	0.4	0.3	1.3	22.3	16.4	_	24.0	3.2	3.2	3.2	2.2	2.2	3.2	3.3	18.2	2.5	3.2	21.6	20.7	22.3	4.6	8.2	3.3	2.7	21.6	25.0	21.6	22.0	21.6	24.0
8	23.0	23.3	23.2	23.6	0.0	11.3	21.3	_	24.5	24.0	24.0	25.0	25.0	26.0	25.0	16.3	25.0	25.0	16.0	14.0	8.0	23.0	25.0	25.5	24.7	15.0	2.0	15.0	12.3	15.0	1.0
9	0.9	0.7	0.5	1.9	23.8	16.7	0.8	22.8	_	1.0	1.0	1.0	1.0	2.0	3.0	17.3	1.5	2.7	21.0	20.5	22.3	7.0	9.0	2.5	2.0	21.0	25.5	21.0	21.6	21.0	24.5
10	1.6	1.3	1.2	2.6	24.0	16.5	1.5	23.0	0.3	_	0.0	1.0	1.0	2.0	3.0	16.3	1.5	2.7	20.0	19.5	21.5	7.0	9.0	2.5	2.0	20.0	25.0	20.0	20.7	20.0	24.0
11	1.6	1.3	1.2	2.6	24.0	16.5	1.5	23.0	0.3	0.0	_	1.0	1.0	2.0	3.0	16.3	1.5	2.7	20.0	19.5	21.5	7.0	9.0	2.5	2.0	20.0	25.0	20.0	20.7	20.0	24.0
12	0.6	0.3	0.2	1.6	25.0	17.5	0.5	24.0	0.3	1.0	1.0	_	0.0	1.0	2.0	17.3	0.5	1.7	21.0	20.5	22.5	6.0	8.0	1.5	1.0	21.0	26.0	21.0	21.7	21.0	25.0
13	0.6	0.3	0.2	1.6	25.0	17.5	0.5	24.0	0.3	1.0	1.0	0.0	_	1.0	2.0	17.3	0.5	1.7	21.0	20.5	22.5	6.0	8.0	1.5	1.0	21.0	26.0	21.0	21.7	21.0	25.0
14	1.6	1.3	1.2	2.6	26.0	18.5	1.5	25.0	1.3	2.0	2.0	1.0	1.0	_	3.0	18.3	1.5	2.7	22.0	21.5	23.5	7.0	9.0	2.5	2.0	22.0	27.0	22.0	22.7	22.0	26.0
15	1.4	0.5	0.3	0.8	24.2	18.7	0.8	23.2	1.5	2.2	2.2	1.2	1.2	2.2	_	19.3	1.8	2.3	23.0	21.5	23.5	7.7	9.7	2.0	1.7	23.0	26.0	23.0	23.3	23.0	25.0
16	13.1	14.0	13.8	15.3	13.0	0.5	12.8	11.7	13.0	12.7	12.7	13.7	13.7	14.7	14.8	_	17.8	18.9	4.7	5.2	9.8	18.7	20.3	18.8	18.3	4.3	17.7	4.3	6.3	4.3	16.7
17	0.5	0.1	-0.1	1.1	24.5	17.5	0.3	23.5	0.3	1.0	1.0	0.0	0.0	1.0	0.5	13.7	_	1.9	21.5	20.8	22.8	6.5	8.5	1.8	1.2	21.5	26.0	21.5	22.1	21.5	25.0
18	0.9	0.0	0.0	0.7	23.9	17.8	0.4	22.9	0.9	1.6	1.6	0.6	0.6	1.6	0.3	14.0	0.2	_	22.4	21.2	23.2	7.4	9.4	2.5	1.8	22.4	26.0	22.4	22.9	22.4	25.0
19	20.4	21.3	21.2	22.6	16.0	1.5	19.9	15.0	20.3	20.0	20.0	21.0	21.0	22.0	22.2	1.0	21.0	21.3	_	2.5	8.5	21.0	22.0	22.5	22.0	1.0	17.0	1.0	4.3	1.0	16.0
20	17.2	17.8	17.7	18.6	12.0	0.0	16.5	10.5	17.3	17.0	17.0	18.0	18.0	19.0	18.2	-1.0	17.8	17.6	0.0	_	8.0	20.5	21.5	21.5	20.8	2.5	15.5	2.5	4.7	2.5	14.5
21	15.2	15.8	15.7	16.6	1.0	0.5	14.1	0.5	15.1	15.0	15.0	16.0	16.0	17.0	16.2	-0.3	15.8	15.6	2.0	-1.0	_	21.5	23.5	23.5	22.8	7.5	8.5	7.5	7.3	7.5	7.5
22	6.6	6.3	6.2	7.6	23.0	17.5	2.9	22.0	6.3	7.0	7.0	6.0	6.0	7.0	6.8	15.0	6.0	6.3	21.0	18.0	15.0	_	6.0	7.5	7.0	21.0	24.0	21.0	21.3	21.0	23.0
23	8.6	8.3	8.2	9.6	26.0	18.7	6.5	24.0	8.3	9.0	9.0	8.0	8.0	9.0	8.8	16.7	8.0	8.3	22.0	19.0	17.0	6.0	_	9.5	9.0	22.0	27.0	22.0	22.7	22.0	26.0
24	0.4	-0.2	-0.3	0.6	24.0	17.5	0.1	23.0	0.3	1.0	1.0	0.0	0.0	1.0	-0.3	13.7	-0.3	-0.1	21.0	17.5	15.5	6.0	8.0	_	1.8	22.5	26.5	22.5	22.9	22.5	25.5
25	0.7	0.0	-0.2	0.6	24.0	17.9	0.3	23.0	0.7	1.3	1.3	0.3	0.3	1.3	0.2	14.0	0.0	0.0	21.3	17.7	15.7	6.3	8.3	-0.3	_	22.0	25.7	22.0	22.4	22.0	24.7
26	19.4	20.3	20.2	21.6	14.0	0.8	18.9	13.0	19.3	19.0	19.0	20.0	20.0	21.0	21.2	-0.3	20.0	20.3	0.0	-1.0	0.0	20.0	21.0	20.0	20.3	_	16.0	1.0	3.7	1.0	15.0
27	25.0	25.3	25.2	25.6	1.0	14.0	23.3	1.0	24.8	25.0	25.0	26.0	26.0	27.0	25.2	14.0	25.5	24.9	17.0	13.0	2.0	24.0	27.0	25.0	25.0	15.0	_	16.0	13.3	16.0	1.0
28	19.4	20.3	20.2	21.6	14.0	0.8	18.9	13.0	19.3	19.0	19.0	20.0	20.0	21.0	21.2	-0.3	20.0	20.3	0.0	-1.0	0.0	20.0	21.0	20.0	20.3	-1.0	15.0	_	3.7	1.0	15.0
29	18.0	18.8	18.7	19.9	9.3	0.9	17.3	8.3	17.9	17.7	17.7	18.7	18.7	19.7	19.5	-0.3	18.6	18.7	1.3	-0.8	-2.2	18.3	19.7	18.4	18.8	-0.3	10.3	-0.3	_	3.7	12.3
30	19.4	20.3	20.2	21.6	14.0	0.8	18.9	13.0	19.3	19.0	19.0	20.0	20.0	21.0	21.2	-0.3	20.0	20.3	0.0	-1.0	0.0	20.0	21.0	20.0	20.3	-1.0	15.0	-1.0	-0.3	_	15.0
31	24.0	24.3	24.2	24.6	0.0	13.0	22.3	0.0	23.8	24.0	24.0	25.0	25.0	26.0	24.2	13.0	24.5	23.9	16.0	12.0	1.0	23.0	26.0	24.0	24.0	14.0	1.0	14.0	9.3	14.0	_
32	1.6	1.3	1.2	2.6	26.0	18.5	1.5	25.0	1.3	2.0	2.0	1.0	1.0	2.0	2.2	14.7	1.0	1.6	22.0	19.0	17.0	7.0	9.0	0.0	1.3	21.0	27.0	21.0	19.7	21.0	26.0
33	1.6	1.3	1.2	2.6	26.0	18.5	1.5	25.0	1.3	2.0	2.0	1.0	1.0	2.0	2.2	14.7	1.0	1.6	22.0	19.0	17.0	7.0	9.0	1.0	1.3	21.0	27.0	21.0	19.7	21.0	26.0
34	0.6	0.3	0.2	1.6	25.0	17.5	0.5	24.0	0.3	1.0	1.0	0.0	0.0	1.0	1.2	13.7	0.0	0.6	21.0	18.0	16.0	6.0	8.0	0.0	0.3	20.0	26.0	20.0	18.7	20.0	25.0
35	19.1	20.0	19.8	21.3	14.0	0.7	18.6	12.7	19.0	18.7	18.7	19.7	19.7	20.7	20.8	-0.6	19.7	20.0	0.7	-1.0	0.0	19.7	20.7	19.7	20.0	-0.7	15.0	-0.7	-0.3	-0.7	14.0
36	23.0	23.3	23.2	23.6	2.0	12.8	21.3	2.0	23.3	23.0	23.0	24.0	24.0	25.0	23.2	12.0	23.5	22.9	15.0	11.0	1.5	22.0	25.0	23.0	23.0	13.0	3.0	13.0	8.8	13.0	2.0
37	19.4	20.3	20.2	21.6	15.0	1.5	18.9	13.0	19.3	19.0	19.0	20.0	20.0	21.0	21.2	0.0	20.0	20.3	3.0	0.0	1.0	20.0	21.0	20.0	20.3	1.0	16.0	1.0	0.7	1.0	15.0
38	25.0	25.3	25.2	25.6	1.0	14.0	23.3	1.0	24.8	25.0	25.0	26.0	26.0	27.0	24.5	14.0	25.0	24.9	17.0	13.0	2.0	24.0	27.0	25.0	25.0	15.0	2.0	15.0	10.3	15.0	1.0

Figure 3.5: (continued)

	32	33	34	35	36	37	38
1	2.6	2.6	1.6	21.1	25.5	20.4	26.0
2	2.0	2.0	1.0	21.7	25.5	21.0	26.0
3	1.5	1.5	0.5	21.2	25.0	20.5	25.5
4	2.8	2.8	1.8	22.5	25.3	21.8	25.8
5	26.0	26.0	25.0	15.0	3.5	15.0	1.0
6	20.9	20.9	19.9	4.0	16.6	3.9	16.4
7	3.2	3.2	2.2	21.3	24.5	20.6	25.0
8	26.0	26.0	25.0	14.7	4.5	14.0	2.0
9	2.0	2.0	1.0	20.7	25.5	20.0	25.5
10	2.0	2.0	1.0	19.7	24.5	19.0	25.0
11	2.0	2.0	1.0	19.7	24.5	19.0	25.0
12	1.0	1.0	0.0	20.7	25.5	20.0	26.0
13	1.0	1.0	0.0	20.7	25.5	20.0	26.0
14	2.0	2.0	1.0	21.7	26.5	21.0	27.0
15	3.0	3.0	2.0	22.7	25.5	22.0	25.3
16	18.3	18.3	17.3	4.1	17.2	3.7	17.7
17	1.5	1.5	0.5	21.2	25.5	20.5	25.5
18	2.7	2.7	1.7	22.1	25.5	21.4	26.0
19	22.0	22.0	21.0	1.7	16.5	3.0	17.0
20	21.5	21.5	20.5	2.5	15.0	2.5	15.5
21	23.5	23.5	22.5	7.5	9.5	7.5	8.5
22	7.0	7.0	6.0	20.7	23.5	20.0	24.0
23	9.0	9.0	8.0	21.7	26.5	21.0	27.0
24	1.5	2.5	1.5	22.2	26.0	21.5	26.5
25	2.0	2.0	1.0	21.7	25.2	21.0	25.7
26	22.0	22.0	21.0	1.3	15.5	2.0	16.0
27	27.0	27.0	26.0	16.0	4.5	16.0	2.0
28	22.0	22.0	21.0	1.3	15.5	2.0	16.0
29	22.7	22.7	21.7	3.7	13.3	3.7	13.3
30	22.0	22.0	21.0	1.3	15.5	2.0	16.0
31	26.0	26.0	25.0	15.0	3.5	15.0	1.0
32	_	2.0	1.0	21.7	26.5	21.0	27.0
33	2.0	_	1.0	21.7	26.5	21.0	27.0
34	1.0	1.0	_	20.7	25.5	20.0	26.0
35	20.7	20.7	19.7	_	15.5	1.3	16.0
36	25.0	25.0	24.0	13.0	_	15.5	4.5
37	21.0	21.0	20.0	0.3	14.0	_	16.0
38	27.0	27.0	26.0	15.0	3.0	16.0	_