

CHROMOSOMAL EVOLUTION IN THE VLEI RAT
OTOMYS IRRORATUS

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To the late Jurgens Anthony "Waldo" Meester

"...I am a great lover of these methods of division and collection as instruments which enable me to speak and to think, and when I believe that I have found in anyone else the ability to discuss unity and plurality as they exist in the nature of things, I follow in his footsteps..."

(Phaedrus, 266: Plato *ca.* 370 B.C.)

PREFACE

This study was carried out in the Department of Biology, University of Natal, Durban, from 1988 to date, under the joint supervision of Professor J. A. Meester, Dr. G. K. Campbell and Dr. J. H. Grace.

This thesis represents original work by the author and has not been submitted in any form to another university. Where use was made of the work of others it has been duly acknowledged in the text.

Durban, 1996

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Giancarlo Contrafatto

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ABSTRACT

Proponents of the recognition concept of species hold that isolating mechanisms, including chromosome rearrangements, play no role in speciation while the more commonly accepted biological species concept proposes that isolation mechanisms are instrumental in the formation of new species. Moreover, some adherents of the biological concept of species, reject the hypothesis that chromosomal rearrangements can be instrumental in causing reproductive isolation and, hence, speciation.

Evidence to the causative role played in speciation by chromosome changes can be obtained from cytogenetic investigations of sibling species, in parallel with analyses of gene products, DNA polymorphism and premating behaviour.

This study reports the results of a cytogenetic investigation of 97 specimens of the vlei rat *O. irroratus*, from 18 South African localities, and 11 samples of the Angoni vlei rat *O. angoniensis* from two geographically distant populations. All *O. angoniensis* individuals showed a constant karyotype with 56 acrocentric chromosomes but extensive variation was detected in *O. irroratus*. Five cytotypes could be recognized within the latter. In the south-eastern parts of its South African range, *O. irroratus* had a diploid number ($2n$) of 30 chromosomes in which all autosomes were acrocentric (cytotype A) while further east (cytotype A2), the diploid number was 30-32 with, again, acrocentric autosomes. A further acrocentric cytotype (A1) with $2n = 24-27$ occupied the southern and south-eastern slopes of the Drakensberg range. A type with $2n = 28-30$ (cytotype B), with eight pairs of biarmed autosomes, was found in the southern Cape region while in the Cape of Good Hope and in the north-eastern parts of South Africa, *O. irroratus* had $2n = 28$ with only four pairs of biarmed autosomes (cytotype C). Most of the numerical changes were due to variation in the number of copies of B-chromosomes which were small, biarmed and partly heterochromatic.

C-banding analysis revealed that the short arms of biarmed autosomes were totally heterochromatic. On the other hand, G-banding patterns of acrocentric autosomes were, with two exceptions (A1 and A2 types), similar in all cytotypes while G-banding of the long arms

of banded chromosomes matched the pattern of their homologues in acrocentric cytotypes. A potentially heterotic rearrangement was detected in the A1 localities where a unique acrocentric autosome was identified as the product of a fusion between chromosomes 7 and 12.

The geographic distribution of these groups of karyotypes correlated, by Discriminant Function Analysis, with bioclimatic regions of South Africa. The A1 cytotype was shown to occupy the coldest and wettest region of the montane Drakensberg while the B type is found in the hot area of the eastern Cape with an unpredictable rainfall pattern: group C occupies regions of intermediate climate.

Gene product analysis was carried out using the novel approach of subjecting liver homogenates to "Western blotting". This method was first assessed at supraspecific level using specimens of various southern African rodents, and allowed the generation of phylogenies essentially similar to those produced by allozyme studies of the same taxa. At intraspecific level, immunoblotting analysis did not reveal synapomorphies congruent with karyotype groups. This was interpreted, in conjunction with available allozyme data from the same populations, as evidence of low genetic differentiation between *O. irroratus* cytotypes. A measure of genetic divergence was indicated in two populations from the Cape province and this was in agreement with existing data from allozyme electrophoresis and mitochondrial DNA polymorphism.

The cytogenetic results were related to available data on breeding and premating behaviour concerning some of the *O. irroratus* populations investigated here. The presence of the 7/12 chromosome fusion in the A1 cytotype correlated with a dramatic reproductive impairment of F1 individuals originated from A1/A2 and A1/B cytotype crosses. Evidence of partial premating behavioural barriers has been reported by others, but information on premating behaviour between populations which are not chromosomally isolated is lacking. Therefore, it was not possible to establish if behavioural premating barriers preceded, or followed, the fixation of negatively heterotic chromosomal rearrangements. It was, nevertheless, suggested that the existence of such impaired mate recognition may be an example of reproductive character displacement which may have followed the fixation of the t(7:12) typi-

cal of the A1 populations.

In conclusion, the existence of chromosome changes in the A1, and possibly A2, populations accompanied by low genetic divergence and severely impaired hybrid reproductive success, are consistent with a hypothesis whereby chromosomal reproductive isolation causes speciation. Nonetheless, other speciation mechanisms mediated by genetic divergence and/or mate recognition failure, are possible in other populations where no chromosome changes of negatively heterotic potential were found.

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CHAPTER 1

INTRODUCTION

This work is concerned with the genetic variability of the vlei rat *Otomys irroratus* (Brants, 1827). Documenting the genetic variability which is often involved in speciation events whereby new species are formed, is essential in providing insight into concepts such as species, speciation modes and isolation mechanisms.

◆ *Species, speciation and isolation*

Several concepts of species have been proposed over the years (see King, 1993 for a recent review). However, with the exception of the advocates of the recognition concept of species (Patterson, 1978; 1985), most evolutionary biologists, particularly those studying mammals, subscribe to a concept which defines a species as a group of populations which is capable of interbreeding but is reproductively isolated from other such groups of populations. This isolation concept of species (*sensu* Vrba, 1985) and its attendant processes of speciation are best typified by the work of Mayr (1942, 1963, 1970, 1982, 1988).

The most commonly accepted mode of speciation, allopatric speciation, has been eminently discussed by various authors (*inter alia* Mayr, 1942, 1963, 1970, 1982, 1988; White, 1978a, 1978b, 1982) and is usually understood as the divergence of two or more populations which have become separated, often by geographic barriers, from the main distribution area of the species. Because they no longer can come in contact, therefore, they no longer interbreed. In addition to this, White (1982) recognizes sympatric speciation (i.e. separation through adaptation to different habitat and/or niches within the same area), stasipatric speciation (i.e. chromosomal separation) and various mechanisms which lead to parapatric distribution patterns and which are, sometimes, referred to as parapatric

speciation (White, 1978a).

Whatever the mode of speciation, the biological concept of species requires the existence of naturally occurring isolating agencies whose effect is to prevent two populations of the same species from interbreeding. This statement, however, may be somewhat oversimplified since parapatric distribution patterns, for example, postulate that isolated populations or incipient species overlap to some extent and may hybridize, although the hybrids are not expected to be fertile (see White, 1978a).

Since the crucial issue is that of failed interbreeding, the isolating mechanisms can either be pre-mating or post-mating. Among the first type of mechanisms which prevent interspecific crosses, Mayr (1963) listed seasonal and habitat isolation, behavioural isolation and mechanical isolation (unsuccessful copulation). Post-mating isolating mechanisms would include all events whereby reproductive success of interspecific crosses is impaired and, therefore, involve gamete inviability, zygote inviability or mortality, hybrid inviability and hybrid sterility (Mayr, 1963).

◆ *Chromosomal speciation*

The group of post-mating isolating mechanisms is of concern to this investigation since it is through these that chromosomal speciation is expected to occur. Models invoking isolation through the agency of chromosome rearrangements posit that crosses between chromosomally isolated populations would suffer from the negative heterosis caused by carrying the chromosomal neomorph(s) in the heterozygous state. This, generally, implies that the rearrangement(s) must be of a type capable of causing meiotic malsegregation - thus gamete aneuploidy - followed by failed or impaired reproduction. Recently, Capanna and Redi (1994) have provided a critical re-evaluation of these views and attribute a role to interphase nuclear and chromosomal architecture in causing the negative hybrid heterosis required for reproductive isolation to ensue. These authors point out, further, that impaired gametogenesis can be a most prevalent event in structural heterozygotes.

There is, however, little agreement concerning the role played by karyotype evolution in the process of speciation. While many authors (*inter alia* White, 1978a; Capanna, *et al.* 1985; Baker and Bickham, 1986; Nevo, 1991) do not doubt that chromosomal rearrangements can initiate speciation, others maintain that there is no causal relationship between karyotype changes and speciation events (*inter alia* Vrba, 1985; John and Miklos, 1988) and that fixation of chromosomal alterations is merely an accidental by-product of small population size and forced selection for reorganization during speciation (Carson, 1982). Criticism of the concept of chromosomal isolation has originated from two schools: the followers of the recognition concept of species (Patterson, 1980, 1985; Vrba, 1985) and some of the evolutionists who endorse the biological species concept such as Mayr (1963) and Carson (1982) although, at a later stage, Mayr (1969) modified his position and accepted the possibility of chromosomal isolation in special cases of very small peripherally isolated populations.

Criticism from the "Pattersonians" is logically consequent to their concept of species based on recognition of potential mates:

"We can, therefore, regard as a species that most inclusive population of individual biparental organisms which share a common fertilization system."

(Patterson, 1985).

Therefore, as long as their members can recognize one another as potential mates, because they share their fertilization system, two populations are part of the same species whether they interbreed or not: post-zygotic (and geographic) isolation does not play a role in speciation. Both camps, however, use similar arguments to refute the validity of karyotypic isolation. Proponents of these models focus on the fact that before a chromosome rearrangement becomes fixed in a population, it must undergo a stage of chromosomal heterozygosity and that, therefore, structural heterozygotes will suffer from the same type of heterosis as interspecific hybrids. The algebra of population genetics, maintain these authors, predicts instead that these heterozygotes will be rapidly eliminated from the

general population, thus homokaryotypes can never become established (see White, 1978a). However, this need not be the case if the organism in question, for example, displays low vagility or is generally restricted to a fragmented habitat. Both conditions would favour inbreeding with consequent rapid fixation of new chromosomal morphs; meiotic drive may be a further factor facilitating fixation (White, 1978a). Thereafter, selection may operate against the energetic wastage of failed reproduction, which would favour reinforcement or reproductive character displacement of post-zygotic with pre-mating barriers (Butlin, 1987; see also Meester, 1988). An algebraic model for heterozygous disadvantage under random mating has also been used by Patterson (1978) to demonstrate the impossibility of chromosomal rearrangements acting as reproductive barriers upon secondary contact. However, Robinson and Roux (1985) have demonstrated that judicious use of such algebra - under the assumptions of limited sympatry and/or low migration rates - results in stable equilibria and selection for positive assortative mating.

Although the views expressed, amongst others, by White (1978a; 1982) and Robinson and Roux (1985) seem to be accepted by most cytotaxonomists, perusal of the relevant literature of the past two decades elicits little consensus as to which mode of speciation pertains to karyotype evolution. Some investigators fit chromosome rearrangements into sympatric or stasipatric speciation models (White, 1982) while others (Mayr, 1982; Bickham and Baker, 1980; Sites, 1983) find little evidence to support such a view and prefer a scenario where chromosomal evolution occurs in allopatry. On the other hand, Bickham and Baker (1979) have proposed an allopatric model of chromosomal speciation (canalization model) which postulates an adaptive significance for karyotypic modifications associated with speciation. These authors have suggested that following chromosomal isolation, incipient species colonize new habitats and undergo further karyotypic changes during adaptation to these environments.

◆ *The hypothesis*

Regardless as to whether these events occur in sympatry or allopatry, Meester (1988) proposed a special case of chromosomal evolution whereby sibling species, phenotypically indistinguishable from one another, are the most likely result of the post-zygotic reproductive breakdown caused by chromosome rearrangements. A corollary of this hypothesis is that formation of sibling species is compatible with both sympatric and allopatric modes of speciation.

"It is frequently overlooked that any event, such as a chromosomal mutation, affecting some but not all individuals within a population, is in fact occurring sympatrically within that population, whether or not the population has a closely related allopatric relative. Fixation of a chromosomal mutation, therefore, is a sympatric event..." (Meester, 1988).

Meester, however, pointed out that it is more parsimonious to postulate an allopatric isolation. This would facilitate overcoming the bottle-neck of negative heterosis by favouring inbreeding - hence fixation - in small peripheral isolates, in accordance with Mayr's (1982) peripatric speciation model (i.e. allopatric speciation). At this stage, Meester (1988) emphasized, there should be little or no detectable genetic divergence between the incipient species, a phenomenon already noted by White (1982) in association with sympatric and stasipatric speciation. The reproductive isolation established by chromosome changes, coupled to reproductive character displacement, would then hasten the accumulation of the genetic differences associated with the conventionally understood allopatric speciation mode and usually considered to precede speciation. This may be particularly applicable if, as indicated earlier, the sibling species in question also display low migration rates. A possible example of "sibling speciation" may be found in the case of *Mastomys natalensis* and *M. coucha*, which were long regarded as a single species but have emerged as sibling species differing in diploid number (Hallett, 1979), G-banding pattern (Lyons *et al.*, 1977, 1978), haemoglobin electrophoretic migration (Robbins *et al.*, 1983)

and sperm morphology (Gordon and Watson, 1986). Other, less studied, possible test species could be within *Aethomys chrysophilus*, between *Thallomys paedulus* and *T. nigricauda*, as well as *Otomys angoniensis* and *O. irroratus* (Meester, 1988).

◆ *Experimental aspects and scope of the study*

Meester's hypothesis of sibling speciation has two major merits. Firstly, because it is not in conflict with recognized modes of speciation, it can also provide insights and indirect evidence for more general models of chromosomal speciation such as the stasipatric and the canalization ones. Secondly, it is more amenable than other hypotheses to testing, because it predicts a lack of genetic differentiation - as well as little phenotypic difference and poorly developed pre-mating isolation systems - in the populations being investigated. Methods to acquire the necessary information for testing, such as differential chromosome staining and allozyme starch gel electrophoresis, have been available for more than two decades while new and more sensitive methods have been developed in more recent times (see Hillis and Moritz, 1990 for an anthology and manual) or are being developed (see chapter 5).

Predictions of Meester's hypothesis would, thus, be fulfilled should chromosome rearrangements be detected in concomitance with low inter-population genetic divergence, as measured by electrophoretic methods which detect gene product variation. Similarly, mating behaviour, as observed in the laboratory, should be such that interbreeding between population members is still possible and the successful matings should result in offspring of limited fertility and/or limited viability. This should then be taken as indication that, by the time an isolating homokaryotype becomes established, interbreeding is still possible. Barring the chromosomal differences, therefore, lack of genetic impediments to the production of healthy and fertile offspring would be implied. The existence of interfertile populations, within the species distribution range, carrying different and "non-isolating" chromosomal rearrangements can - if they are detected - confirm such a conclusion. This would allow the establishment of a temporal sequence of events leading to sibling

speciation. Also, if populations with greater numbers of isolating karyotypic differences are uncovered, their levels of genetic divergence and pre-mating behavioural differences should also be greater. Furthermore, should natural hybridization zones be found between chromosomally diverse populations within the species distribution range, the theoretical requirements for the more general mode of stasipatric speciation would be met. On the other hand, the possible finding of correlation between the distributions of intraspecific cytotypes and habitat and/or climatic patterns, may provide indirect support for the canalization model of chromosomal speciation.

Consequently, the scope of the investigation presented here is articulated according to the following lines.

1. Sampling within the distribution range of the species *O. irroratus*.
2. Karyotype analysis of the specimens by conventional Giemsa staining, G- and C-banding following established methods.
3. Analysis of the load of genetic mutation by methods designed to detect variation amongst gene products.
4. Correlation of the data obtained from the two types of analysis.
5. Correlation of such data with environmental parameters.
6. Correlation of such data with pre-mating behaviour of the specimens as studied by Pillay *et al.* (1992, 1993).
7. Correlation of all data with those from mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) analysis as reported by Rimmer *et al.* (1996) and those from analysis of randomly amplified polymorphic nuclear DNA sequences (RAPD) Dace (1995).

◆ ***Layout of the manuscript***

The aspects listed above are treated as follows. The next section provides a review of the general biology of the test species. The cytogenetical findings with related methods and

discussion are detailed in Chapter 2 which incorporates suitably edited versions of two papers¹ published on the subject in 1992.

Chapter 3 ("Environmental correlates") deals with statistical analysis and correlations of karyotype distribution with climatic and habitat parameters and it is an edited version of a paper² published in 1994.

Immunoreactivity of gene products both at generic and intraspecific levels is detailed and discussed in Chapters 4 and 5 respectively which include edited versions of two papers³ on the subject. The last section of the manuscript, Chapter 6, provides a synthesis of the experimental data and discussions presented in previous chapters. Moreover, aspects which are not dealt with in previous sections are discussed therein.

◆ *The test organism*

As indicated in page 5 of this chapter, Meester (1988) suggested *O. angoniensis* and *O. irroratus* as likely candidates to show evidence for sibling chromosomal speciation. Meester based his suggestion on the fact that these two species are morphologically indistinguishable apart from the shape of the posterior petrotympanic foramen (slit-like in *O. angoniensis* and round in *O. irroratus*) and the diploid number of the two karyotypes: $2n=56$ in *O. angoniensis*, $2n=28$ in *O. irroratus*. However, the consideration that the two rodents were already recognized as good, albeit sibling species, and of the great difference in respective diploid numbers, suggested that they may have already diverged sufficiently as to make the task of demonstrating chromosomal speciation rather difficult. On the other

¹Contrafatto G., Meester J.A., Willan K., Taylor P.J., Roberts M.A. & Baker C.M. 1992a. Genetic variation in the African subfamily Otomyinae (Muridae) II: chromosomal changes in some populations of *Otomys irroratus*. *Cytogenetics and Cell Genetics* 59: 1-10. Contrafatto G., Meester J., Bronner G., Taylor P.J. & Willan K. 1992b. Genetic variation in the African rodent subfamily (Muridae) IV: chromosome G-banding analysis of *Otomys irroratus* and *O. angoniensis*. *Israel Journal of Zoology* 38: 277-291.

²Taylor, P.J., G. Contrafatto & K. Willan. 1994. Climatic correlates of chromosomal variation in the African vlei rat, *Otomys irroratus*. *Mammalia*, 58(4): 624-634

hand, Robinson and Elder (1987), in reporting lack of G-banding homology between the karyotype of *O. irroratus* and those of *O. unisulcatus* and *Rattus norvegicus*, rightly inferred that the vlei rat must have undergone an extensive genome reorganization during its evolutionary history. This suggested that *O. irroratus* itself might be the better species to be used as a test case for a chromosomal speciation hypothesis.



Figure 1.1 - *Otomys* photographed in captivity (courtesy of K. Willan and N. Pillay).

The general biology of this species of vlei rat has been exhaustively reviewed by De Graaff (1981) and Skinner and Smithers (1990). Therefore, only a synthesis of these authors' contributions is presented here, covering the aspects which may

be necessary to peruse this manuscript. Figure 1 shows a representative of this species.

Otomys irroratus is a medium sized vole-like rodent endemic to Southern Africa. It is stocky, blunt-nosed with shaggy pelage of long and soft hair. The ears are round and large - hence the generic name - and well haired. The tail, much shorter than in common rodents, is about 60 per cent of the length of body and head. Total animal length averages 240 mm while body mass is on average 122 g in males and 114 g in females. Coat colour is geographically variable and, in the dorsal parts, is a grizzled dark slate-gray tinged with buff or brown. The flanks and under parts are paler and grayer, while the tail is brown in the upper surface and buffy below, the feet dark gray.

The incisors are large and grooved while the molars, as in all other Otomyines, are laminated with the upper third molar having four to seven laminae and the lower first molar showing only four. This feature is used as a key character to distinguish *O. angoniensis* and

³ Contrafatto G, David D. & Goossens-Le Clerq V. 1994. Genetic variation in the African rodent subfamily Otomyinae (Muridae) V: phylogeny inferred by immunoblotting. *Durban Museum Novitates* 19: 1-7.

O. irroratus from other members of the genus (Meester *et al.*, 1986) while a molar series longer than 8.8 mm is the character that separates *O. irroratus* from *O. saundersiae*. The species is similar in appearance to its partly sympatric sibling species *O. angoniensis*.

Distinguishing characters include the already mentioned shape of the posterior petrotympanic foramen (see page 8) and the hind foot length of 29-34 mm in *O. irroratus* and 25-28 mm in *O. angoniensis*. A distinct ring of orange coloured hair around the eye of *O. angoniensis* which is absent in *O. irroratus*, is also listed as a useful identification feature (Skinner and Smithers, 1990) but does not appear to be reliable. Of several unidentified *Otomys* from the north-eastern part of the *O. irroratus* range of distribution - where it is sympatric with its sibling species - all of those that showed such an orange ring around the eye (K. Willan, pers. comm.) had an *O. irroratus* karyotype (Pers. obs.).

The species distribution illustrated in figure 1.2, is within the limits of the Southern African region.

In addition to the areas within the South African political confines, it is also recorded as an isolated population from the eastern parts of Zimbabwe and

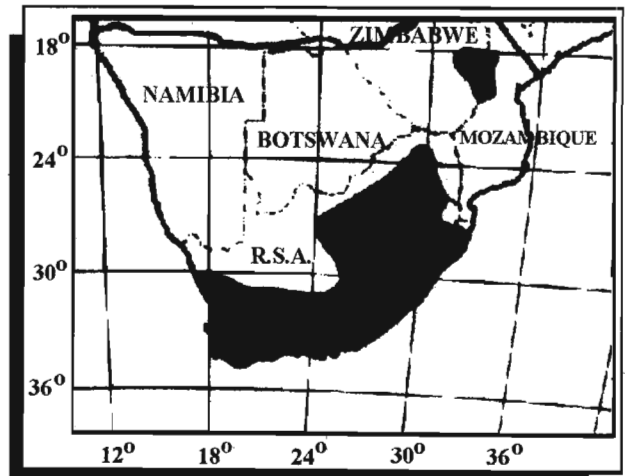


Figure 1.2 - Map of Southern Africa and distribution of *O. irroratus* (After Skinner and Smithers, 1990).

adjacent Mozambique, south of the Zambesi river. Although catholic in dis-

tribution as it occurs throughout grasslands, *O. irroratus* prefers moist habitats associated with rivers and vleis, hence its common name of vlei rat. However, in montane areas these animals occur on grassy hillsides and ridgetops at a distance from water (Rowe-Rowe and Meester, 1982).

The vlei rat is of crepuscular habits and socially territorial (see Pillay, 1993); it

Contrafatto, G, van den Berg, J R & Grace, J H, Genetic variation in the African rodent subfamily Otomyinae (Muridae). VI: electrotransfer of liver proteins of some *Otomys irroratus* (Brants 1827) populations. *Tropical Zoology*. 9:000-000 (in press)

seldom, if ever, burrows and normally constructs variously shaped nests under grass cover. Home range size in the north-eastern part of the species distribution has been reported to be 1730 m² for males and 1252 m² for females. Pillay (1993) measured average distances (Av.D.) travelled between successive captures, as an index of adult home range size, varying from 10.1 to 18.8 m in two KwaZulu/Natal populations. Home range size decreases marginally in autumn and winter (Skinner and Smithers, 1990; Pillay, 1993). *O. irroratus* is a herbivorous species and, thus, exhibits specializations to this diet such as transverse lophs in the teeth and large and complex caecum. *O. irroratus* uses grass both, as a food source and as cover. Some grass species, such as *Eragrostis curvula*, are considered unpalatable to vlei rats (Willan and Bigalke, 1982). These rodents, nonetheless, have been shown in some habitats to associate with *E. curvula* in preference to grasses of higher nutritional value (i.e. *Themeda triandra*) since the former offers better opportunities for cover as indicated by readings of ground level light penetration in the KwaZulu/Natal Midlands (Pillay, 1993).

Reproduction, in areas of summer rainfall, peaks during the warm wet summer months but has been reported to begin as early as August and continue through to May. Pillay (1993), however, reports a short reproductive season in one of two populations from KwaZulu/Natal. Females are polyoestrous and, since the gestation period is 40 days, they can produce up to seven litters, with a mean size of 2.33, each season. Their precocial pups show nipple-clinging behaviour up to 14 days of life. Pillay (1993) suggests that nipple-clinging, which is common to all Otomyines, correlates with the species lifestyle of nesting on the surface rather than burrowing.

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CHAPTER 2

THE KARYOTYPE OF *O. IRRORATUS*

◆ Introduction

The genus *Otomys* is endemic to Africa and is, for the most part, confined to Southern Africa with only four of its 10 species entirely extralimital (Misonne, 1974). Some of the Southern African species are clearly distinct from each other, and were previously assigned to two further genera (Roberts, 1951): *Myotomys* (*O. unisulcatus*; *O. sloggetti*) and *Lamotomys* (*O. laminatus*). All of these are currently considered synonyms of *Otomys* (Meester *et al.*, 1986). On the other hand, *O. irroratus* and the frequently sympatric *O. angoniensis* are difficult to distinguish, being described as sibling species (Misonne, 1974), whereas the extralimital *O. tropicalis* is regarded by some (Honacki *et al.*, 1982) as a synonym of *O. irroratus*, in spite of a marked geographic discontinuity between their ranges. Meester *et al.* (1986) provisionally listed eight subspecies of *O. irroratus* and stated that too many subspecies are probably recognized. Allozyme analysis (Taylor *et al.*, 1989a) of four otomyinae has highlighted the distinctiveness of *O. irroratus*, supported the hypothesis of a diphyletic evolution of this subfamily and also suggested the inclusion of the bush Karoo rat, *O. unisulcatus*, within the genus *Parotomys*.

The range of taxonomic relationships found within *Otomys* and the difficulties of distinguishing between phenotypically very similar taxa within the genus, suggest the need to go beyond morphological taxonomic approaches to chromosomal and biochemical techniques in dealing with these problems. The karyotypes of *O. angoniensis* ($2n = 56$) and *O. unisulcatus* ($2n = 28$) have been described (Matthey, 1964) but a G-banded karyogram of *O. angoniensis* has not yet been published. Matthey (1964) referred to *O. unisulcatus* as '*O. irroratus*', but others (Robbins

and Baker, 1978; Robinson and Elder, 1987) have corrected this misidentification. Robinson and Elder (1987) provided karyotypes of *O. irroratus* and *O. unisulcatus*, and pointed out that although their diploid number was the same ($2n = 28$), chromosome morphology was markedly different and no homologies in chromosome G-banding patterns were apparent between these two species. The karyotype of *O. irroratus* described by these authors comprises eight pairs of submetacentric autosomes, one pair of metacentric and four pairs of acrocentric autosomes. The Y chromosome is a small acrocentric whereas the X chromosome is submetacentric.

Meester *et al.*, (1992) suggested that chromosomal speciation may be occurring in this species by post-zygotic isolation. The aim of this chapter, therefore, is to describe the chromosomes of 18 populations of *O. irroratus*, as well the G-banded karyotype of *O. angoniensis* and to uncover possible homologies between the chromosomes of the two species. In turn this may enable one to establish what phylogenetic relationships may exist between the various *O. irroratus* populations and to provide some evidence in favour of a speciation model which envisages chromosomal changes leading to speciation by post-zygotic reproductive isolation.

◆ *Materials and methods*

Chromosome preparations were obtained from 97 specimens of *O. irroratus* (and 11 of *O. angoniensis*) from various Southern African localities encompassing most of the distribution range of this species. Fourteen of these animals were born in captivity in the animal-housing facilities of the Department of Biology. Details of animals, localities and habitats are given in Table 2.1. The localities of the specimens studied are mapped in Figure 2.1. Voucher specimens of these rodents have been deposited in the mammal collections of the Durban Natural Science Museum and their accession numbers are provided in the appendix at the end of chapter 2.

Chromosome preparations were obtained from cultured spleen lymphocytes,

cultured skin or spleen fibroblasts and by direct harvesting of bone marrow samples. Spleens were minced in Dulbecco's Modified Eagles Medium (DMEM, Highveld Biological, R.S.A.) with forceps and scissors and the resulting cell suspension and tissue fragments were then cultured at 37 °C with phytohaemagglutinin (PHA) (Wellcome, U.K.) for 72 hours.

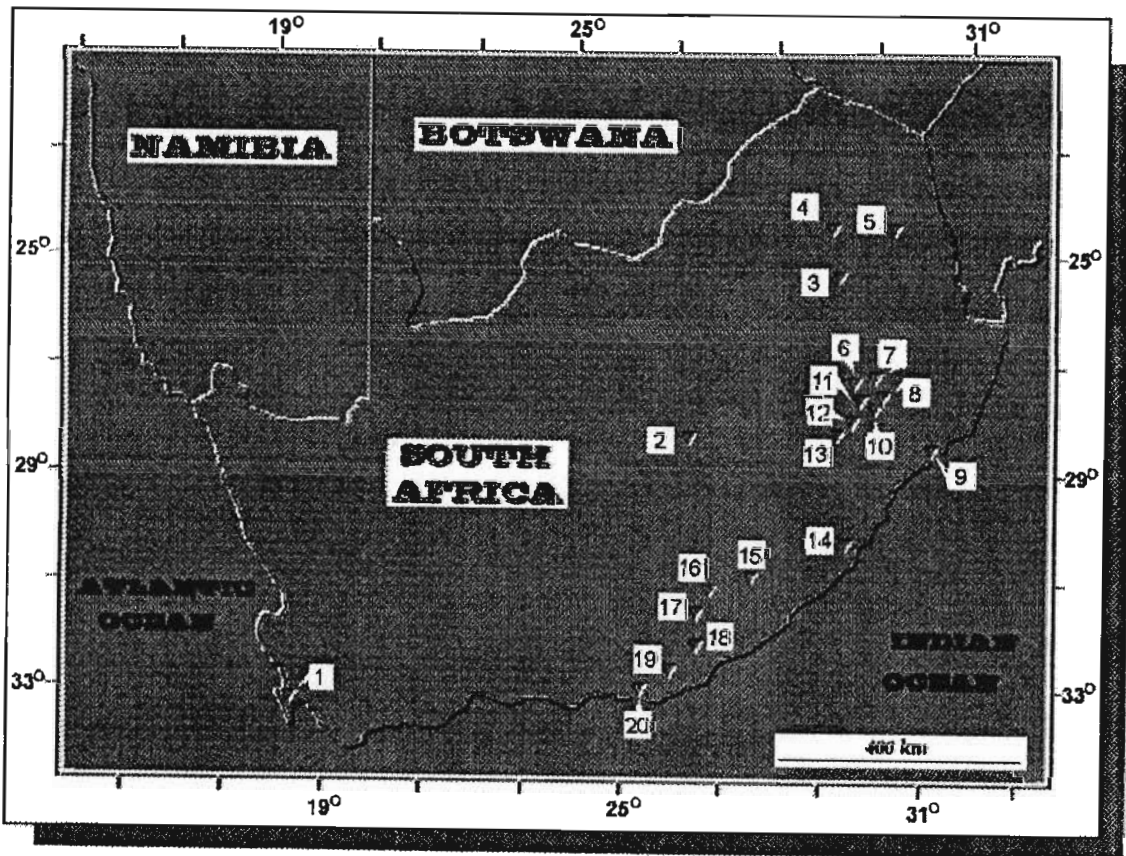


Figure 2.1 - Map of South Africa with the approximate position of the localities where the Otomyine specimens reported here were collected. 1=Constantia, 2=Bloemfontein, 3=Rietvlei, 4=Nylsvlei (*O. angoniensis*), 5=Mariepskop*, 6=Garden Castle*, 7=Dargle Station*, 8=Karkloof, 9=Durban (*O. angoniensis*), 10=Umgani Valley Nature Reserve, 11=Loteni Nature Reserve*, 12=Kamberg Nature Reserve, 13=Vergelegen Nature Reserve, 14=Umtamvuna*, 15=Stutterheim, 16=Hogsback, 17=Alice, 18=Committees Drift, 19=Grahamstown, 20=Port Elizabeth. (Asterisks refer unpublished observations).

Table 2.1. Sample size and localities data for the *O. irroratus* populations presented in this study. Veld types: Acocks (1975).

| Locality and Grid Reference | n | Sex | Veld Type and Number |
|--|--------|--------|--|
| Alice (Eastern Cape) 32°47'S, 26°50'E | 5 7 | F M | False thornveld: (21) |
| Captive born from Alice specimens | 2 | M | |
| Bloemfontein (Orange Free State) 29°06' S, 26°14' E | 2 | M | Dry <i>Cymbopogon-Themeda</i> veld (50) |
| Captive born from Bloemfontein specimens | 1 | F | |
| Committee's Drift (Eastern Cape) 33°05'S, 26°46'E | 2 3 | F M | Valley bushveld: (23) |
| Constantia (Western Cape) 34°00' S, 18°30' E | 4 | M | Coastal macchia (47) |
| Dargle (KwaZulu/Natal) 29°30' S, 30°01' E | 1 | M | Ngongoni veld of the Natal mist belt: (45) |
| Garden Castle Nature Reserve (KwaZulu/Natal) 29°45' S, 29°15' E | 3 1 | F M | Highland sourveld: (44A) |
| Grahamstown (Eastern Cape) 33°17' S, 26°33' E | 3 3 | F M | False macchia (70) |
| Hogsback (Eastern Cape) 32°33'S, 26°57'E | 3 6 | F M | Highland sourveld: (44A) |
| Kamberg Nature Reserve (KwaZulu/Natal) 29°23' S, 29°43' E | 4 | M | Highland sourveld (44A) |
| Karkloof (Natal) 29°21'S, 30°13'E | 5 8 | F M | Ngongoni veld of the Natal mist belt: (45) |
| Captive born from Karkloof specimens | 1 3 | F M | |
| Loteni N. R. (KwaZulu/Natal) 29°27' S, 29°32' E | 4 | F | Highland sourveld: (44A) |
| Mariepsskop (Northern Province) 24°35' S, 30°35' E | 1 | F | Sourish mixed bushveld: (19) |
| Port Elizabeth (Eastern Cape) 33°55'S, 25°40'E | 2 1 | M F | Alexandria forest: (2) |
| Rietvlei (Northern Province) 25°51' S, 28°18' E | 4 6 | F M | Sourish mixed bushveld (19) |
| Stutterheim (Eastern Cape) 32°30' S, 27°17' E | 7 | F | Dohne sourveld: (44B) |
| Umgeni Valley Nature Reserve (KwaZulu/Natal) 29°28' S, 30°15' E | 1 | F | Ngongoni veld of the Natal mist belt: (45) |
| Umtamvuna (KwaZulu/Natal) 31°03' S, 30°10' E | 2 | F | Dohne sourveld: (44B) |
| Vergelegen Nature Reserve (KwaZulu/Natal) 29°32' S, 29°27' E | 1 1 | F M | Highland sourveld (44A) |

The cultures were then transferred to conical centrifuge tubes and incubated for a further 50 minutes with 25 ng/ml of colchicine (BDH Chemicals, U.K.). Fibroblasts that remained attached to the bottom of the culture flasks (25 cm², NUNC, Denmark) were cultured further. Primary fibroblast cultures were established from small ear biopsies (<0.5cm²). Colchicine treatment, in such cases, was at a concentration of 50 ng/ml for 25 minutes while bone marrow preparations were obtained following established procedures (Shiloh and Cohen, 1978). DMEM (Highveld Biological, R.S.A.) with 20 percent foetal bovine serum (Highveld Biological, R.S.A.) was used in all culturing procedures.

Karyograms were constructed from one individual per population unless microscope observation suggested the existence of significant intra-population variability. For comparative purposes, the classification and nomenclature used by Robinson and Elder (1987) were adopted in the preparation of the karyograms. Thus, chromosomes whose banding patterns did not match those of the published karyotype were generally not assigned pair numbers.

Chromosome G-banding was carried out by trypsin treatment (Seabright, 1971) or by incubation in Sorensen's phosphate buffer at pH 8.0 for 22 hours at 65 °C. C-banding was unsuccessfully attempted on preparations from Alice and Stutterheim by incubation in 50 ml of saturated barium hydroxide for 10-20 minutes followed by 2 x SSC buffer incubation. In subsequent chromosome preparations from other populations C-bands were obtained following, *pari passu*, a method described by Modi (1987). Differential staining similar to C-banding was also performed by treatment with the restriction endonuclease *A_{lu}I* (Miller et al., 1983; Mezzanotte et al., 1983a, Mezzanotte et al., 1983b). In order to analyze heterochromatin in more detail, a technique which is under development (Contrafatto, unpublished), was employed on some preparations by incubating them in a phosphate buffer (pH 8.4) at 65 °C for 24 hours prior to Giemsa staining.

Cladistic analysis of the cytogenetic data employed the PAUP (Phylogenetic Analysis Using Parsimony) computer programme of David Swofford (Version 2.4), while

the same data were also subjected to phenetic analysis with the programme BIOSYS of Swofford and Selander (Release 1.7) coding each chromosome pair as a locus as described by others (Porter and Sites, 1986). In the case of cladograms, trees were rooted from the outgroup (*O. angoniensis*).

◆ *Results*

No differences were observed in the modal chromosome number in the same individual when more than one culturing method was used on tissue from the same animal. When direct bone marrow preparations were used in parallel with culturing techniques, modal numbers consistently matched those found in cultured cells (P. Taylor, Pers. Com.). In some cases, chromosome preparations from fibroblast cultures showed a high frequency of aneuploid spreads.

The G-banded chromosomes of *O. angoniensis* are shown in Figure 2.2. This confirms earlier reports of a diploid number of $2n = 56$ and a karyotype comprising 28 pairs of acrocentric chromosomes (Matthey, 1964). The X-chromosome is the second largest member of the karyotype while the Y-chromosome is the smallest (see Table 2.2). No prominent chromosomal alterations were detected in the specimens from this species. Numerical and morphological karyotype variability, however, occurred between populations and within some of the populations of *O. irroratus* and between these karyotypes and that of Tsitsikama published by Robinson and Elder (1987). The diploid numbers found in my study are listed in Table 2.3. Biarmed cytotypes with at least seven large submetacentric autosomes with heterochromatic short arms (B cytotype) were observed at Committee's Drift, Port Elizabeth, Alice and Grahamstown.

Table 2.2 - Mean relative chromosome lengths (MRL) and standard error (SE) from 10 spreads of *O. angoniensis* expressed as percentage of the haploid karyotype.

| Chr | MRL | SE | Chr | MRL | SE | Chr | MRL | SE |
|-----|------|------|-----|------|------|-----|------|------|
| 1 | 7.17 | 0.21 | 11 | 3.70 | 0.16 | 21 | 2.56 | 0.11 |
| 2 | 5.37 | 0.02 | 12 | 3.60 | 0.16 | 22 | 2.51 | 0.11 |
| 3 | 5.16 | 0.09 | 13 | 3.13 | 0.14 | 23 | 2.40 | 0.10 |
| 4 | 5.00 | 0.07 | 14 | 2.89 | 0.13 | 24 | 2.38 | 0.10 |
| 5 | 4.96 | 0.06 | 15 | 2.89 | 0.03 | 25 | 2.28 | 0.10 |
| 6 | 4.78 | 0.21 | 16 | 2.89 | 0.13 | 26 | 2.28 | 0.10 |
| 7 | 4.39 | 0.19 | 17 | 2.85 | 0.13 | 27 | 1.95 | 0.08 |
| 8 | 4.38 | 0.19 | 18 | 2.85 | 0.13 | X | 6.21 | 0.17 |
| 9 | 4.37 | 0.19 | 19 | 2.71 | 0.12 | Y | 1.60 | 0.15 |
| 10 | 4.19 | 0.18 | 20 | 2.57 | 0.11 | | | |

Acrocentric karyotypes lacking heterochromatic short arms (A cytotype) were detected at Stutterheim and Umtamvuna while acrocentric karyotypes carrying a tandem fusion of autosomes 7 and 12 (A1 cytotype) were detected in specimens from Hogsback, and at the Nature Reserves of Kamberg, Vergelegen, Loteni and Garden Castle. A further acrocentric karyotype without the t(7:12) but showing two unique pairs of supernumerary medium-sized acrocentrics (A2 cytotype) was seen in specimens from Karkloof, Umgeni Valley Nature Reserve and Dargle. In addition, the populations at Constantia, Bloemfontein, Rietlei and Mariepskop (C cytotype) exhibited intermediate karyotypes: only four of the largest autosomes carried heterochromatic short arms. As shown in Figure 2.6 (page 33), the G-banding pattern of the acrocentric cytotypes corresponded to that of the long arms in the biarmed cytotypes while complete homology was seen between the acrocentric small chromosomes (pairs 11-14).

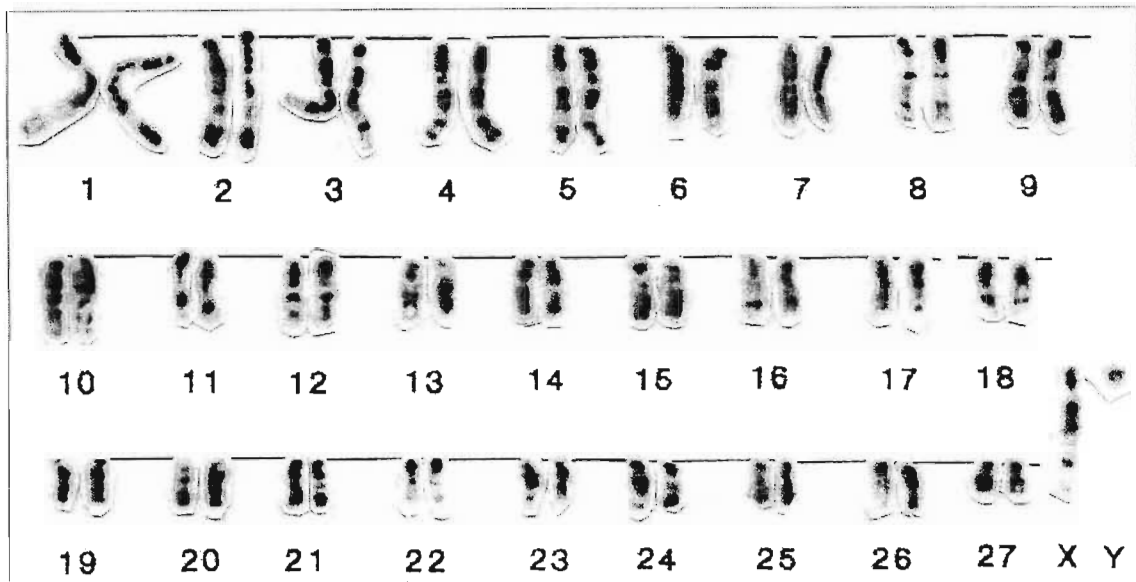


Figure 2.2 - G-banded karyotype of the Angoni vlei rat *O. angoniensis* ($2n=56$, $NF=56$).

The sex chromosomes were similar in all cytotypes so far studied: the X-chromosome was a medium-sized submetacentric carrying a heterochromatic short arm of variable length while the Y-chromosome is a wholly heterochromatic small acrocentric. Intrapopulation numerical differences were due mostly to variation in the number of copies (from zero to four) of two small heterochromatic biarmed autosomes (chromosomes 8 and 9).

- *Cytotype A*

All the specimens from Stutterheim and Umtamvuna (see Fig. 2.1) were karyotypically consistent having 30 chromosomes, including three small biarmed autosomes (pairs 8 and 9). The remaining autosomes were all acrocentric (Figure 2.3-C;

One of the small acrocentrics did not match any other member of the karyotype (see 'a' in Figures 2.3-C, page 25 and 2.6 page 33) and it is possible that this chromosome represented a uniarmed heteromorph of pair 9 without the heterochromatic short arm. The X chromosome was a medium sized submetacentric while the Y chromosome was a small acrocentric. A G-banded karyogram typical of these populations

Table 2.3 - Diploid and fundamental numbers in the *O. irroratus* populations studied.

| Population | Cytotype | 2n | NF | M | F |
|-------------------|----------|----|----------|--------|---|
| Stutterheim | A | 30 | 33 | | 7 |
| Umtamvuna | A | 30 | 34 | | 2 |
| Garden Castle | A1 | 24 | 24 | 1 | 3 |
| Hogsback | A1 | 23 | 24 | | 1 |
| | | 24 | 24 | | 1 |
| | | 25 | 26 | 3 | 1 |
| | | 26 | 28 | 1 | |
| | | 27 | 30 | 2 | |
| Kamberg | A1 | 24 | 24 | 1 | |
| | | 25 | 26 | 2 | |
| | | 26 | 28 | | |
| | | 27 | 30 | 1 | |
| Loteni | A1 | 24 | 24 | | 4 |
| Vergelegen | A1 | 24 | 24 | 1 | 1 |
| Dargle | A2 | 30 | 30 | 1 | |
| Karkloof | A2 | 29 | 30 | | 1 |
| | | 30 | 30 | 5 | 3 |
| | | 31 | 32 | 3 | 1 |
| | | 32 | 34 | 3 | 1 |
| Umgeni Valley | A2 | 32 | 34 | 1 | |
| Alice | B | 28 | 44 | 3 | 2 |
| | | 29 | 46 | 2 | 1 |
| | | 30 | 48 | 4 | 2 |
| Committee's Drift | B | 28 | 46 | 3 | 2 |
| Grahamstown | B | 28 | 42 43 | 2 1 | 3 |
| Port Elizabeth | B | 28 | 44 | 2 | 1 |
| Bloemfontein | C | 29 | 38 | 2 | |
| | | 30 | 40 | | 1 |
| Constantia | C | 28 | 42 | 3 | |
| | | | 41 | 1 | |
| Mariëpskop | C | 28 | 36 | 1 | |
| Rietvlei | C | 28 | 36 | 6 | 4 |

is depicted in Figure 2.3-A. Apart from the lack of heterochromatic short arms, the banding pattern matched that of the Tsitsikama population studied by Robinson and Elder (1987) but an extra autosomal pair (14) was present which was not reported in the Tsitsikama specimens.

• Cytotype A1

Ten of the eleven individuals from Hogsback had modal numbers ranging from 24 to 27 chromosomes with 22 acrocentric autosomes. A further specimen exhibited a diploid number of 23 with 20 acrocentric autosomes and a large metacentric which originated from the centric fusion of chromosomes 1 and 3 (Figure 2.3-D, upper inset). Small polymorphic and partially heterochromatic chromosomes similar to pairs 8 and 9 from other populations were not observed in three specimens. However, four individuals had a pair 9, one carried a pair 9 and one member of pair 8 while the remaining three had only one member of pair 9.

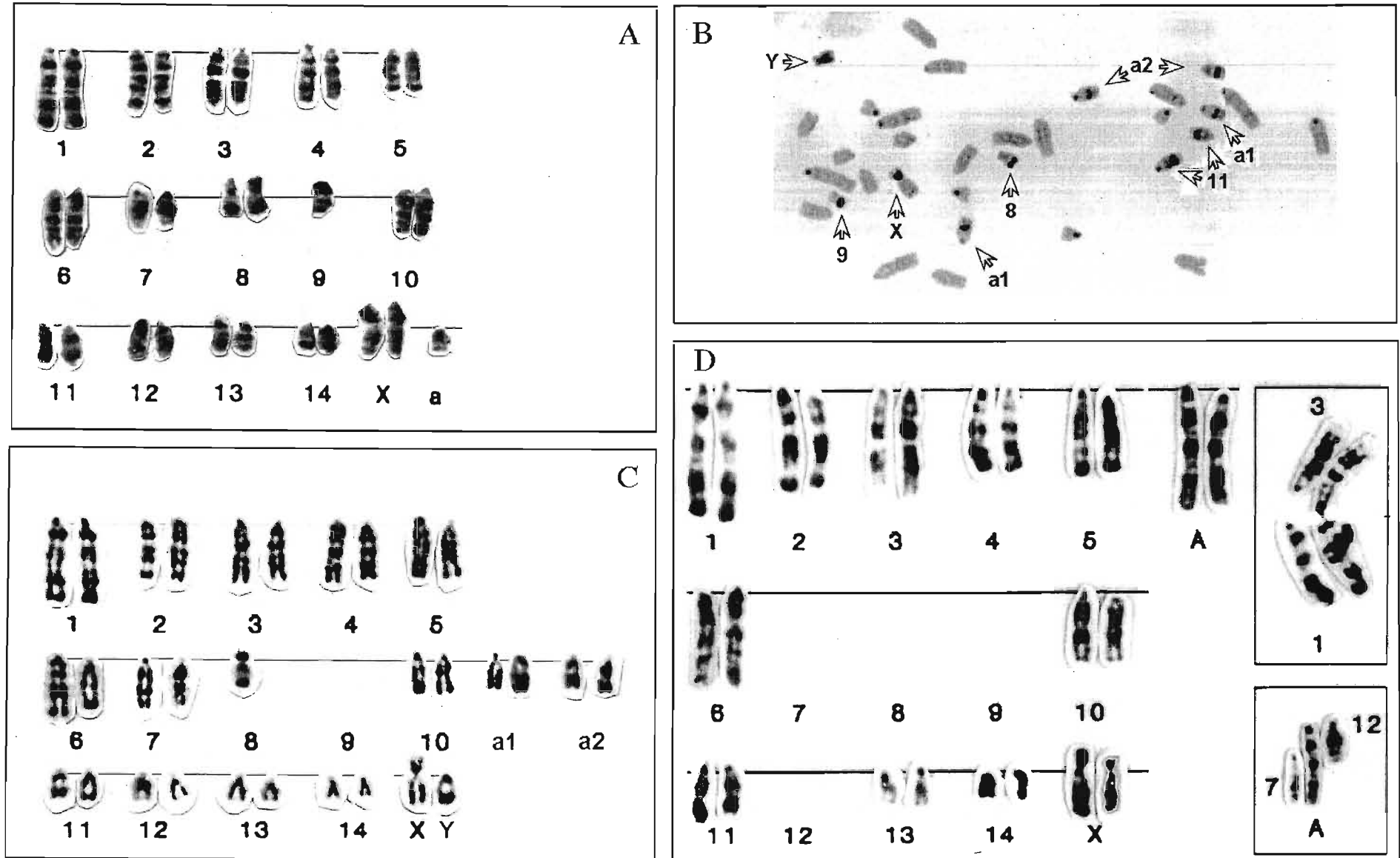


Figure 2.3 - Cytotypes A, A1 and A2. Frame A: G-banded karyogram from Stutterheim (type A). Frames B and C: C- and G- banding, respectively, from Karkloof (type A2). Frame D: G-banded karyogram from Hogsback (type A1). Upper inset in frame D: centric fusion of autosomes 1 and 3 from a Hogsback specimen. Lower inset in frame D: tandem fusion of chromosomes 7 and 12 typical of the A1 cytotype.

The X-chromosomes were similar to those of other *O. irroratus* cytotypes. All specimens were characterized by the presence of a unique autosomal pair (pair 'A' in Figure 2.3-D) and the absence of pairs 7 and 12. The G-banding pattern indicated that this chromosome resulted from a tandem fusion of autosomes 7 and 12 (Figure 2.3-D, lower inset), which were present in an unfused state in most other populations.

As expected in view of the close proximity of these localities (see Table 2.1 page 19 and Figure 2.1 page 18), Kamberg ($2n = 24-27$), Vergelegen, Loteni ($2n=24-25$) and Garden Castle ($2n = 24$) Nature Reserves specimens yielded the same karyotypes which were all-acrocentric and similar to that from the widely separated Hogsback locality. The banding pattern of these acrocentric chromosomes matched that of the long arms of their homologues in the biarmed cytotypes. Pairs 7 and 12 were missing and were replaced by a large acrocentric with bands matching those of the Hogsback tandem fusion (see inset in figure 2.3-A page 25 and chromosome "A" in figure 2.6 page 33). No members of pairs 8 and 9 were found in the preparations from the single Vergelegen specimen or two from Kamberg. Of the other two Kamberg animals, one exhibited both members of pair 9 and one number 8 while the other had only one of each pair. Only one of the Loteni specimens showed one of each pair whereas none of these chromosomes were found in the four specimens from Garden Castle.

- *Cytotype A2*

Karkloof specimens had diploid numbers ranging from 29 (one individual) to 32 (three individuals) with intermediate numbers of 30 (four individuals) and 31 (six specimens; see Table 2.2). All these karyotypes displayed 14 pairs of acrocentric autosomes (pairs 1-7, 10-14, 'a1' and 'a2' in Figure 2.3-C) and the X- and Y-chromosomes were similar to those of the other cytotypes. Most of the differences in diploid number were due to variation in the number of small biarmed chromosomes (pairs 8 and 9), which varied from zero in the $2n = 30$ karyotypes to one in the $2n = 31$ and two in the $2n = 32$ types.

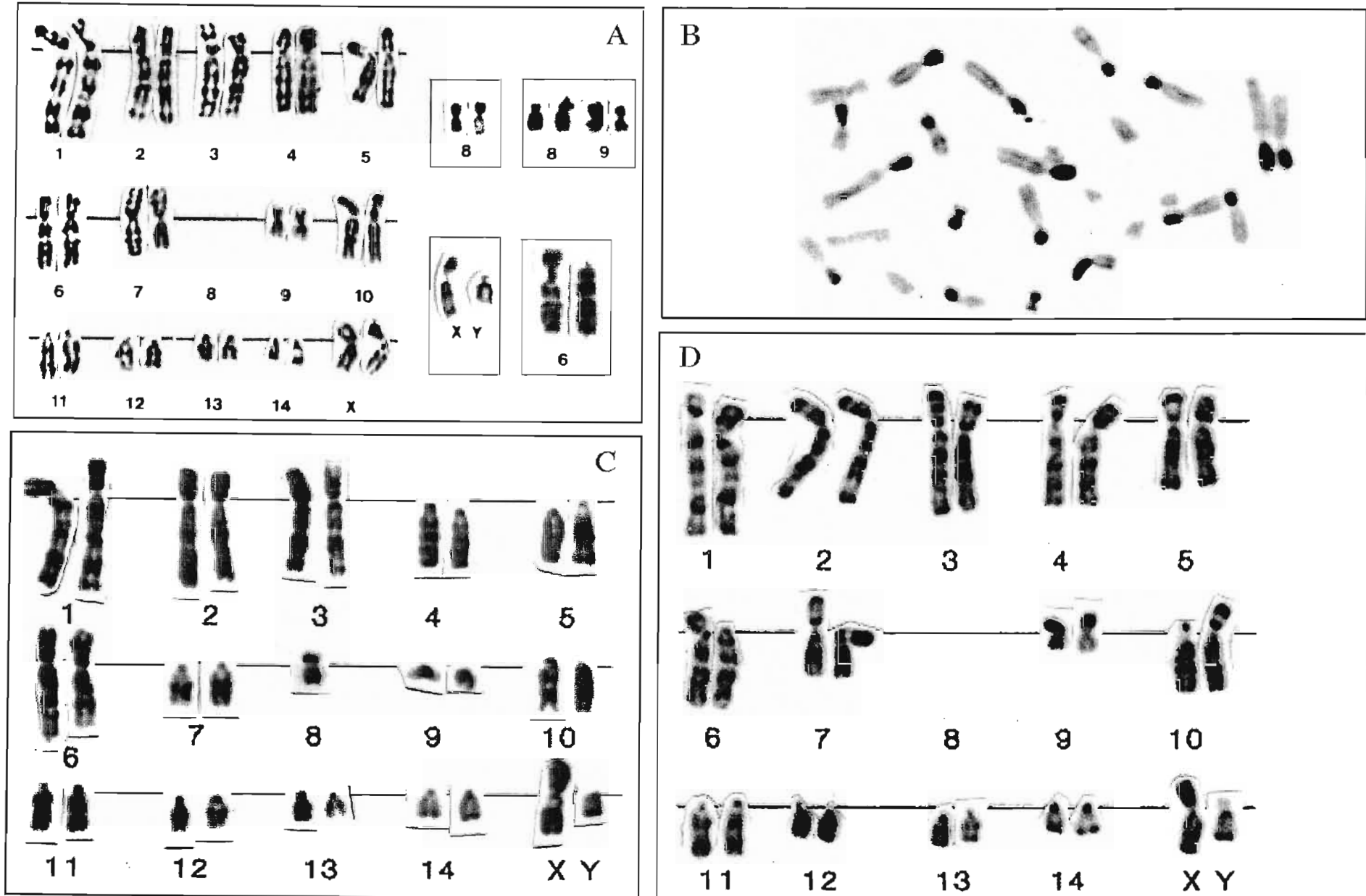


Figure 2.4 - Cytotypes B and C. Frame A: G-banded karyogram from Port Elizabeth (type B). Upper inset: polymorphism of pairs 8 and 9 at Alice. Lower right inset: pericentric inversion of autosome 6 in one Alice individual. Frame B: C-banded metaphase spread from Port Elizabeth. Frame C: G-bands

In all $2n = 31$ specimens there was a single biarmed autosome (number 8) whereas in one of the $2n = 32$ karyotypes, both biarmed elements were identified as members of pair 9. However, in the other two animals with a diploid number of 32, one small biarmed chromosome was classified as a chromosome 9 while the other was similar to the chromosome 8 observed in other cytotypes (see Figure 2.4-A). Three of the $2n=31$ *O. irroratus* specimens originated, in captivity, from the mating of a $2n = 32$ male with a $2n = 30$ female; similarly, one $2n = 30$ individual was an offspring of a $2n = 31$ male and a $2n = 30$ female. Further evidence for the unusually polymorphic nature of these heterochromatic chromosomes was obtained from the three captive-born siblings with $2n = 31$, whose mother had one copy each of chromosomes 8 and 9. These offspring all carried one copy of chromosome 8 and no number 9. Thus preferential segregation of chromosome 8 was apparent in these matings.

A C-banded metaphase spread from an individual with 32 chromosomes is shown in Figure 2.3-B (page 25). This showed very small amounts of centromeric heterochromatin in most large acrocentrics, and interstitial C-bands in three pairs of medium sized chromosomes (a1, a2 and 11). The C-banding pattern of chromosome 8 appeared to be different from that of chromosome 8 in the Tsitsikama cytotype (Robinson and Elder, 1987); namely because its short arm had no heterochromatin but this was, instead, present in the terminal portion of the long arm. The X-chromosome and autosome 9 had heterochromatic short arms while the Y-chromosome was fully heterochromatic and acrocentric. In the Karkloof specimen with 29 chromosomes the small biarmed autosomes were absent while one member of pair 1 had undergone a centric fusion with one member of pair 3. This rearrangement was also found in one specimen from Hogsback (see page 24 and inset in Figure 2.3-D page 25). Therefore, this diploid number can be derived from the 30-chromosome cytotype by centric fusion involving one homologue each from pairs 1 and 3.

A single specimen trapped at the Umgeni Valley Nature Reserve, near Karkloof,

yielded a karyotype in all respects similar to that of Karkloof ($2n = 32$ with pair 8) and a juvenile from the Dargle area showed a $2n = 30$ karyotype similar to that of Karkloof.

• *Cytotype B*

Karyotypes of the Committee's Drift, Port Elizabeth, Alice and Grahamstown specimens bore the closest resemblance to that described by Robinson and Elder (1987) for the Tsitsikama locality. Individuals from Committee's Drift and Port Elizabeth exhibited an identical karyotype with a diploid number of 28 (Figure 2.4 A page 27) comprising seven pairs of submetacentric autosomes (pairs 1-6 and 10), one pair of small metacentrics (9), one pair of medium metacentrics (7) and four pairs of acrocentrics (11-14). The X-chromosome was submetacentric and the Y-chromosome was a small acrocentric. The autosomes assigned by Robinson and Elder to pair 8 were absent in these specimens, which exhibited instead an extra acrocentric pair (14). Figure 2.4-B depicts a C-banded spread from the Port Elizabeth individual, in which most biarmed chromosomes had heterochromatic short arms and lacked C-band positive chromatin in the acrocentric autosomes.

Three karyotypic variants were seen in the Alice *O. irroratus* population (see Table 2.3 page 24 and Fig 2.4 A insets page 27) and these exhibited diploid numbers of 28, 29 and 30. In general, while these karyotypes were similar to those of Port Elizabeth and Committee's Drift, the variation in diploid numbers reflected the presence or absence of members of pairs 8 or 9 or both. The $2n = 30$ animals carried a pair of small submetacentric autosomes not present in the $2n = 28$ specimens (see top right inset in Fig 2.4-A page 27). These submetacentric chromosomes could be considered homologous to pair 8 of the Tsitsikama cytotype only in size and general morphology, as their G-banding often appeared featureless. The two members of the presumed pair 8 in one of the $2n=30$ specimens exhibited different amounts of chromatin in the short arm (see upper right inset in Figure 2.4-A). Pair 7 of this individual was submetacentric, as in the Tsitsikama

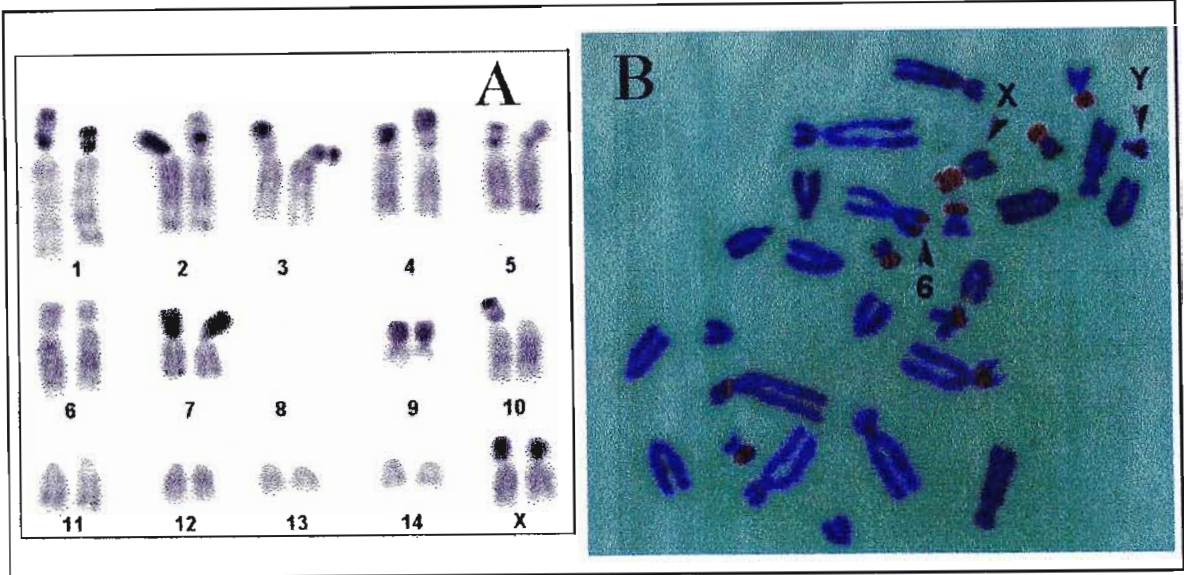


Figure 2.5 - Heterochromatin in *O. irroratus*. A: C-banding-like pattern produced by *Afu I* restriction enzyme on a metaphase from a Grahamstown animal. B: metaphase plate from a Constantia specimen after high pH treatment.

population, in which the short arm of pair 7 was shown to be polymorphic (Robinson and Elder, 1987), rather than fully metacentric as in the other specimens from Alice, Port Elizabeth and Committee's Drift. One specimen with $2n = 29$, displayed three small biarmed chromosomes (not shown in Fig 2.4-A page 27) which banded poorly and were variable in length and arm ratio so that their homology to pairs 8 and 9 could not be established. One individual, with a modal chromosome number of 28, carried a pericentric inversion involving one member of pair 6, similar to that found in an animal from Tsitsikama (Robinson and Elder, 1987) (see lower right inset in Figure 2.4-A page 27). As shown by C-banding, all short arms in these biarmed cytotypes, except those of pair 6, were entirely heterochromatic (Figure 2.4-B page 27).

The Grahamstown population ($2n = 28$) had an essentially similar karyotype to that of the Port Elizabeth and Committee's Drift animals, in which autosome pairs 1-7 and 10 were submetacentric while pairs 11 to 14 were acrocentric. In the specimens studied, both members of pair 9, and neither member of pair 8, were found. However, the Grahamstown karyotype was heterozygous for the inverted chromosome 6 and presence of

chromosomes 6 and 10: three individuals out of six carried one acrocentric member in each of these two pairs (Figure 4-D page 27). Of the remaining three individuals, one was homozygous for the biarmed morph in both pairs, one was homozygous biarmed in pair 10 and one was homozygous acrocentric in pair 6. Treatment with the restriction enzyme *Alu I* produced differentiation in staining intensity within all heterochromatic short arms except those of pair 9 and of the X-chromosome which were totally heterochromatic (Figure 2.5-A page 30) while all euchromatic regions were weakly G-banded. As shown in this illustration, a feature of this technique was its ability to reveal short arm heteromorphism between members of the same pairs. This staining method showed that sizeable portions of most heterochromatic short arms in *O. irroratus* are high repeats of the AG \uparrow CT recognition sequence of this endonuclease.

• *Cytotype C*

O. irroratus from the Rietvlei, Mariepskop, Bloemfontein and Constantia populations exhibited karyotypes intermediate between the biarmed ones of the Eastern Cape and the more easterly all-acrocentric ones, in which only four of the largest autosomes carried heterochromatic short arms.

Two individuals from Bloemfontein had a diploid number of 29. Only pairs 1, 2, 3 and 6 were biarmed, while pairs 7 and 10 to 14 were acrocentric. Only one member of pair 8 was observed in these specimens and a supernumerary pair of small acrocentrics of the same size and with staining properties as the short arm of chromosome 9 was seen (see Figure 2.4-C, pair 9, page 27). One individual from this population had, besides the two uniarmed 9, both members of pair 8 ($2n = 30$).

The specimens from Rietvlei and the one from Mariepskop, had a diploid number of 28. Like at Bloemfontein, only pairs 1, 2, 3 and 6 were biarmed and pair 9 was acrocentric.

The Constantia *O. irroratus* also exhibited a karyotype similar to that of Rietvlei,

with a diploid number of 28. In this population, however, the heterochromatic short arms, in other cytotypes associated with pairs 8 and 9, were seen on pairs 9, 13 and 14. This karyotype, therefore, included three pairs of small biarmed, four pairs of large biarmed and only six pairs of acrocentric autosomes (see Figure 2.6- page 33). One individual from Constantia, was heterozygous for the pericentric inversion of chromosome 6, as in the case of several Grahamstown specimens. Figure 2.5-B (page) shows a metaphase spread, from the Constantia individual heterozygous for the autosome 6 pericentric inversion, treated with a high pH buffer (see page 20). This technique stained the chromosomes light blue while the heterochromatic short arms of pairs 9, 13 and 14 were highlighted by taking up a magenta-red colour. The paracentromeric regions of the short arm of pairs 1 to 3 showed less prominent spots of magenta stained chromatin. A prominent magenta-red band could be observed on the distal portion of the short arm of the biarmed autosome 6 but not in its acrocentric homologue.

A comparison of G-banding patterns between the *O. irroratus* karyotypes described above is given in Figure 2.6 (page 33). Because there was complete homology between some of the karyotypes, only one population per cytotype is presented here. With the exception of pairs 'a1' and 'a2' (present at Karkloof only) and pairs 7 and 12 (absent at Hogsback but replaced by pair 'A'), full banding homology was evident for the chromosomes from the acrocentric cytotypes; these matched the banding patterns of the long arms of their biarmed morphs in the western and northern cytotypes.

The dendrograms generated by cladistic and phenetic analyses are presented in Figure 2.7 (page 34). Parsimony analysis produced five equally parsimonious trees which differed from one another by the branching of the acrocentric cytotypes. This uncertainty was borne out by the consensus tree shown in Figure 2.7B, whose topology showed this node as an unresolved dichotomy.

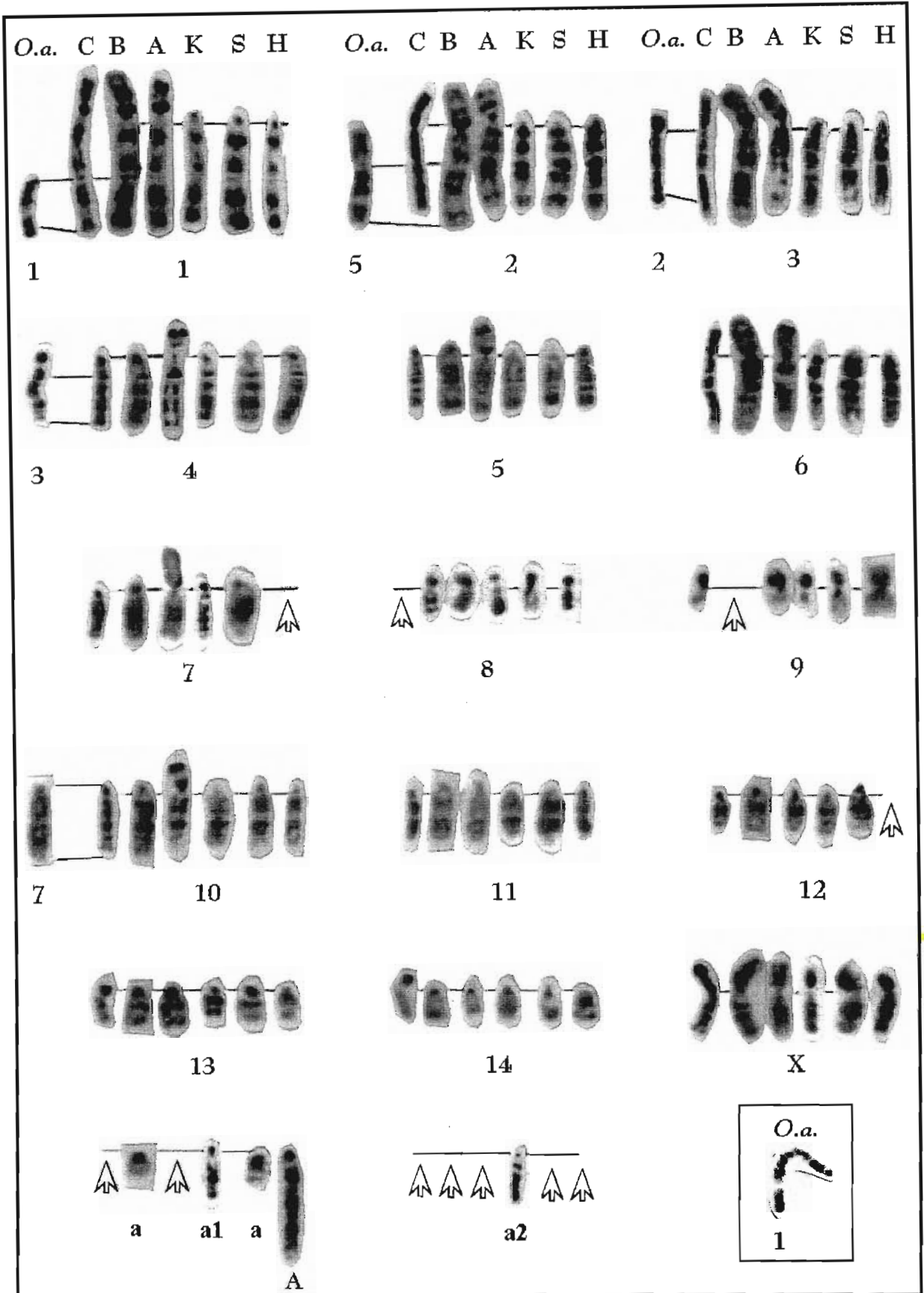


Figure 2.6 - *O. irroratus* chromosomes showing G-band homologies between cytotypes and *O. angoniensis*. Inset: complete *O. angoniensis* autosome 1. Arrows indicate chromosomes absent at that locality. C=Constantia, B=Bloemfontein, A=Alice, K=Karkloof, S=Stutterheim, H=Hogsback.

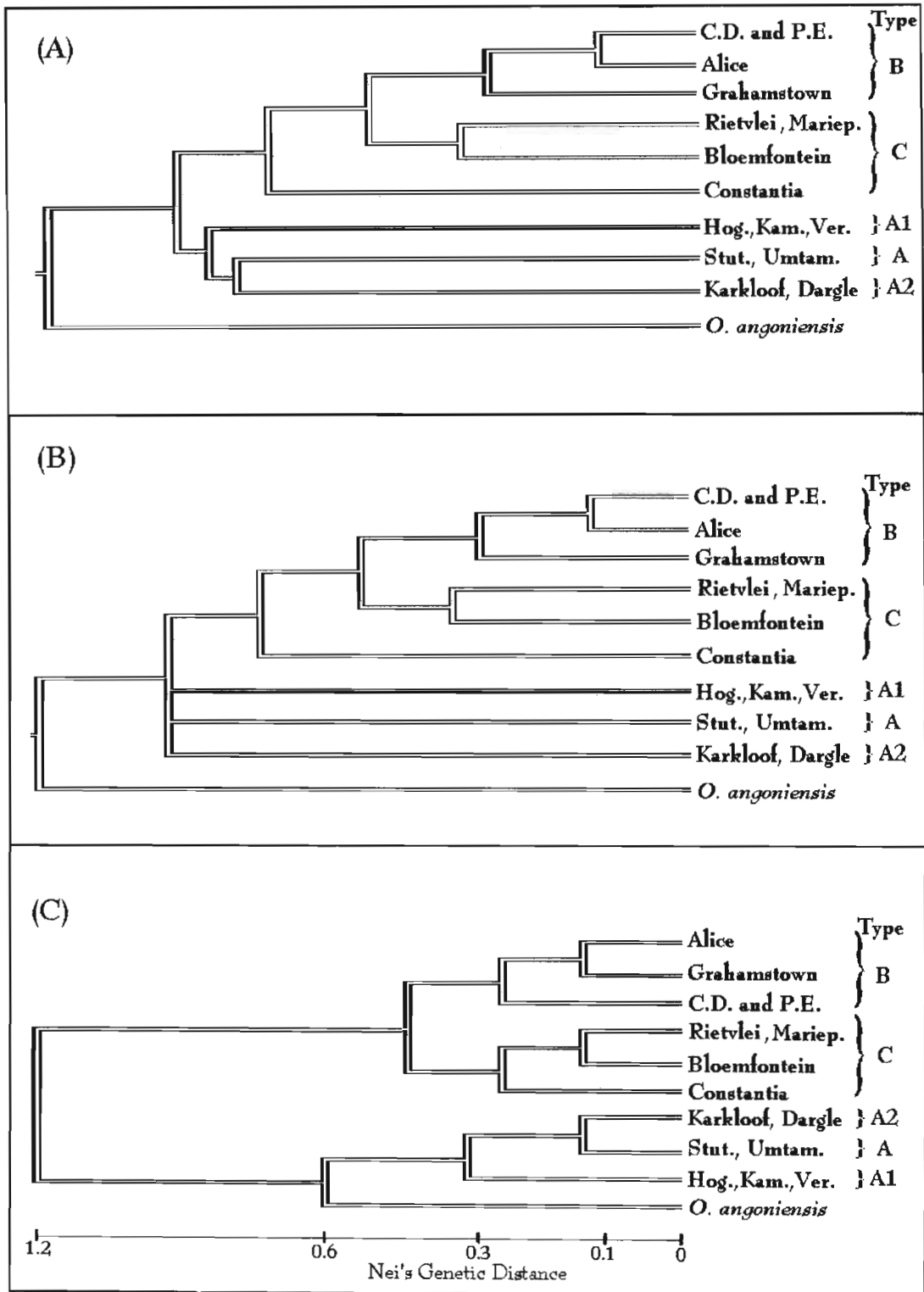


Figure 2.7 - Dendrograms summarizing the relationships between various *O. irroratus* populations and *O. angoniensis*. A: one of the five equally most parsimonious cladograms obtained from the BANDB algorithm included in the PAUP programme of Swofford, consistency index = 0.889. B: consensus tree from the same programme (both ADAMS and STRICT consensus trees were identical), consensus information = 0.680. C: UPGMA phenogram (from BIOSYS), co-phenetic correlation = 0.687.

◆ *Discussion*

The *O. angoniensis* chromosomes tended to be much shorter than those of *O. irroratus*. Notwithstanding the use of synchronized cultures and short colchicine treatment times, I obtained very few metaphase spreads with sufficiently extended chromosomes to allow a detailed comparison of band sequences. It is, thus, possible that future implementation of high-resolution banding methods to material from this species may reveal further homologies. On the basis of the considerable difference in diploid numbers, it may be expected that many *O. irroratus* chromosomes would have derived from those of *O. angoniensis* by whole chromosome fusions. In comparing G-banding homologies, however, no obvious rearrangements which could explain the derivation of the chromosomes of the former species from those of the other were detected. This may imply that these two species diverged from a hypothetical ancestor, maintaining only a few shared primitive characters, while all others may be derived. The choice of *O. angoniensis* as an outgroup appears to be justified not only because these two groups are considered sibling species (Meester, 1988) but also because both allozyme data (Taylor et al., 1989a; Taylor et al., 1989b) and immunoblot analysis (see chapter 5) clearly indicate that, among the Otomyinae, *O. angoniensis* is the closest sister group to *O. irroratus*.

A comparison of G-banding patterns between the published Tsitsikama karyotype (Robinson and Elder, 1987) and the B cytotypes reveals no apparent difference between chromosomal pairs with the exception of chromosome 14, which was absent from Tsitsikama, and possibly chromosome 8 which, in my preparations, appears to be polymorphic (see Figure 2.6, page 33 and upper insets in Figure 2.3-A, page 25). In this cytotype pair 7, although metacentric in the Alice population, has a heterochromatic short arm, but its long arm appears to be homologous to that of Tsitsikama pair 7. Similarly, chromosome 10 is banded with a heterochromatic short arm in the B cytotype, but the bands of its long arm and those of chromosome 10 of the Tsitsikama specimen show complete homology. Furthermore, the banding pattern of the chromosomes in the all-

acrocentric cytotypes matches that of the long arm of their homologues from Alice and Tsitsikama. Exceptions to this are pair 6, which has undergone a pericentric inversion, and pairs 'A' at Hogsback, 'a1' and 'a2' at Karkloof, which are unique to these cytotypes. The lower right inset in Figure 2.4-A (page) shows that the acrocentric autosome 6 is shorter than its biarmed homolog. The banding pattern suggests that the postcentromeric region of the biarmed autosomal short arm has been lost from the acrocentric morph. An analogous region was shown by Robinson and Elder (1987) to be heterochromatic in the Tsitsikama cytotype. Another finding of interest concerns the polymorphism of chromosome pairs 6 and 10 in the Grahamstown population (see Figure 2.4-D, page): the frequency of the pair 6 heterozygosity for the pericentric inversion was considerably higher than that found in the Alice population. Further, the heterozygosity of the acrocentric morph in pair 10 has not been detected in any other population. Although the heterozygote frequencies do not deviate significantly from Hardy-Weinberg equilibrium (chromosome 6: $\chi^2=0.024$, $DF=1$ and $P=0.877$; chromosome 10: $\chi^2=1.071$, $DF=1$ and $P=0.301$), they appear high for a data-set (chromosomes) whose norm is to be homozygous. There is no ready explanation for this result; it does not appear to be due to hybridization with acrocentric populations, as the other biarmed chromosomes did not show this kind of polymorphism. A more detailed discussion of this aspect of *O. irroratus* chromosomes can be found in Chapter 6 (pages 112-117).

The most striking morphological difference between the A, B and C cytotypes lies in the presence or absence of short arms. Such short arms, however, are totally heterochromatic, with the exception of pair 6 (Figure 2.3-B and Robinson and Elder, 1987), although they reveal a different staining pattern when treated with the restriction endonuclease *Alu I* (Figure 2.4-C). This may indicate the presence of an *Alu*-like family of sequences in most heterochromatic blocks of this species.

Similar chromosomal rearrangements (i.e. gain or loss of whole heterochromatic arms) have been previously reported in arvicolid rodents (Modi, 1987) and in the genera

Gerbillus (Volobouev *et al.*, 1988) and *Thomomys* (Patton and Sherwood, 1982). Such rearrangements, therefore, appear to be common in the differentiation of rodent karyotypes, although their significance in terms of speciation and cellular physiology is obscure.

Some authors (Viegas-Pequignot *et al.*, 1986) seem to consider biarmed chromosomes with totally heterochromatic short arms as being acrocentric, presumably because constitutive heterochromatin contains highly repeated non-coding sequences (Hsu, 1973; Vosa, 1973). Others (John and Miklos, 1988), consider this type of chromatin to be devoid of recognizable function. Nevertheless, the available evidence (John and Miklos, 1979; John, 1988) suggests that not all heterochromatin is non-coding and that, in some organisms, the presence of partly or wholly heterochromatic B-chromosomes influences the frequency of interchromosomal recombination events. Therefore, a possible role may exist for constitutive heterochromatin in increasing genetic variability and, consequently, rates of speciation (Fredga and Mandahl, 1973).

Polymorphism of the small biarmed autosomes was common in all populations, as shown in Figure 2.3-A (upper insets) and 2.3-C (pair 8) and provided a possible explanation for the lack of C-banding homology between chromosomes 8 of the Karkloof and Tsitsikama populations.

The presence or absence of the small heterochromatic biarmed chromosomes (pairs 8 and 9), and of the heterochromatic short arms in some of the larger members of a cytotype, is not associated with any overt morphological change in the animals. The origin of pairs 8 and 9 is not clear. If they originated from small acrocentrics by acquisition of heterochromatic short arms, their presence in any one cytotype would be expected to correlate with the absence of an equivalent number of small acrocentric chromosomes. This may be the case at Stutterheim where a single presumed chromosome 9 was consistently found with an unpaired small acrocentric, or at Tsitsikama where the presence of pair 8 was associated with the absence of pair 14. However, such associations were not observed in the other cytotypes. Numerical variation is unlikely to be an artefact of the harvesting method

because chromosomal numbers were consistent in preparations from both cultures and bone marrow, which had been prepared by two different workers. The karyotypes of the Rietvlei and Bloemfontein populations suggest that chromosome 9 may originate from a supernumerary small acrocentric showing no obvious G-bands. The karyotype of the Constantia animals suggests that chromosome 8 may originate from pair 14 as, in this population, autosome 14 carries a heterochromatic short arm. I am, therefore, unable to explain the origin of these two pairs. Indeed, because of their numerical variability, partly heterochromatic appearance and absence of overt phenotypic effect, these small chromosomes (pairs 8 and 9) are reminiscent of B-chromosomes of some plants, grasshoppers, and crickets. Similar supernumerary B-chromosomes have been reported, though uncommonly, in mammals (Voloboujev, 1981; Switonski *et al.*, 1987; see also John and Miklos, 1988).

The short arms of these chromosomes appear to consist of a different type of heterochromatin which is resistant to *Alu-I* digestion and stains differently from the short arms of other chromosomes after incubation in high pH buffers (see page 20). This technique reveals magenta-red staining on the short arm of chromosomes 2 and 6, against a background of uniformly stained blue chromosomes (Figure 2.5-B, page 30). The short arms of these small chromosomes and that of the X-chromosome appear to be composed exclusively of this type of heterochromatin. These magenta-red regions do not correspond to silver staining nucleolar organizing regions (NOR) because, in this species, these have been shown to occur paracentrally on the short arms of autosomes 3 and 7 and distally on the long arm of autosome 3 (Robinson and Elder, 1987). The remote possibility that these positive regions may, nevertheless, represent ribosomal gene clusters cannot be ruled out because silver staining is specific for transcriptionally active NORs (Miller *et al.*, 1976; Croce *et al.*, 1977) and not all NORs, and not always the same ones, are active in different cells (Ferraro and Pranter, 1988). Since lack of transcription can correlate with base methylation (Voet and Voet, 1990), it is possible to speculate that the short arms of these

B-chromosomes could represent methylated (i.e. inactivated) NORs.

A noticeable karyotypic difference between the A1 cytotype and all other populations was the absence of pairs 7 and 12, together with the presence of a pair of large acrocentric chromosomes (Figure 2.3-D, pair 'A', page 25) which was unique to this cytotype. Although comparing the banding patterns of these two chromosomes from other cytotypes with that of pair 'A' does not produce a totally convincing match (Figure 2.3-D lower inset), the most likely interpretation is that chromosome 'A' derives from a tandem fusion of chromosomes 7 and 12. The lack of complete homology in band sequences is due to the presence of a prominent band of undetermined origin, present halfway along the length of chromosome 'A', near the presumed point of fusion. It is possible that further rearrangements (i.e. insertion) may have occurred here subsequent to the fusion. Crossing animals from Hogsback with those from other cytotypes would then produce hybrids which are heterozygous for Pairs 'A', 7 and 12. In turn, this may result in the formation of aneuploid gametes through malsegregation of meiotic multivalents, as described by others (Moritz, 1986) in the genus *Gehyra* and thus cause reduced fertility. Breeding experiments involving captive specimens from Hogsback indicate that while these animals are able to reproduce successfully with individuals from all the other localities studied, back-crosses to either parental stock have no breeding success (Pillay *et al.*, 1992). In interpopulation experiments (involving animals from Hogsback, Kamberg, Karkloof and Committee's Drift), the presence of this translocation correlated with a drastic reduction in breeding success. Unsuccessful breeding occurred in backcrosses between the F1 hybrids involving A1 cytotype animals with parental stock from all three localities (Pillay *et al.*, 1992). As detailed in this chapter, the Karkloof population lacked this tandem fusion and instead carried two unique pairs of medium-sized acrocentrics (see Figure 2.3-C) whose origin could not be determined. Pillay (1991) noted that reproduction of F1 hybrids between Karkloof and Committee's Drift parents was also impaired though to a lesser extent than backcrosses involving Hogsback animals. In the same crosses, a high pre-weaning mortality,

particularly of males, was also recorded (Pillay, 1991).

As shown in Figure 2.3-A (lower inset, page 25), pair 6 of the acrocentric cytotypes displayed complete homology with one member of pair 6 from the Tsitsikama individual which had undergone a pericentric inversion (see Robinson and Elder, 1987). This rearrangement was also detected in one of the specimens from Alice, as shown in Figure 2.4-A (lower right inset, page 27) and was present in 50 per cent of the Grahamstown specimens studied and is, thus, comparatively common in the 'biarmed' cytotypes. Because this pericentric inversion has the potential to cause post-zygotic isolation, through meiotic bivalent loop formation, the finding can be regarded as evidence that this type of chromosomal mutation can reach stable equilibria under sympatric conditions.

A further point which may be relevant to chromosomal evolution is the presence of a centric fusion involving chromosomes 1 and 3 in one individual each from two geographically well separated populations such as Hogsback and Karkloof (see Figures 2.1, page 18 and 2.3-C and D, page 25). Should this be detected in other specimens of *O. irroratus*, it would suggest that some chromosomes of this species are more prone than others to undergo Robertsonian translocations. In turn, this would suggest a propensity for chromosomal orthoselection in accordance to Bickham and Baker's (1979) canalization model of chromosomal speciation.

On the basis of the present study and a previous report (Robinson and Elder, 1987) *O. irroratus* can be divided into three major chromosomal categories. The first group (group A in Figures 2.6 and 2.7 pages 33 and 34) comprises cytotypes with acrocentric chromosomes (Stutterheim, Umtamvuna, Hogsback, Kamberg, Vergelegen, Loteni, Garden Castle, Karkloof, Umgeni Valley and Dargle) while a second type (group B in Figures 2.6 33) includes karyotypes with at least seven pairs of large biarmed autosomes (Alice, Port Elizabeth, Committee's Drift and Grahamstown). The third kind (group C in Figure 2.6 page 33), so far found at Mariepskop, Rietvlei, Bloemfontein and Constantia,

includes cytotypes with four pairs of large biarmed chromosomes. Grouping along these lines, however, does not carry a sufficient amount of phylogenetic information. While groups B and C can interbreed successfully, reproductive biology (Pillay, 1991) and chromosome rearrangements suggest that group A can be divided into three meaningful subgroups. One of these (A1) is represented by the cytotypes from Hogsback, Kamberg, Vergelegen, Loteni and Garden Castle in which the tandem fusion of autosomes 7 and 12 was detected (see inset in Figure 2.3-D) while group A2 (Karkloof, Umgeni Valley and Dargle) do not carry the fusion but have a pair of unique supernumerary autosomes. Moreover, group A - so far including the localities of Stutterheim and Umtamvuna but, probably, extending further north-east into KwaZulu/Natal - can be described as a B cytotype without heterochromatic short arms.

Both cladistic and phenetic analyses of the chromosomal data included in Figure 2.7, reflect the relationships here suggested. It should, however, be stressed that this analysis is based on the assumption that karyotype evolution in the Otomyinae proceeded from high to low diploid numbers and from acrocentric to biarmed karyotypes. Such reduction in chromosome numbers may have occurred by orthoselection via tandem fusions followed by inversions or reciprocal translocations. Robertsonian fusions do not appear to have played a major role: only one, $t(1,3)$ in the heterozygous state, was detected in two of a total of 98 animals studied. A second stage of orthoselection, through acquisition of heterochromatic short arms, would follow with the establishment of group C cytotypes as a first step. Thus, because it carries two extra chromosome pairs, the Karkloof cytotype would be the likely ancestor to all other populations studied with Stutterheim ancestral to Constantia which, in turn, would give rise to groups B and C. This suggested phylogeny corresponds to the branching pattern of the cladogram shown in Figure 2.7-A (page 34), one of the five equally most parsimonious (BANDB algorithm of PAUP) trees used to construct the consensus tree in Figure 2.7-B. In both dendrograms, the acrocentric cytotypes are separated from the biarmed ones, which are further divided into groups with

lower and higher numbers of biarmed autosomes (groups B and C). However, the separation between Hogsback, Karkloof and Stutterheim is not resolved in the consensus tree. Figure 2.7-C (page 34) shows a phenogram based on the unweighted pair group method for averages (UPGMA) cluster analysis of Nei's (1978) genetic distances. This topology is similar to the cladogram (Figure 2.7-A) and consensus tree (Figure 2.7-B) but the node involving group A cytotypes is resolved.

Meester (1988) proposed that speciation can occur through the agency of chromosomal rearrangements which can cause reproductive isolation without the phenotypic changes usually associated with this process. Most of the chromosomal changes detected in *O. irroratus* involve an increase or decrease in the amount of heterochromatic material and are unlikely to cause the disturbances in meiotic pairing and segregation which are consistent with post-zygotic reproductive isolation. However, the correlation between the reduced reproductive success and the cytogenetic findings in the A1 cytotypes is consistent with incipient speciation and supports Meester's hypothesis of chromosomal speciation. This is particularly true in view of the results of allozyme electrophoresis (Taylor *et al.*, 1992) which contrast with the chromosomal picture of this species. These authors point out that the population genetic structure of *O. irroratus* does not agree with that of a Wrightian model consisting of small isolated demes, which would be in accord with a model of stasipatric speciation. Moreover, allozyme analysis reveals high heterozygosity, low F_{IS} values indicative of lack of inbreeding, relatively low F_{ST} values and low genetic distances. On the other hand, if these parameters are calculated using the available chromosomal data ($H=0.015$, $F_{IS}=-0.145$, $F_{ST}=0.935$ and high genetic distances) a fragmented Wrightian population, rather than panmictic, is suggested although the negative F_{IS} value somewhat blurs this picture. It can be deduced, therefore, that chromosome rearrangements in *O. irroratus* proceed more rapidly than detectable gene mutations with the result that they can initiate reproductive isolation, and speciation, before phenotypic divergence has developed.

◆ *Acknowledgements*

The assistance from co-authors of the two papers incorporated into this chapter is hereby acknowledged. Dr. P.J. Taylor, for performing bone marrow preparations on a number of specimens and thus confirming diploid modal numbers of some cytotypes. Dr. K. Willan for the capture and care of most live specimens. Dr. G. Bronner for the capture of the live specimens from Rietvlei. Mr. M.A. Roberts for one Karkloof specimen karyogram used as an illustration in one of the publications. The late J. Meester for reviewing the manuscripts.

◆ *Appendix*

With the exception of the specimens listed by collector's numbers (KW or GCC) voucher specimens were deposited with the Durban Natural Science Museum where they were allocated the following accession numbers.

Alice: 1985, 2026, 2029, 2031, 2945, 2946, 2947, 2948, 2950, 2951, 3032, 3318,
KW960, KW961.

Bloemfontein: 1995, 3073, KW844.

Committee's Drift: KW360, KW494, KW549, KW571, KW709.

Constantia: 3074, 3075, 3076, 3077.

Dargle: GCC1.

Garden Castle Nature Reserve: 3151, 3152, 3153, 3154.

Grahamstown: 1991, 2002, 2010, 2019, 3080, 3085.

Hogsback: 2932, KW302, KW303, KW309, KW335, KW339, KW365, KW366,
KW370.

Kamberg: 3016, 3019, KW467, KW558.

Karkloof: 1993, 1994, 1996, 2007, 2011, 2901, 2902, 2904, 2905, 2907, 3041,
3093, 3095, KW346, KW347, KW348.

Loteni Nature Reserve: 3146, 3148, 3149, 3150.

Mariepskop: KW1136.

Port Elizabeth: 3006, 3007, KW418.

Rietvlei: 1992, 2004, 2006, 3058, 3061, 3062, 3065, 3068, KW791, KW952.

Stutterheim: 2912, 2914, 2918, 2920, 2925, 2926, 2991.

Umgeni Valley Nature Reserve: KW502.

Umtamvuna: KW1134, KW1135.

Vergelegen Nature Reserve: 3032, KW603.

O. angoniensis (Durban): 2877, 2878, 2881, 2961, 3021, KW98, KW476, KW477 (9)
(Nylsvlei): 3078, 3079.

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CHAPTER 3

CLIMATIC CORRELATES OF *O. IRRORATUS* CYTOTYPES

◆ *Introduction*

Many workers have postulated an adaptive role for chromosomal rearrangements in the process of speciation. Bickham and Baker (1979) suggested that an individual's karyotype contributes directly to its fitness in a given environment. According to these authors, non-Robertsonian chromosomal mutations (i.e. inversions, translocations, etc.) provide a possible source of genetic regulatory change which may alter individual fitness. Therefore, selective advantage may overcome the disadvantage of negative heterosis, allowing fixation of a new chromosomal arrangement in reasonably large populations. Baker *et al.*, (1983) demonstrated positive chromosomal heterosis in pocket gophers, and concluded that differential fitness of chromosomal phenotypes may be an important force in chromosomal evolution.

The stasipatric and cascading models of White (1978) and Hall (1973) emphasize the importance of drift acting in small demes, as requisite mechanism for evolution of new chromosomal complements. However, White (1978) proposed an adaptive role for chromosomal mutations in explaining the establishment and spread of new cytotypes, whereby coadapted gene complexes may be protected from introgression from neighbouring populations by means of chromosomal sterility barriers. Concerning the fixation of new chromosomal rearrangements, White (1982) commented that "...it must be supposed that the new homozygous type has a biological advantage".

Nevo (1991) described parallel cases in *Spalax eherenbergi* in Israel and *Spalax leucodon* in Turkey of a positive correlation between diploid numbers and increasing aridity and climatic unpredictability. He suggested that a high diploid number may have been selected for in unpredictable climates due to the higher recombination index (associated with a greater

level of genetic recombination and heterozygosity) provided by the higher $2n$. In Israel, the ranges of four chromosomal species ($2n = 52, 54, 56, 60$) correspond closely with four climatic regions defined on the basis of temperature and humidity, and multivariate analysis demonstrated a close association between chromosomal and climatic variation (Nevo, 1985).

Sharman *et al.* (1990) found a close correspondence between bioclimatic variables and the distribution of karyotypically defined, parapatric species and races of rock wallabies (*Petrogale*) in Australia. Although selection acting on different chromosome races occupying diverse climatic regimes could explain the origin and subsequent spread of new chromosomal forms of *Petrogale*, it could not be established whether different karyotypes contributed to individual fitness or they were simply incidental byproducts of allopatric divergence.

The vlei rat *Otomys irroratus* is widely distributed in mesic habitats in South Africa, Mozambique and Zimbabwe. As detailed in Chapter 2, extensive chromosomal variation has been found in South African populations of this species. Variation resulted mainly from the addition of heterochromatic short arms, the presence or absence of small B-chromosomes, as well as tandem fusion and pericentric inversion events. Cladistic analysis of chromosomal changes (see page 34) recognized three major, allopatrically-distributed, cytotypes defined on the basis of the number of heterochromatic short arms (Figure 3.1; Table 3.1): (A) an all-acrocentric form which can be further subdivided into two subtypes: (A1) a form occurring in the Drakensberg Mountain range in KwaZulu/Natal and Eastern Cape, carrying a tandem fusion of autosomes 7 and 12; and (A2) a further acrocentric cytotype without the tandem fusion but with two extranumerary autosomal pairs, occurring at lower altitudes in the KwaZulu/Natal midlands; (B) a further Eastern Cape form having at least seven pairs of biarmed autosomes; and (C) a form occupying the highveld of the Orange Free State, Northern Province and Western Cape, carrying an intermediate number (four) of large, biarmed autosomes.

This chapter details a multivariate statistical approach to investigate possible

climatic correlates of chromosomal variation in *O. irroratus*. Principal component analysis was used to explore climatic relationships of known-cyotype localities. Climatic separation among the four recognized cytotypes was assessed statistically by means of discriminant analysis.

◆ *Materials and Methods*

Procedures for obtaining karyotypes have been described in Chapter 2 (pages 18-35) together with details of karyotypes in 18 *O. irroratus* populations. A summary of the chromosomal characteristics of each population is shown in Table 3.1.

Table 3.1 - Summary of chromosomal characteristics of 18 *O. irroratus* populations.

| Locality | Geographical coordinates | 2n ¹ | B-chromosomes | NF | Race |
|------------------------|--------------------------|-----------------|---------------|-------|------|
| Stutterheim (9) | 32°34'S 27°26'E | 26 | 4 | 24 | A |
| Umtamvuna (8) | 31°03'S 30°10'E | 26 | 4 | 24 | A |
| Garden Castle (5) | 29°45'S 29°15'E | 24 | 0 to 3 | 22 | A1 |
| Hogsback (1) | 32°33'S 26°57'E | 24 | 0 to 3 | 22 | A1 |
| Kamberg (2) | 29°23'S 29°43'E | 24 | 0 to 3 | 22 | A1 |
| Loteni (3) | 29°27'S 29°32'E | 24 | 0 to 3 | 22 | A1 |
| Vergelegen (4) | 29°32'S 29°27'E | 24 | 0 to 3 | 22 | A1 |
| Dargle | 29°30'S 30°01'E | 30 | 0-- | 28 | A2 |
| Karkloof (6) | 29°21'S 30°13'E | 30 | 0 to 2 | 28 | A2 |
| Umgeni Valley (7) | 29°28'S 30°15'E | 30 | 2 | 28 | A2 |
| Alice (10) | 32°47'S 26°50'E | 26 | 0 to 4 | 40 | B |
| Committee's Drift (11) | 33°05'S 26°46'E | 26 | 2 to 4 | 40 | B |
| Grahamstown (13) | 33°17'S 26°33'E | 26 | 0 to 4 | 36-40 | B |
| Port Elizabeth (12) | 33°55'S 25°40'E | 26 | 2 | 40 | B |
| Bloemfontein (14) | 29°06'S 26°14'E | 26 | 2 to 3 | 32 | C |
| Constantia (15) | 34°00'S 18°30'E | 26 | 4 | 34 | C |
| Marqeposkop | 24°35'S 30°35'E | 26 | 2 to 3 | 32 | C |
| Rietvlei (16) | 25°51'S 28°18'E | 26 | 2 to 3 | 32 | C |

¹ Diploid numbers excluding B-chromosomes. Numbers in brackets refer to localities in Fig. 2.1 (p. 18).

Climatic data recorded by the Weather Bureau station nearest to each locality were taken from the Weather Bureau publication WB40 (1986). Fourteen of the 18 localities had nearest weather stations situated in the same or adjacent quarter degree grid squares (i.e

within approximately 25 km, usually much closer); in the remaining four localities, the nearest weather station was separated by a single grid square (i.e. within approximately 50 km).

Initially, 21 climatic variables including altitude were obtained for each locality. Disregarding redundant variables and those with missing data, this number was reduced to a group of 13 variables:

- 1 - mean annual temperature ($^{\circ}\text{C}$, MAT)
- 2 - mean monthly maximum temperature ($^{\circ}\text{C}$, MONMAXT)
- 3 - mean monthly minimum temperature ($^{\circ}\text{C}$, MONMINT)
- 4 - absolute maximum temperature ($^{\circ}\text{C}$, ABSMAXT)
- 5 - absolute minimum temperature ($^{\circ}\text{C}$, ABSMINT)
- 6 - mean annual precipitation (mm, MAP)
- 7 - minimum annual precipitation (mm, MINANNP)
- 8 - maximum annual precipitation (mm, MAXANNP)
- 9 - maximum precipitation in 24 hours (mm, MAX24H)
- 10 - highest maximum monthly precipitation (mm, MONMAXP)
- 11 - months with potentially zero rainfall (MONZEROP)
- 12 - average days with > 10 mm rainfall (DAYS10P)
- 13 - altitude (m, ALT).

Climatic data for each locality are shown in Table 3.2. Major regions (after Schulze, 1965) are shown in Figure 3.1.

Two different multivariate techniques were used to analyse the relationships between climatic and chromosomal variation. The distribution of the 18 localities with respect to climatic variation was analysed using principal component analysis (PCA) of standardized data for the 13 climate variables. PCA also allowed analysis of correlations between different climatic variables. Discriminant analysis (DA) was used to further analyse the extent of climatic differentiation between the five recognized cytotypes. Discriminant analysis was performed using a reduced set of 7 variables (selected on the basis of the eigenvector matrix from PCA), as analyses based on larger sets of variables failed due

Table 3.2 - Altitudinal and climatic data for 18 populations of *O. irroratus* included in present analysis (see text for explanation of variables abbreviations). Values for ABSMINT were transformed (+13) prior to statistical analysis to avoid negative values.

| Population (weather station) | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|------------------------------------|------|------|------|------|-------|------|------|------|-----|-----|----|------|------|
| Stutterheim (Dohne) | 16.2 | 21.9 | 10.6 | 40.3 | -4.5 | 759 | 537 | 1205 | 122 | 304 | 2 | 23.1 | 899 |
| Umtamvuna (Paddock) | 18.1 | 22.5 | 13.6 | 41.1 | 1.7 | 1224 | 892 | 1985 | 337 | 586 | 3 | 32.1 | 514 |
| Garden Castle (Waterford) | 13.2 | 20.9 | 5.5 | 33.5 | -12.6 | 1093 | 808 | 1500 | 85 | 444 | 3 | 34.1 | 1860 |
| Hogsback (Evelyn Valley) | 13.9 | 18.9 | 8.9 | 40.6 | -6.1 | 1618 | 1257 | 2050 | 203 | 427 | 0 | 48.3 | 1450 |
| Kamberg (Waterford) | 13.2 | 20.9 | 5.5 | 33.5 | -12.6 | 1093 | 808 | 1500 | 85 | 444 | 3 | 34.1 | 1700 |
| Loteni (Waterford) | 13.2 | 20.9 | 5.5 | 33.5 | -12.6 | 1093 | 808 | 1500 | 85 | 444 | 3 | 34.1 | 1500 |
| Vergelegen (Waterford) | 13.2 | 20.9 | 5.5 | 33.5 | -12.6 | 1093 | 808 | 1500 | 85 | 444 | 3 | 34.1 | 1643 |
| Dargle (Cedara) | 16.2 | 22.4 | 9.9 | 37.3 | -7.6 | 861 | 567 | 1462 | 179 | 558 | 3 | 26 | 1076 |
| Karkloof (Nottingham rd.) | 13.7 | 21.1 | 6.2 | 35 | -10.5 | 853 | 516 | 1271 | 84 | 319 | 5 | 27.2 | 1438 |
| Umgeni Valley (Cedara) | 16.2 | 22.6 | 9.9 | 38.3 | -6.1 | 868 | 568 | 1470 | 121 | 385 | 3 | 26.1 | 1076 |
| Alice (Lovedale) | 17.8 | 25.2 | 10.4 | 45.8 | -3.9 | 574 | 227 | 967 | 118 | 211 | 5 | 16.1 | 536 |
| Committee's Drift (Tyefu) | 19.7 | 26.0 | 13.4 | 43 | 0.0 | 401 | 318 | 554 | 87 | 186 | 3 | 11.3 | 119 |
| Grahamstown (Grahamstown Vet.) | 16.4 | 22.9 | 10.0 | 42.3 | -4.0 | 681 | 401 | 998 | 129 | 308 | 4 | 16.6 | 539 |
| Port Elizabeth (H. F. Verwoerd) | 17.5 | 22.2 | 12.7 | 40.7 | -0.5 | 662 | 407 | 1068 | 429 | 468 | 0 | 17.8 | 60 |
| Bloemfontein (Tempe) | 16.2 | 23.8 | 8.6 | 37.9 | -9.2 | 558 | 378 | 999 | 110 | 207 | 6 | 17.9 | 1422 |
| Constantia (Table Mt. House) | 12.8 | 16.1 | 9.4 | 36.4 | -1.7 | 1690 | 1025 | 2205 | 129 | 522 | 2 | 49.3 | 761 |
| Mariepskop (Graskop) | 15.4 | 24.9 | 5.5 | 32.7 | -3.1 | 1480 | 933 | 2144 | 209 | 707 | 4 | 41.3 | 1432 |
| Rietvlei (Rietvlei, Pretoria) | 16.2 | 23.4 | 9.0 | 36.1 | -8.0 | 720 | 424 | 1084 | 126 | 284 | 6 | 23.0 | 1524 |

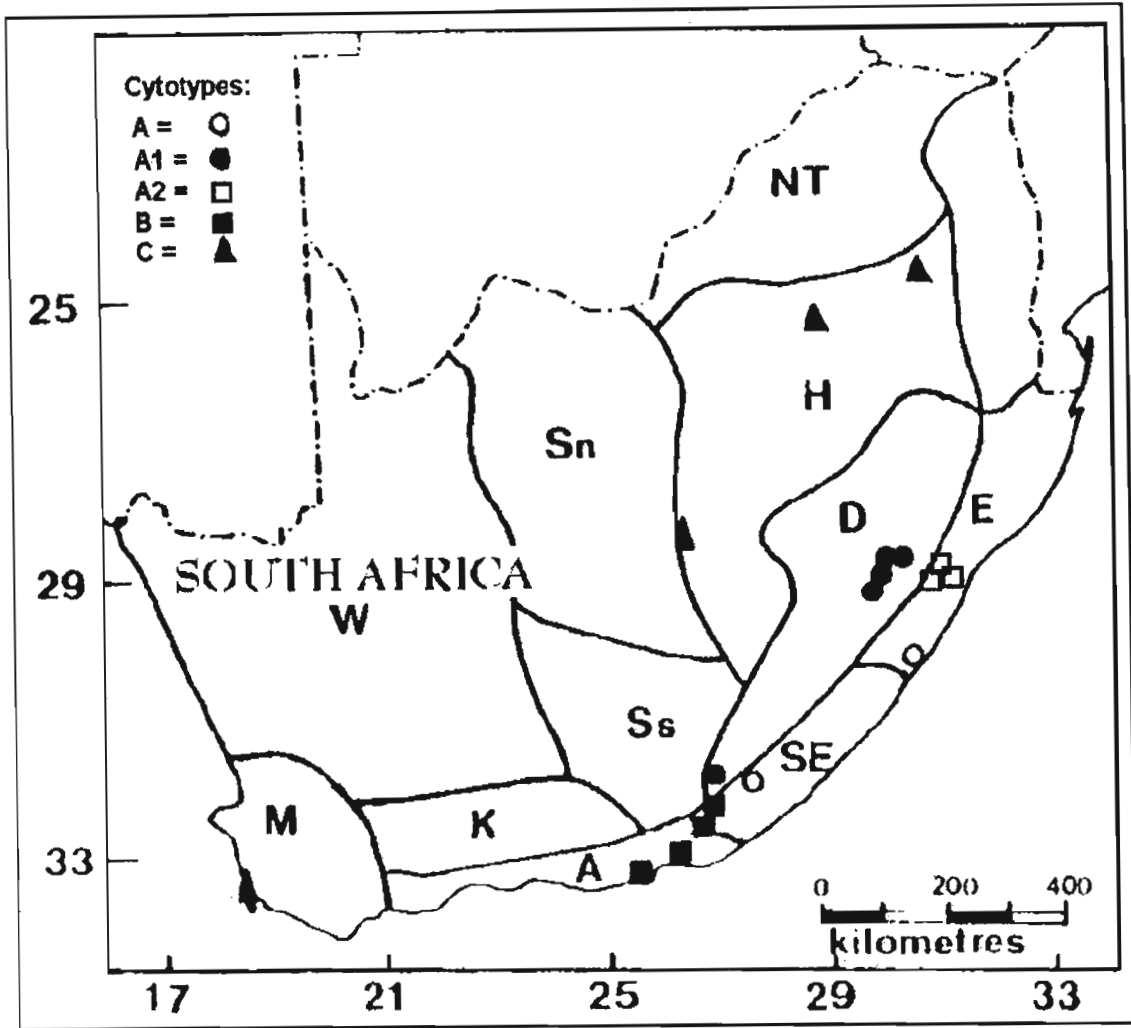


Figure 3.1 - Distribution of the *O. irroratus* cytotypes in South Africa superimposed on Schulze's (1965) climatic regions (indicated by letters).

to unacceptably high levels of inter-variable correlation coefficients. Further discussion of the multivariate procedures used in this study can be found in Blackith and Reyment (1971), Neff and Marcus (1980) and Pimentel and Smith (1986). All analyses were performed using the statistical programme STATGRAPHICS 7.0 PLUS.

◆ Results

Figure 3.1 shows the distribution of the five cytotypes in relation to Schulze's (1965) climatic regions of South Africa. Cytotype A1 occupies the montane

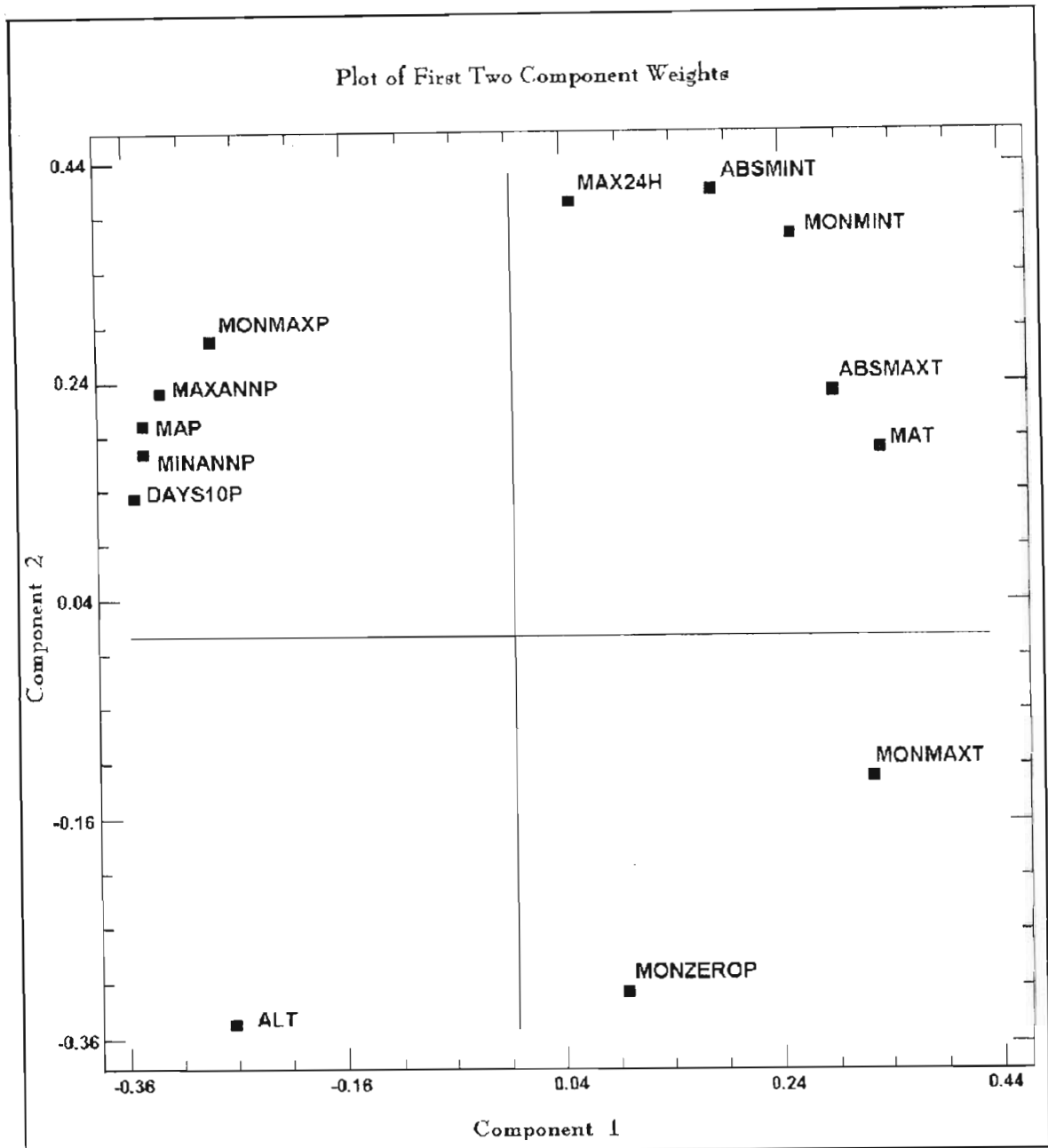


Figure 3.2 - Plot of variable eigenvector loadings on the first two components from a principal component analysis of 13 climate variables for 18 populations of *O. irroratus*. Abbreviations of variables explained in text.

Drakensberg (D) region; cytotypes A and A2 occupy an essentially warm, humid subtropical climate (E and SE regions); cytotype B occupies the cool, temperate afro-montane (A) region and borders with the more semi-arid scrub/grassland (Ss) and Karoo (K) regions; and cytotype C occupies both Mediterranean (fynbos) (M) and highveld

grassland (H) ecosystems. Patterns of correlations between 13 climatic variables are summarized by the plot of eigenvector loadings on the first two principal components from a PCA of all 18 populations (Figure 3.2, page 55).

A strong negative correlation is obtained between all five temperature variables (positive high loadings on the first component) and five measures of precipitation (negative high loadings on first component). Altitude is weakly correlated with precipitation (low negative loading on first component).

Figure 3.3 (page 57) shows a scatterplot of the first two principal components from a PCA of all 18 populations based on 13 climate variables. The first component explains 54.5% of the total variance and the second component 29.5%.

Based on eigenvector loadings of individual variables (Figure 3.2, page 55), the first component is explained by an inverse relationship between rainfall and temperature. Variables with highest loadings on the second component include maximum precipitation in 24 hours, absolute minimum temperature (positive values), altitude and number of months with potentially zero rainfall (negative values). Cytotypes are not grouped together on the PCA scatterplot although the seven metacentric group (cytotype B) occupies a somewhat drier, hotter climate than the other chromosomal races (i.e. distributed on the right of the first component).

Figure 3.4 (page 58) shows a plot of two discriminant functions from DA of five cytotypes (A, A1, A2, B and C). Based on the discriminant functions obtained from seven climatic variables, most individual localities were correctly assigned to their appropriate cytotypes with the exception of those belonging to groups A and C. The two localities from group A are incorrectly assigned to group C 25 % of the times.

Table 3.3 shows the standardized coefficients corresponding to the two discriminant functions shown in Figure 3.4. The first discriminant function contrasts absolute minimum temperature with minimum annual precipitation, while the second contrasts absolute minimum temperature with mean annual precipitation. This clearly reflected in the separation observed in Figure 3.4. Cytotypes A1 and B were well separated from the

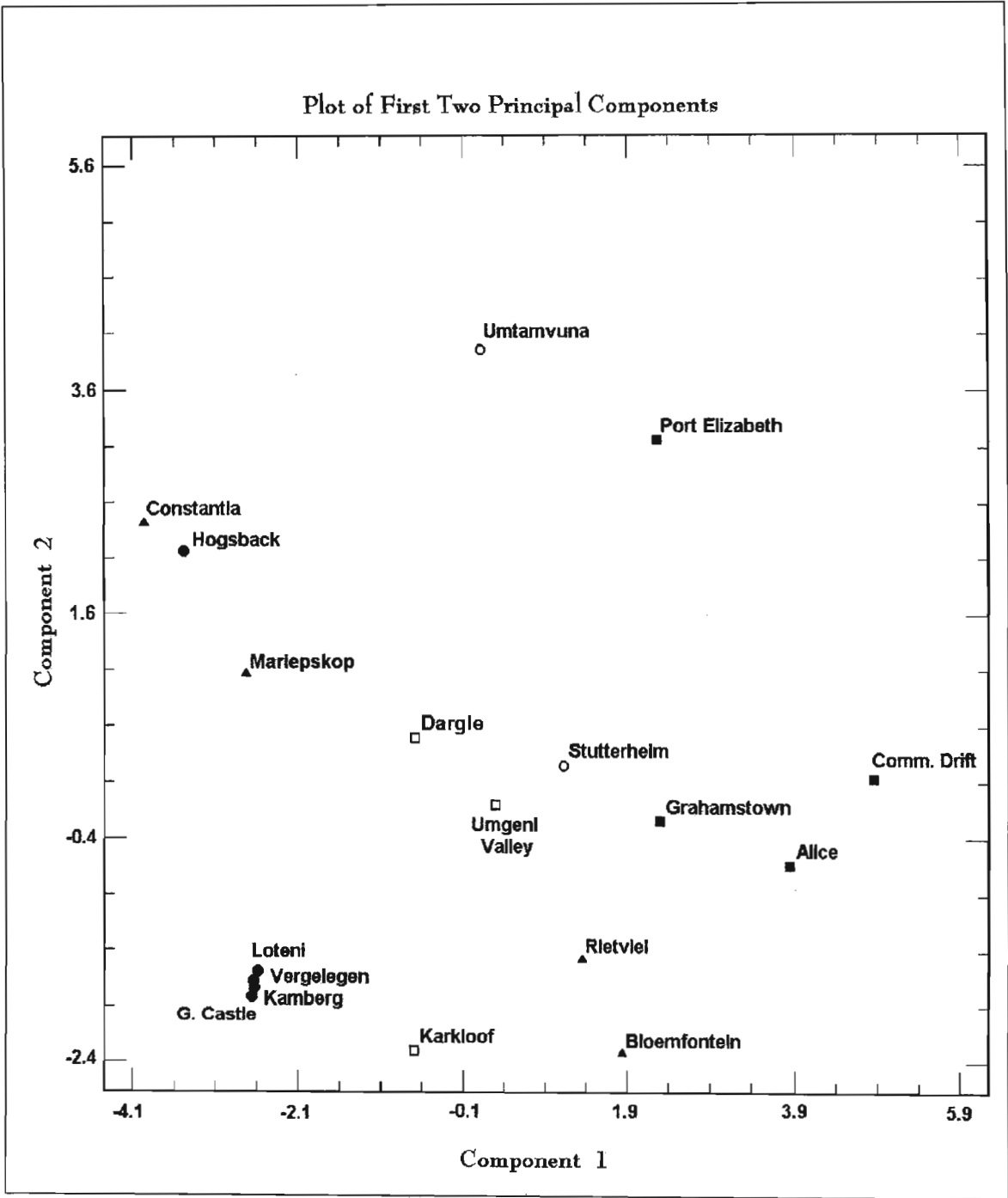


Figure 3.3 - Plot of *O. irroratus* populations on the first two principal components from a principal component analysis of 13 climatic variables. Symbols are as in Figure 3.1.

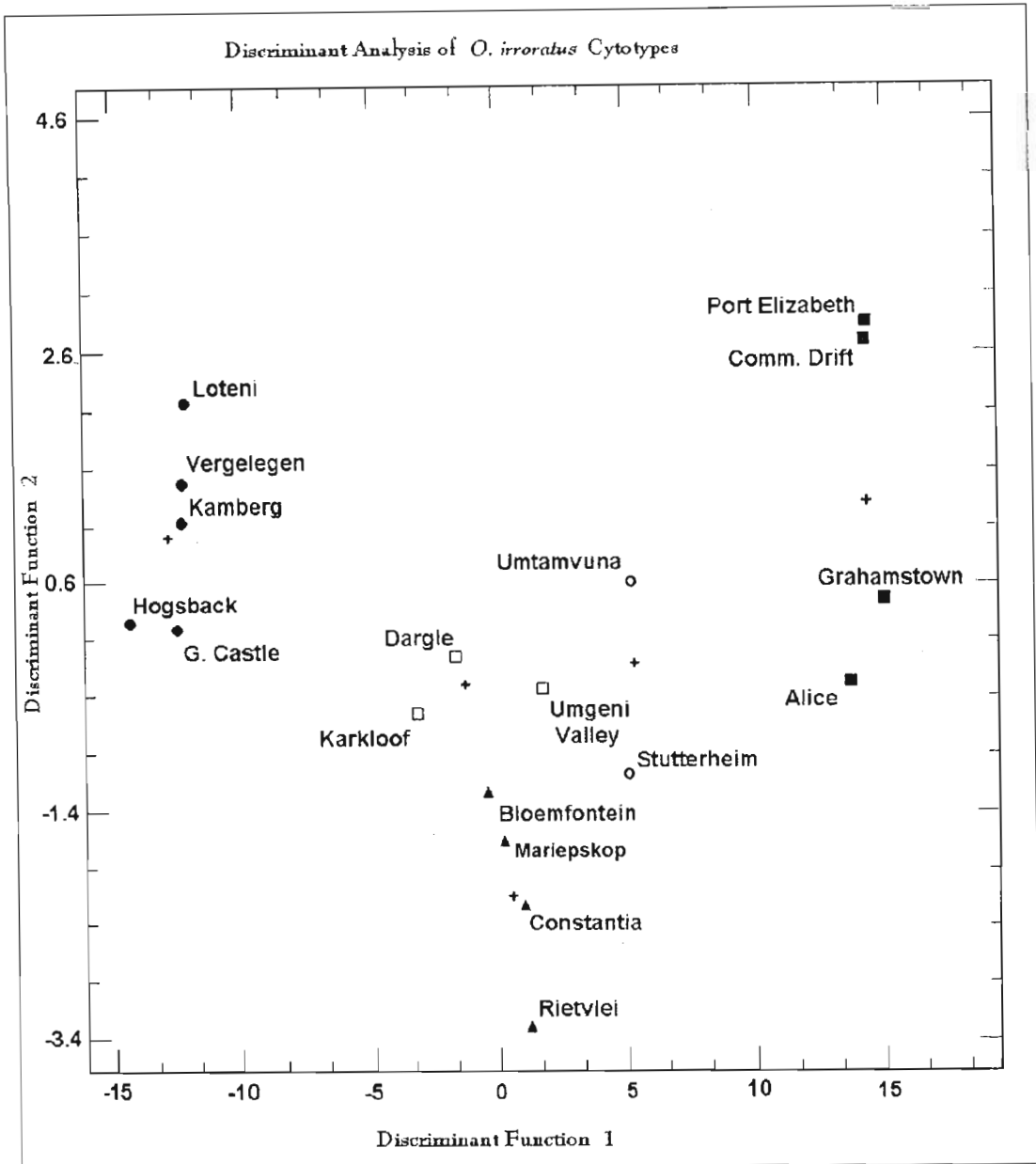


Figure 3.4 - Discriminant analysis of five recognized cytotypes of *O. irroratus*, based on seven climate variables. Symbols are as in Figure 3.1. Crosses represent mean centroids for each cytotype.

other cytotypes on the first axis, with group A1 occupying a climate subject to extremely low temperatures but with a more predictable moisture regime. The seven-metacentric group (B) appeared to occupy the most arid climate (lowest minimum annual precipitation),

subject to the lowest absolute minimum temperatures. The remaining cytotypes (A, A2 and C) were only partially separated on the second axis and seemed to occupy a climate intermediate between those of A1 and B.

Table 3.3 - Results of a five group discriminant analysis of *O. irroratus* cytotypes (A, A1, A2, B and C; abbreviations explained in text).

| Variable (units) | Mean Values (\pm standard deviation) | | | | | Standardised discriminant function coefficients | | |
|--------------------------------|---|--------------|--------------|-------------|--------------|---|-------|-------|
| | A (n=2) | A1 (n=5) | A2 (n=3) | B (n=4) | C (n=4) | 1 | 2 | 3 |
| Mean annual temperature (°C) | 17.1 (1.34) | 13.3 (0.31) | 15.4 (1.44) | 17.8 (1.37) | 15.1 (1.61) | -0.19 | -1.25 | -3.31 |
| Mean annual precipitation (mm) | 991 (328.8) | 1198 (234.8) | 861 (7.5) | 580 (127.8) | 1112 (556.8) | -0.43 | -3.50 | 0.39 |
| Minimum annual precip. (mm) | 714 (251.0) | 898 (200.8) | 550 (29.7) | 338 (84.6) | 690 (336.3) | -5.00 | 1.55 | -0.83 |
| Absolute minimum temp. (°C) | 11.6 (4.38) | 1.7 (2.90) | 4.9 (2.24) | 10.9 (2.15) | 7.5 (3.66) | 6.51 | 4.50 | 2.25 |
| Altitude (m) | 706 (272.2) | 1630 (163.8) | 1196 (209.0) | 320 (266.7) | 1284 (352.2) | 2.29 | 2.71 | 0.25 |
| Months with zero precip. (°C) | 2.5 (0.70) | 2.4 (1.34) | 3.7 (1.15) | 3.0 (2.16) | 4.5 (1.91) | 1.17 | 1.38 | -0.53 |
| Monthly maximum temp.(°C) | 22.2 (0.42) | 20.5 (0.89) | 22.0 (0.81) | 24.1 (1.81) | 22.1 (4.01) | -3.28 | -1.50 | 2.97 |

◆ Discussion

From PCA, most inter-locality variation (54.5 % of the total) can be explained by an inverse relationship between precipitation and temperature (Figures 3.2 and 3.3 pages 55 and 57). However, this combination of variables is clearly not effective in separating cytotypes (Figure 3.3). On the other hand, from DA (which seeks to maximise inter-group relative to intra-group variance), at least three *O. irroratus* cytotypes can be predicted with 100 % accuracy based on a combination of variables which emphasize extreme rather than mean values of temperature and precipitation and which, therefore, seem to be related to climatic unpredictability (Table 3.3 and Figure 3.4, page 58). The first discriminant function in particular can be conveniently labelled as an axis of climatic predictability (high coefficients for extreme values and low coefficients for mean values of temperature and precipitation) with unpredictability increasing from left (e.g. constant, high rainfall

Drakensberg localities) to right (e.g. semi-arid Karoo-type) which is typified by Committee's Drift, where there is a strong correlation between decreasing precipitation and decreasing reliability of precipitation (Werger, 1978).

The A1 cytotype which comprises all the populations carrying the 7-12 fusion, and which is associated with impaired hybrid fertility when mated with A2 and B cytotypes (Pillay *et al.*, 1992) (A1-C and A1-A cytotype pairings have not been attempted but are also likely to show impaired hybrid fertility), is associated with a predictable moisture regime (minimum annual precipitation of 808 mm for Drakensberg localities) and a temperature regime subject to cold fluctuations as low as -12 °C (Table 3.2, page 53; Figures 3.1 and 3.4, pages 54 and 58). From the evidence at hand it is unclear whether the fixation of the tandem fusion occurred in allopatry as a result of stochastic factors acting in a hypothetical small ancestral A1 population, or whether natural selection has favoured the spread of this chromosomal mutation throughout the montane Drakensberg region due to new or different regulatory gene functions induced by the rearrangement (i.e. position effect; see Bickham and Baker, 1979).

The significance of observed heterochromatic changes in *O. irroratus* cytotypes is even more obscure. Heterochromatin has been suggested to heighten karyotypic variability and rates of speciation (Fredga and Mandahl, 1973). There is experimental evidence suggesting a correlation between the presence of B-chromosomes and/or heterochromatic knobs and increased frequency of recombination events at meiosis in maize (Chang and Kikudome, 1974; Nel, 1973; Rhoades, 1978). Whichman *et al.* (1991) have presented evidence suggesting that the position rather than quantity of heterochromatin may be important; interstitially located heterochromatin appeared to enhance karyotypic variability by providing "safe" break points for chromosomal rearrangements without the risk of damage to euchromatic portions of the genome.

Nevo (1991) suggested that a higher recombination index in *Spalax ehrenbergi* and *S. leukodon* (associated with higher diploid numbers) may have been selected for in unpredictable (e.g. arid) environments. This follows from the niche-width variation

hypothesis that increased genetic variation may be an advantage in heterogeneous environments (see Nevo, 1978 for a discussion of this hypothesis).

Since (a) the presence and/or position of heterochromatin may increase karyotypic and genetic heterogeneity (Chang and Kikudome, 1974; Nel, 1973; Rhoades, 1978; Whichman *et al.*, 1991), and (b) genetic heterogeneity may be an adaptation to heterogeneous climates, according to the niche-width variation hypothesis, it could be postulated that increased heterochromatin in the short arms of the B cytotype of *O. irroratus* may be an adaptation to the relatively arid and unpredictable climate experienced by this race. The acrocentric cytotypes, having the least amount of heterochromatin occupy the most predictable climates such as Drakensberg (D) and subtropical-humid (E and SE in Figure 3.1). However, due to lack of conclusive data, it is equally plausible that cytotypes having different numbers of heterochromatic short arms became fixed separately in different founder populations due to stochastic factors. Adaptation to different climatic regimes could have then followed the fixation of chromosomal differences, in which case heterochromatic chromosomal changes need have no adaptive value.

The adaptive hypothesis outlined above predicts increased genetic heterozygosity in populations with karyotypes containing greater amounts of heterochromatin. Thus it would predict a decrease in heterozygosity from the B cytotype to C cytotype to A cytotypes. Based on available allozyme data (Taylor *et al.*, 1992), average heterozygosity (H) of cytotypes decreases from A1 (H=0,085) to B (H=0.076) to A2 (H=0,062) to C (H=0.030). Allozyme data, therefore, argue against the adaptive hypothesis. Clearly, further studies are necessary to test the possible adaptive nature of chromosomal differences between the five cytotypes: the apparent correlation between *O. irroratus* chromosomal types and climatic variation cannot be assumed to imply causality.

Geographical isolation has clearly played a role in the distribution of vlei rat cytotypes, as witness the sequential arrangements of the A (including A1 and A2), B and C groups in a ring surrounding the high Drakensberg massif. In this respect, *O. irroratus* seems to behave as a "ring species". The distribution of the A's, B and C cytotypes

corresponds closely with the "KwaZulu/Natal-Lowveld", "Eastern Cape" and "Karoo" genetic races of another ring species, the widely distributed tree *Acacia karoo* (Brain, 1989). However, as *O. irroratus* is distributed widely throughout the High Mountains (> 3000 m) of Lesotho (Lynch, 1994), there is potential for gene flow across the Drakensberg Massif. Further work is needed to determine the extent of the cytotypes distribution, and to establish whether geographic or ecological barriers have played a role in isolating the A1 race from the neighbouring cytotypes.

Finally, there is also an apparent correlation between body size and climatic variables in *O. irroratus*, with larger body size being associated with lower temperatures at higher altitudes, in accordance with Bergmann's Rule (Hoffman, 1990). There is also an apparent relationship between body size and karyotype with acrocentric populations having a greater body and skull size than biarmed populations (Hoffman, 1990). Further research is needed to establish causality of demonstrated correlations between karyotype, climate and body size in this species.

◆ *Acknowledgements*

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CHAPTER 4

INTERSPECIFIC IMMUNOREACTIVITY

◆ *Introduction*

Recent taxonomic interpretations (Meester *et al.*, 1986; Skinner and Smithers, 1990) recognize, in the Southern African subregion, the following Otomyine species: *O. angoniensis*, *O. irroratus*, *O. laminatus*, *O. saundersiae*, *O. sloggetti*, *O. unisulcatus*, *Parotomys brantsii* and *P. littledalei*. The number of laminations of the molars together with body size, bulla size, shape of the posterior petrotympanic foramen and nasal width distinguish between species. While most *Otomys* species are phenotypically similar, some (*O. unisulcatus* and *O. sloggetti*) share habitat, skull, dental and genetic affinities with the two *Parotomys* species (Taylor *et al.*, 1989a and 1989b; Van Dyk *et al.*, 1991) giving rise to uncertainty regarding their correct taxonomic status (Taylor *et al.*, 1989a). These authors suggested that genetic distances between species did not support the recognition of the two genera *Otomys* and *Parotomys* but, if two genera were to be retained, *O. unisulcatus* should be grouped with the *Parotomys* species. The biochemical data further indicated the existence of two evolutionary lineages of Otomyines, one essentially arid-adapted and the other mesic-adapted. The existence of these lineages was also supported on ecological, morphological and paleontological grounds.

At least one species, *Otomys irroratus*, has been shown to be chromosomally variable. Three groups of karyotypes differing in the number of chromosomes carrying heterochromatic short arms have been documented, as well as a fully acrocentric cytotype bearing a tandem fusion (see Chapter 2). However, Taylor *et al.* (1992) established by electrophoresis that liver iso-enzymes from this species do not show a high degree of polymorphism and that the phylogeny inferred from cytogenetic data (see Chapter 2, Figure 2.7 page 34) did not match those obtained from allozyme data.

A further taxonomic uncertainty concerns the inclusion of the Otomyines in either the family Cricetidae as suggested by Misonne (1974) and Lavocat and Parent (1985), or the family Muridae (Meester *et al.*, 1986), or Nesomyidae (Lavocat, 1978). It has also been suggested that the sub-family Otomyinae should be elevated to family status (Roberts, 1951).

It appears, therefore, that additional studies of genetic diversity within this rodent group are necessary to substantiate phylogenetic relationships at intra-specific as well as specific, generic and family levels. Because current measures of genetic diversity best resolve phylogenetic relationships at different taxonomic levels (see Murphy *et al.*, 1990; Werman *et al.*, 1990; Dowling *et al.*, 1990) I have developed a method capable of detecting genetic polymorphism at all levels of taxonomic hierarchy to confirm the significance of genetic changes in allozyme and chromosome patterns. This method involves separation of liver homogenates on Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) followed by 'Western Blotting' (Towbin *et al.*, 1979) and detection using a specific antiserum. Similar methods have been used to characterize antigenic molecules of different parasite sub-species (Pratt *et al.*, 1984; Boustouller *et al.*, 1986) but, to my knowledge, not in phylogenetic studies of mammals. This approach combines the high resolving power of SDS-PAGE exploited by Qumsiyeh *et al.* (1990) with the high sensitivity of immunological reactions, as it allows the identification of electrophoretically separated antigenic proteins by Enzyme Linked Immuno-sorbant Assay (ELISA) (see Tsang *et al.*, 1983). The results of a preliminary study undertaken to assess the potential of this technique are presented in this chapter.

◆ *Materials and Methods*

Two specimens each of the Otomyine species *O. irroratus*, *O. angoniensis*, *O. unisulcatus*, *O. sloggetti* and one specimen each of *O. laminatus*, *Parotomys brantsii* and *P. littledalei* were studied. At inter-family level, I examined two samples each of *O. irroratus*, representative of the cricetines (*Mesocricetus auratus*), a representative of the gerbillines

(*Tatera leucogaster*), the Murine genus *Mastomys*, and one specimen each of the following African Murines: *Rhabdomys pumilio*, *Grammomys dolichurus* and *Dasymys incomtus*. Samples of liver stored in liquid nitrogen in the Biology Department of the University of Natal (Durban) were used. Voucher specimens have been deposited with the Durban Natural Science Museum, accession numbers are included in the appendix.

- *Preparation of antiserum*

Livers from three specimens of *O. irroratus* from Karkloof were homogenized in phosphate buffered saline (PBS, pH 6.8) with an Ultra Turrax (Janke and Kunkel, Germany) homogenizer. Cellular debris was discarded by spinning twice for 30 min at 3000 rpm in a GPR centrifuge (Beckman, USA). Total protein concentration in the supernatant was determined by the Biuret method.

Polyvalent rabbit antiserum against *O. irroratus* liver proteins was developed by the staff of the Biomedical Resource Centre of the University of Durban-Westville. Immunization was by intravenous injection of 50 mg total protein in 250 μ l sterile PBS and 250 μ l Freund's incomplete adjuvant (Difco, USA), followed by two booster injections (50 mg protein in 500 μ l PBS) three and four weeks later. Samples of rabbit serum (200-500 μ l each), obtained before each immune challenge were tested for antibody activity against *O. irroratus* liver homogenate by 'dot-blot' ELISA (Hawkes, 1983) using a rabbit antibody detection kit (Amersham, UK). Immunization was discontinued after the second boost when the antibody titer was approximately 3000 antibody units.

- *Sample preparation*

Liver samples of 50-100 mg each were washed twice in cold PBS and homogenized in 100 μ l each of PBS using a Teflon micro-pestle and mortar (200 μ l volume) attached to a Janke and Kunkle homogenizer head. Cellular debris was removed by centrifugation for 30 min at 11 000 rpm in a Sepatech Biofuge B (Heraeus, Switzerland). This was followed, after 1:10 dilution in PBS, by filtration through 0.22 μ m pore size membrane

filters (Dynaguard, UK). Protein concentration was estimated by comparison of spectrophotometric readings at 280 nm with a liver homogenate of known protein concentration.

- *Electrophoresis*

Each sample was diluted in Tris-HCl buffer (pH 6.8) containing 43 % (v/v) glycerol and 4 % (w/v) SDS and loaded onto 12.5 % SDS-Polyacrylamide (BDH Chemicals, UK) gels to a total protein content of 50-60 μg per lane. A negative control consisting of 30 μg each of azocasein and ovalbumin was also included.

Electrophoresis was performed, on duplicate gels, in a Protean dual vertical gel apparatus (Bio-Rad, USA) at a constant voltage of 150 V for 5 to 6 hours. One of the gels was stained with Brilliant Blue G-250 (BDH Chemicals, UK), the other was used for immuno-electro-transfer as described below.

- *Electro-transfer and detection*

The second gel was soaked for three to five minutes in transfer buffer (25 mM Tris, 193 mM glycine and 20 % v/v methanol) then blotted onto a nitrocellulose membrane (Hybond-C, Amersham, UK) using a TE70 SemiPhor horizontal transfer apparatus (Hoefer Scientific Instruments, USA) at 118 mA for 90 min.

Detection of immunogenic liver proteins, using a rabbit antibody detection kit (Amersham, UK) containing a biotinylated second antibody and streptavidin conjugated alkaline phosphatase, was performed according to the manufacturer's instructions.

- *Analysis of the immuno-blots*

Bands which, on the nitrocellulose sheets, showed positive reaction to the anti-*O. irroratus* antiserum were scored so as to generate a multistate character matrix. Therefore, each band showing strong reactivity to the antiserum was assigned a character state of zero, while progressively higher state numbers were given to electromorphs with

weaker or no reactivity. Table 4.1 summarizes the criteria used in character state assignment.

Table 4.1 - Assignment of character states to electromorphs according to reactivity to anti-*O. irroratus* antiserum.

| Migration Change | Antibody Reactivity | Character State |
|-----------------------------|---------------------|-----------------|
| No | Strong | 0 |
| No | Weak | 1 |
| No | Absent | 2 |
| Yes | Strong | 3 |
| Yes | Weak | 4 |
| Yes | Absent | not found |
| Greater than states 3 and 4 | Strong | 5 |
| Greater than states 3 and 4 | Weak | not found |
| Greater than states 3 and 4 | Absent | not found |

Two matrices were constructed, for the Otomyines and the non-Otomyine rodents respectively, and analyzed cladistically using the implicit enumeration algorithm of the computer programme Hennig86 (Farris, 1988). Characters of equivocal interpretation were inactivated before analysis. Bootstrap confidence intervals of the resulting phylogenies, were estimated with the programme BOOT in the PHYLIP computer package (Felsenstein, 1989). The two matrices were then combined (see Table 4.2) to determine the relationships among all taxa. Nei's (1978) unbiased genetic distances were also estimated using the programme BIOSYS (Swofford and Selander 1989), coding each character as a locus and each state of such a character as a different allele.

Table 4.2 - Multi-state character matrix of all species. (Characters 4-8,11,12,14,19,20 were inactivated due to equivocal interpretation).

| | Character | | | | | | | | | | | | | | | | | | | | |
|----------------------|-----------|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
| <i>O.irroratus</i> | ? | ? | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| <i>O.angoniensis</i> | ? | ? | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 2 | 0 | 1 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 5 | 5 |
| <i>P.brantsii</i> | ? | ? | 1 | 0 | 1 | 0 | 1 | 0 | 2 | 2 | 2 | 2 | 0 | 1 | 0 | 0 | 0 | 2 | 3 | 5 | 5 |
| <i>O.sloggetti</i> | ? | ? | 0 | 0 | 1 | 0 | 1 | 0 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 2 | 3 | 2 | 2 |
| <i>O.unisulcatus</i> | ? | ? | 1 | 0 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 2 | 2 | 1 | 2 | 0 | 0 | 4 | 1 | 2 | 2 |
| <i>O.laminatus</i> | ? | ? | 0 | 0 | 2 | 1 | 1 | 0 | 0 | 1 | 0 | 2 | 2 | 0 | 2 | 0 | 0 | 1 | 0 | 2 | 2 |
| <i>P.littledalei</i> | ? | ? | 2 | 0 | 2 | 0 | 1 | 1 | 2 | 0 | 0 | 2 | 2 | 0 | 2 | 0 | 0 | 2 | 1 | 2 | 2 |
| <i>R.pumilio</i> | 2 | 1 | 0 | 0 | ? | 2 | 1 | ? | 3 | 0 | ? | ? | 1 | ? | 2 | 2 | 4 | 0 | 1 | ? | ? |
| <i>G.dolichurus</i> | 4 | 2 | ? | 0 | ? | 4 | 3 | ? | 2 | ? | ? | 2 | 1 | ? | 2 | 2 | 3 | 0 | 2 | ? | ? |
| <i>Mastomys sp.</i> | 1 | 1 | 0 | 0 | ? | 4 | 2 | ? | 2 | 2 | ? | ? | 2 | ? | 2 | 2 | 2 | 2 | 2 | ? | ? |
| <i>D.incomtus</i> | 0 | 3 | ? | 0 | ? | 1 | 0 | ? | 3 | ? | ? | 0 | 1 | ? | 3 | 2 | 2 | 2 | 2 | ? | ? |
| <i>M.auratus</i> | 4 | 4 | ? | 1 | ? | 2 | 1 | ? | 0 | ? | ? | 2 | 2 | ? | 4 | 1 | 3 | 2 | 3 | ? | ? |
| <i>T.leucogaster</i> | 2 | 4 | ? | 1 | ? | 2 | 0 | ? | 4 | ? | ? | 4 | 3 | ? | 2 | 2 | 2 | 2 | 4 | ? | ? |

◆ Results

Prominent variability in band migration and reactivity to antibody was observed in the blot of the Otomyines as shown in Figure 4.2 (page 73); the most striking variation was in the 14-16 KD region (possibly analogous to the α and β chains of human haemoglobins). Here the *O. sloggetti* and *P. brantsii* lanes showed strong reactivity as well as faster migration compared with the *O. irroratus* samples; *O. angoniensis* displayed weaker reactivity to antibody and the *O. unisulcatus* specimens exhibited very low reactivity and slow migration. The two fastest bands corresponding to MW of 10-12.8 KD were not consistently detected in all blots. These bands were, therefore, excluded from the parsimony analysis.

Further variations were observed in the 55-97 KD region. At the migration distance of glutamate dehydrogenase (see Figure 4.1, page 73), for example, antibody avidity was high against *O. irroratus*, *O. angoniensis*, *O. sloggetti* and *P. brantsii* samples but

very low against the other species of Otomyines. The opposite (weak reactivity in *O. irroratus*, *O. angoniensis* and *P. brantsii*) was the case with two heavier proteins of about 60 KD. Comparison with the duplicate gel indicated that these proteins were present at low concentrations in *O. irroratus*, *O. angoniensis* and *P. brantsii* (i.e. representing differences in gene expression).

Similarly striking differences were observed in the blot showing the supra-generic relationships which included various Murines as well as one representative each of Gerbillinae and Cricetinae (see Figure 4.3, page 74). A pertinent aspect of the phylogenetic analysis was the need to determine the most appropriate outgroup for the Otomyines. To this end, a phenetic analysis of the available non-Otomyine rodents and of *O. irroratus* samples was undertaken. As shown in Table 4.3 (page 74), the lowest estimate of Nei's unbiased genetic distance was that between *R. pumilio* and *O. irroratus*. Therefore, I took this to indicate that the genetically closest rodent to *O. irroratus* was *R. pumilio* and used this species as the outgroup to root the Otomyine phylogenetic tree(s).

Figure 4.4 depicts the most parsimonious tree of the non-Otomyine rodents whereas the shortest tree representing the Otomyine phylogeny is shown in Figure 4.5 (page 75). The topology of the tree in Figure 4.4 indicates an early separation of *R. pumilio* and *O. irroratus* from the other rodents while a close relationship between *Otomys* and the Cricetine is not indicated. The Otomyine cladogram shows two evolutionary lineages: a group including species occupying mesic habitats, such as *O. irroratus*, *O. angoniensis* and *O. laminatus*, and a second one including the arid-adapted species. Figure 4.6 (page 76) presents the Nelson consensus tree from the two shortest cladograms found including all the taxa used in this study. The relationships implied in Figures 4.4 (page 75) and 4.5 are broadly confirmed here.

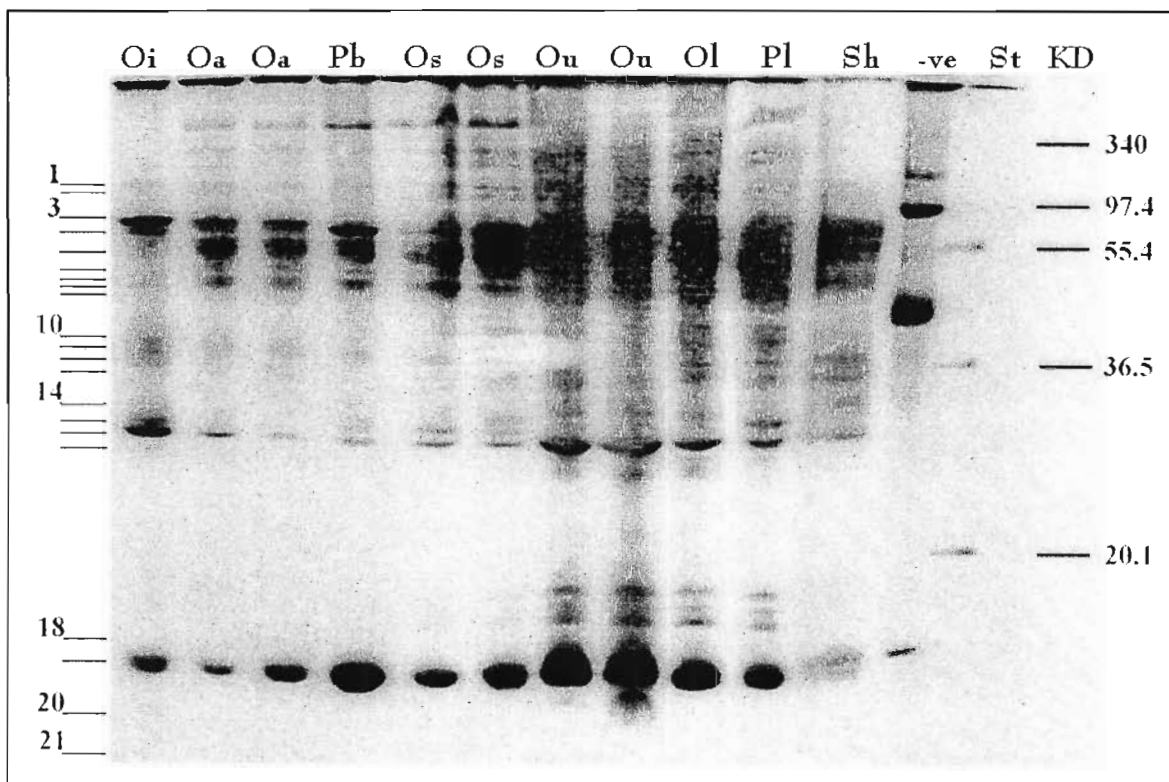


Figure 4.1 - SDS-gel of Otomyines samples. Oi=*O. irroratus*, Oa=*O. angoniensis*, Pb=*P.brandtsii*, Os=*O. sloggetti*, Ou=*O. unisulcatus*, Ol=*O. laminatus*, Pl=*P. littledalei*, Sh=ovine liver homogenate, St=calibration proteins. Combitech calibration proteins: 340 KD= α_2 -macroglobulin, 170 KD= α_2 -macroglobulin (reduced), 97.4 KD=phosphorylase b; 55.4 KD=glutamate dehydrogenase, 36.5 KD=lactate dehydrogenase, 20.1=trypsin inhibitor. Numbered lines to the left of the illustration represent location of characters.

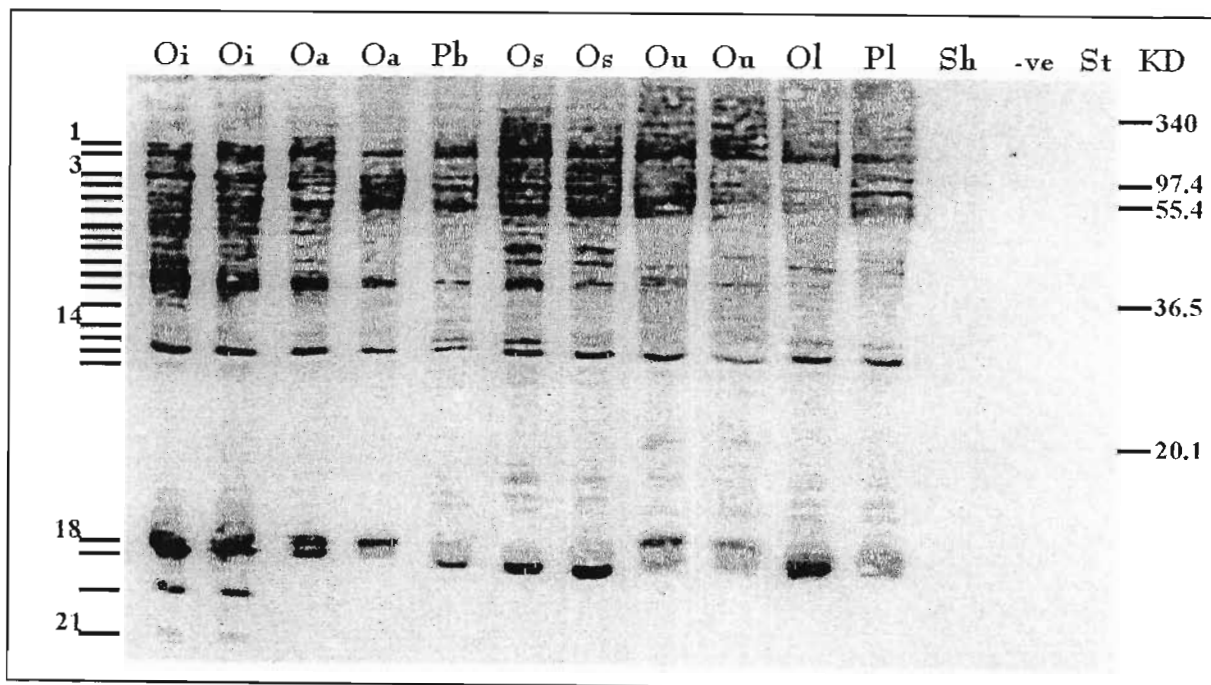


Figure 4.2 - Western-blot of duplicate gel shown in Figure 4.1. Legend as in Figure 4.1.

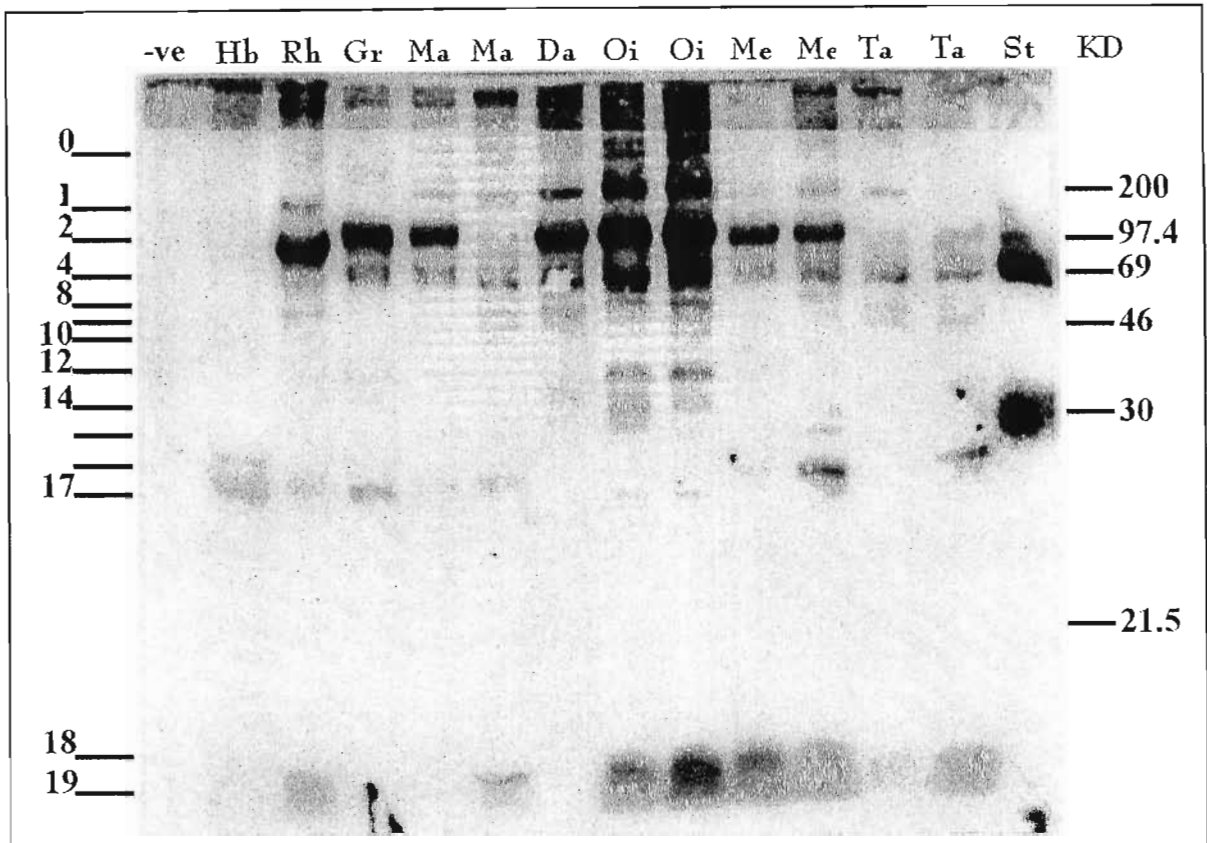


Figure 4.3 - Western-blot of non-Otomyine rodents. Hb= bovine haemoglobin, Rh=*R. pumilio*, Gr=*G. dolichurus*, Ma=*Mastomys sp.*, Da=*D. incomtus*, Oi=*O. irroratus*, Me=*M. auratus*, Ta=*T. leucogaster*, St=calibration proteins. Amersham rainbow calibration proteins: 200 KD=myosin, 97.4 KD=phosphorylase b, 69 KD=albumin, 46 KD=ovalbumin, 30 KD=carbonic anhydrase, 21.5 KD=trypsin inhibitor.

Table 4.3 - Estimated Nei (1978) unbiased genetic distances below diagonal and unbiased genetic identities above diagonal.

| Taxon | <i>O.irror.</i> | <i>R.pum.</i> | <i>G.dolic.</i> | <i>Mastom.</i> | <i>D.inc.</i> | <i>M.aur.</i> | <i>T.leuko.</i> |
|----------------------|-----------------|---------------|-----------------|----------------|---------------|---------------|-----------------|
| <i>O.irroratus</i> | | 0.476 | 0.429 | 0.381 | 0.333 | 0.442 | 0.401 |
| <i>R.pumilio</i> | 0.742 | | 0.524 | 0.571 | 0.476 | 0.541 | 0.693 |
| <i>G.dolichurus</i> | 0.847 | 0.647 | | 0.667 | 0.619 | 0.455 | 0.438 |
| <i>Mastomys</i> | 0.965 | 0.560 | 0.405 | | 0.762 | 0.602 | 0.620 |
| <i>D.incomtus</i> | 1.099 | 0.742 | 0.480 | 0.272 | | 0.639 | 0.620 |
| <i>M.auratus</i> | 0.816 | 0.615 | 0.788 | 0.507 | 0.448 | | 0.602 |
| <i>T.leukogaster</i> | 0.913 | 0.367 | 0.826 | 0.478 | 0.478 | 0.507 | |

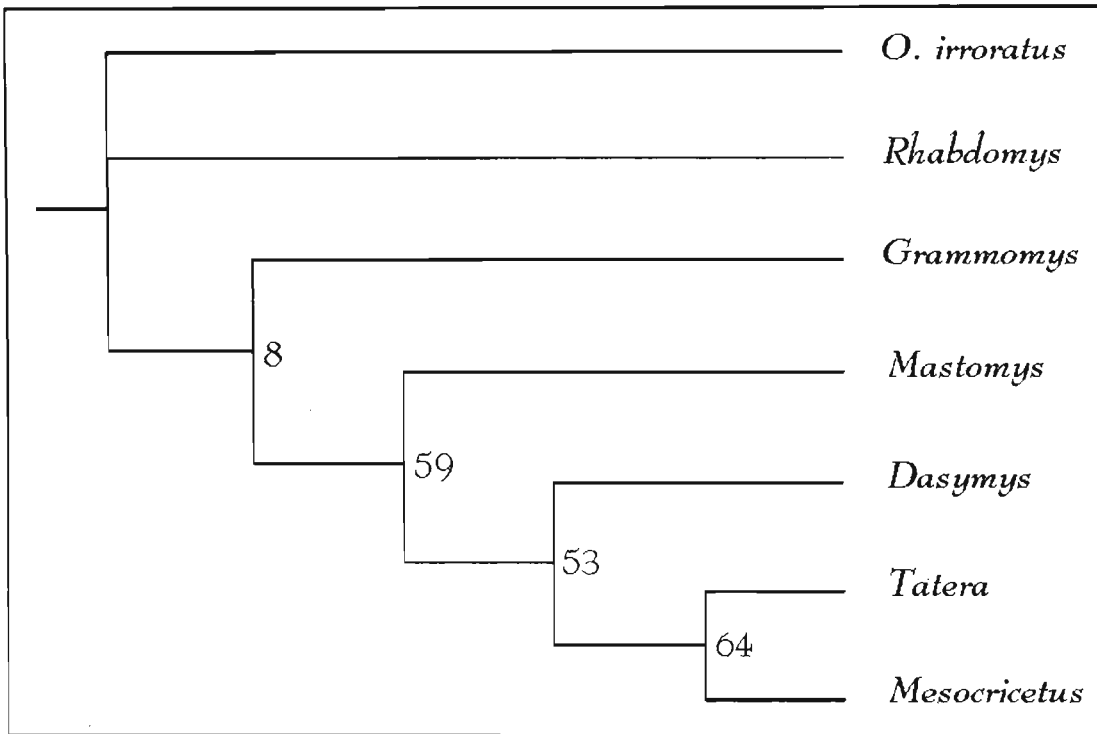


Figure 4.4 - The most parsimonious tree (length = 68) of non-Otomyine rodents from Hennig86 implicit enumeration. bootstrapping estimates from 100 replicates (BOOT from Phylip) shown at nodes.

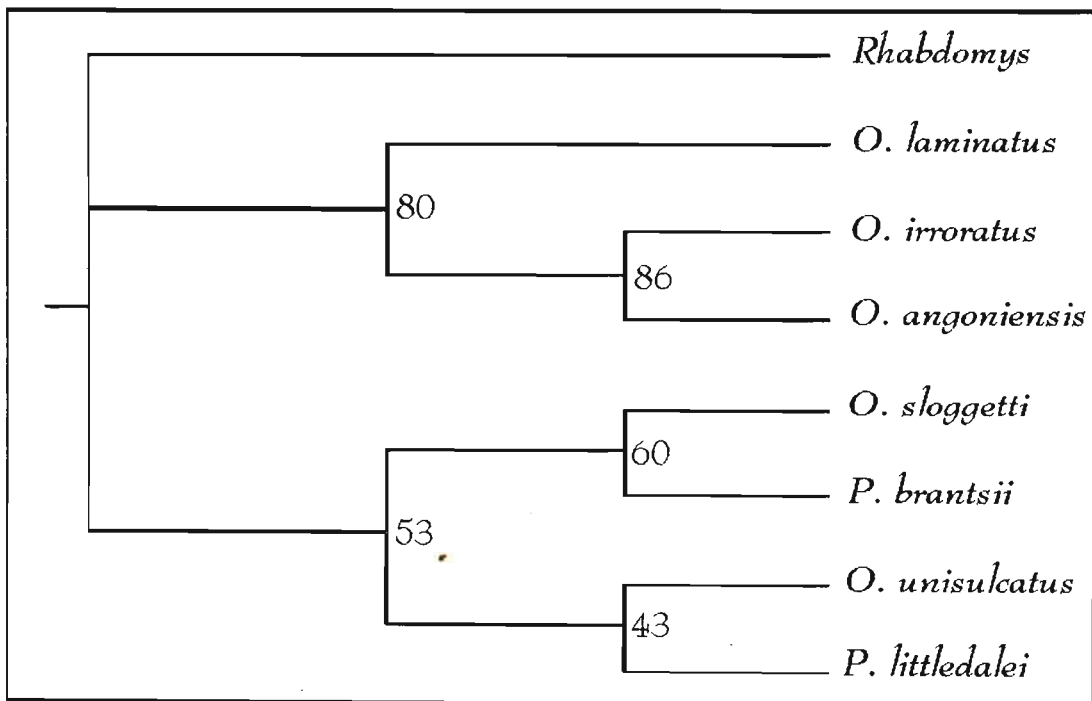


Figure 4.5 - Shortest Otomyine tree (length = 57) obtained using the same programme options as in Figure 4.4.

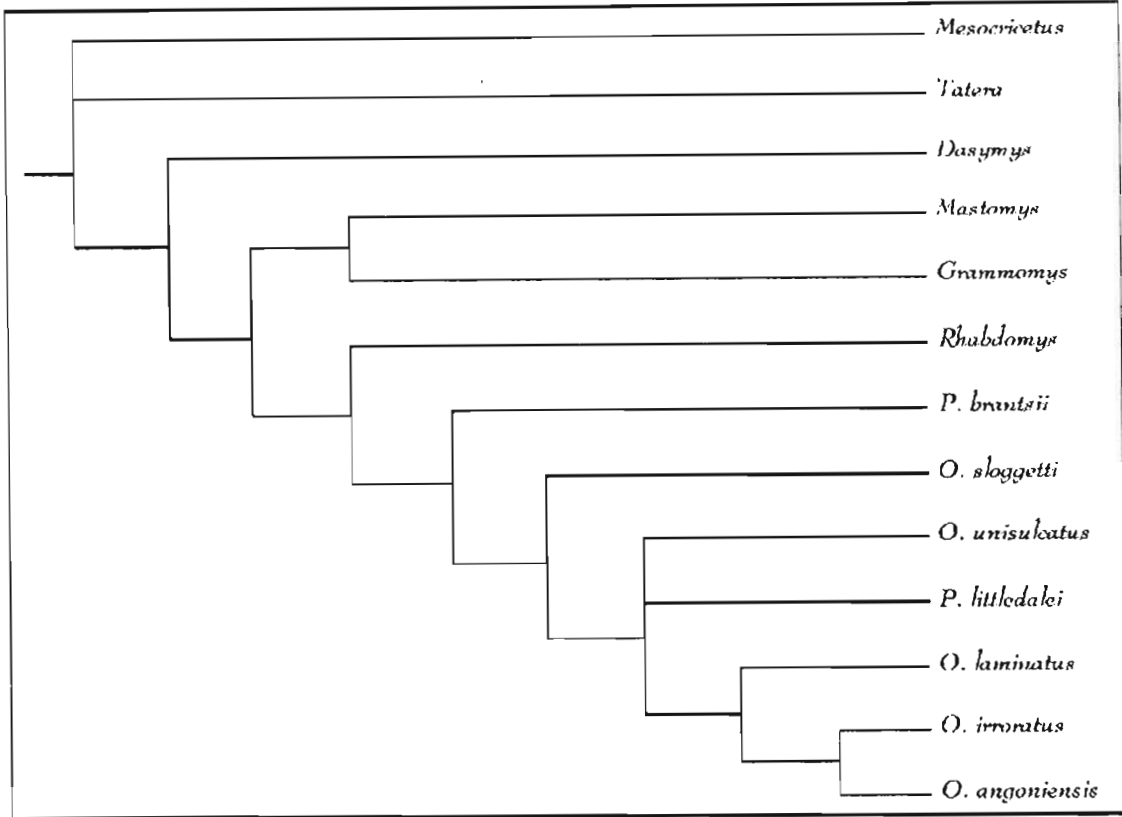


Figure 4.6 - Consensus tree of all taxa constructed from the two most parsimonious cladograms (length = 108).

◆ Discussion

• Immunoblotting

Our major concern was to develop a technique capable of detecting polymorphism at various taxonomic levels. Although allozyme electrophoresis, for example, has been successfully employed in intra-specific investigations, its greatest phylogenetic usefulness is at the species level or among closely related genera (Murphy *et al.*, 1990). DNA-DNA hybridization studies tend to involve inter-specific and higher taxon phylogenetic relationships (Werman *et al.*, 1990), whereas mt-DNA restriction fragment length polymorphism (RFLP) seems to be most appropriate at intra-specific levels or, at best, between closely related species (see Dowling *et al.*, 1990 for a review). Mammalian chromosome analysis by G- and C-banding has proven useful at various hierarchical taxonomic levels (Capanna *et al.*, 1976; Capanna, 1988; Eldridge *et al.*, 1991; Bickham and Baker, 1979) but is unpredictable since in some taxa intra-specific chromosomal

variability is inexplicably high while other groups display a high degree of conservatism.

Our results indicate that the techniques exploited in this study can detect polymorphism between sub-families, genera and species of rodents. However, convergence due to multiple mutations, resulting in similar net charge in proteins from unrelated taxa and similar migration, could still be a problem at higher taxonomic levels. This type of error, nevertheless, would tend to be minimized because SDS-PAGE relies mostly on migration by molecular mass rather than charge. Furthermore, the immunological interaction of epitope and antibody variable region is primarily a function of conformation, making it unlikely that loss of reactivity by an antigenic determinant would be reacquired as a result of multiple mutations. On the other hand, when band reactivity is due to more than one epitope, binding to one or more antibodies, mutations and consequent loss of activity by one antigenic determinant may go undetected thus leading to divergence underestimates. A potential pitfall, due to comigration, is in the interpretation of mobility shifts. A safer approach would be that of using monoclonal or purified antibodies. However, it was felt that, at this stage of technical evaluation, some reliability of interpretation could be traded off for the speed resulting from the ability to detect many characters in a single blot.

This method appears to be informative at several taxonomic levels as it allowed identification of nineteen characters per taxon in any single experiment. This represents a distinct advantage over established methods employing allozyme mobility analysis, since a single blot can yield an amount of information equivalent to that generated by several starch gel slices, each stained with a different allozyme substrate. Moreover, this method offers a benefit over other immunological techniques used in phylogenetic studies (Maxson and Maxson, 1990) that yield exclusively distance data because the anti-serum detected about nineteen electromorphs in the liver homogenates. Therefore, this provided about nineteen data-points per pair of taxa and the information could either be analyzed cladistically or transformed into distance data.

On the other hand, an undesirable side-effect of the method presented here lies in

the fact that the antiserum, as may be expected in such a reagent, contained a number of antibodies of varying specificities. This resulted in the detection of closely spaced bands in regions of the blot close to the origin and, particularly between 50 and 60 KD, making interpretation difficult. Furthermore, I did not readily detect a correspondence between electromorphs and putative alternate alleles since most of these could not be positively identified. Because of this, therefore, as well as the presumed presence of heteromeric enzymes in the samples, a genetic interpretation based on scoring allelic variation was not possible. These factors make it awkward to analyze data sets of this kind by a phenetic approach. A possible solution to this problem could be the use of gradient polyacrylamide gels instead of 12.5 % continuous gels combined with the use of specific antibodies purified from anti-*O. irroratus* serum by affinity chromatography. This need not be a major drawback, however, since - as discussed below - the dendograms generated from the data presented here corresponded closely with both phenograms and cladograms produced by allozyme studies. Ultimately, the choice of method will depend on the investigator's preference for either cladistic or phenetic analyses. Converting multistate characters into alleles and loci, to evaluate genetic distances, may have led to incorrect estimates. However, such a systematic error would have been evenly distributed across all taxa and, therefore, would not have influenced their ranking. I thus justified this practice in this context as it was implemented for the sole purpose of finding which taxon was closest to *O. irroratus*.

- *Phylogenies*

The topology of the cladogram included in Figure 4.5 (page 75) closely resembles that produced by allozyme analysis of the seven Southern African species of Otomyinae (Taylor *et al.*, 1989b) and confirms the suggestion presented by Taylor *et al.* (1989a) of a diphyletic evolution in this group. Their recommendation that *O. unisulcatus* be included in the genus *Parotomys* is also supported by this study. In addition, the results indicate that a similar suggestion can be made regarding *O. sloggetti*.

The family group relationships shown in Figure 4.4 (page 75), as well as the

genetic distances reported in Table 3, indicate that the species *O. irroratus* is more closely related to the true Muridae than to either the Gerbillinae or the Cricetidae. This lends support to modern classifications (Meester *et al.*, 1986) which include this subfamily in the family Muridae. This conclusion is supported by independent experimental evidence including sperm morphology comparison (Bernard *et al.*, 1990), DNA-DNA hybridization (Chevret *et al.*, 1992), cytochrome oxidase sequences (R. L. Honeycutt, pers. comm.) and analysis of long interspersed nuclear repeats (LINES) (K. Usdin, pers. comm.).

The phylogenetic relationships suggested by the cladogram in Figure 4.6 (page 76) should still be considered tentative because of the limited number of non-Otomyine rodents available. Nonetheless, the relationships emerging from the trees depicted in Figure 4.4 and 4.5 are broadly confirmed when all the specimens included in the present study are analyzed as a single multistate matrix (see Figure 4.6). A notable exception, for which I have no ready explanation, is that two of the arid-adapted Otomyines (*P. brantsii* and *O. sloggetti*) are here indicated as being ancestral to the other five Otomyine species.

In conclusion, I believe that immuno-electro-transfer of liver proteins shows high potential as a method of phylogenetic analysis since, in my hands, it has produced results comparable to those obtained from more established techniques. It appears, so far, to be reliable in detecting polymorphism between species, genera and sub-families. An assessment of the potential of this method at intra-specific level, in the species *O. irroratus*, is underway.

◆ *Aknowledgements*

I am grateful to the co-authors of the original publication, D. David and V. Goossens-LeClerq, for carrying out the experiments which yielded the immunoblots used as illustrations. The original manuscript was reviewed by J.H. Grace, P.J. Taylor and the late J.A. Meester.

◆ *Appendix*

With the exception of the two *O. irroratus* specimens (collector's numbers KW 342 and KW 343) from the Karkloof locality (29°21' S, 30°13' E) all voucher specimens were deposited with the Durban Natural Science Museum where they were allocated the following accession numbers. *O. angoniensis*: DM 2966 and DM 2967. *O. laminatus*: DM 2906. *O. unisulcatus*: DM 2980 and DM 2981. *O. sloggetti*: DM 2538 and DM 2539. *P. brantsii*: DM 2975. *P. littledalei*: DM 2983. *Mastomys*: DM 2039 and DM 2035. *Dasymys incomtus*: DM 2033. *Grammomys dolichurus*: DM 2038. *Tatera leucogaster*: DM 2037 and DM 2038. *Mesocricetus auratus*: DM 2044 and DM 2045.

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CHAPTER 5

INTRASPECIFIC IMMUNOREACTIVITY

◆ *Introduction*

Otomys irroratus and its sibling species *O. angoniensis* Wroughton, 1906 (the Angoni vlei rat) have been proposed as likely examples of speciation initiated by chromosomal changes (Meester 1988) and considerable chromosomal variation in *O. irroratus* has been documented (see Chapter 2). As detailed in Chapter 2, a group of cytotypes which include eight pairs of large biarmed autosomes with heterochromatic short arms (see *inter alia* Figure 2.7, page 34, group B) is localized west of 26°57'E while populations east of this longitude have varying diploid numbers (Figure 2.7, group A) and all acrocentric karyotypes. Populations north of the Drakensberg mountains and in the Cape of Good Hope peninsula make up a third group of cytotypes which have only four pairs of large autosomes with heterochromatic short arms (Figure 2.7, group C). More importantly, in the group with acrocentric karyotypes, the three populations at Hogsback, Kamberg and Vergelegen (Figure 2.7, group A1) carry a tandem fusion between autosomes 7 and 12 (7q:12q) which probably explains the break-down in the reproduction of hybrids between individuals from this group and those from other populations. Cladistic and phenetic analyses of cytogenetic data produced dendograms which lent support to this interpretation (Figure 2.7).

These chromosomal variants have been shown by discriminant analysis (DA) to correlate strongly with climatic variables such as temperature and rainfall of the bioclimatic regions of Southern Africa (Taylor *et al.* 1994, see Chapter 3). Allozyme starch gel electrophoresis, however, demonstrated low intraspecific variation and extensive gene flow between all populations which suggested a panmictic genetic structure of this species (Taylor *et al.*, 1992) whereas I indicated in Chapter 2 (page 42) that the cytogenetic findings were consistent with a 'Wrightian', fragmented population. Both sets of results appear to agree with

(*inter alia*) Meester's (1988) hypothesis that speciation can be initiated by chromosomal changes long before genetic changes usually associated with speciation have accumulated.

To substantiate these results, I have developed an alternative method to detect genetic changes by separating liver homogenates on Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) followed by 'Western Blotting' (Towbin *et al.* 1979) and detection by the use of a specific antiserum (see Chapter 4). The phylogenies and genetic distances obtained with this approach on 12.5% continuous gels at interspecific and intergeneric levels were consistent with those obtained by Taylor *et al.* (1989) from allozyme data. This method showed that a number of proteins which separated in the gel region, close to the origin and between 50 and 60 KD, were not sufficiently resolved. Hence this attempt to improve resolution using gradient gels on tissue from several populations of *O. irroratus*, to enhance detection of intraspecific variability.

◆ Materials and Methods

Crude liver homogenates were prepared as described in Chapter 4 (pages 68-69) from one individual each of the *O. irroratus* populations at Alice, Bloemfontein, Hogsback, Grahamstown, Kamberg, Port Elizabeth, Vergelegen; two each from Committee's Drift, Constantia, Rietvlei, Stutterheim, and six from Karkloof; two specimens of *O. angoniensis* served as an outgroup. Figure 2.1 and Table 2.1 (Chapter 2, pages 18 and 19) map and list, respectively, these localities and their associated habitats.

Antiserum production (anti-*O. irroratus* from the Karkloof population) and the procedures employed in blotting and detecting antigenic proteins are described in Chapter 4 (pages 67-71).

SDS-Polyacrylamide gradient gels (7 to 17%) were cast with a peristaltic pump (Pharmacia, Sweden) at a flow rate of 1.5 ml/min. Each electrophoretic lane was loaded with 50 μg of total protein (in 100 μl of sample buffer: see page 69-70) while a negative control comprising azocasein was applied under the same conditions in lanes one or two.

Separation was carried out at 15 V/cm for 6 hrs followed by electrotransfer and immunodetection.

Blot analysis was performed on images digitized using a Coupled Charged Device (CCD) video camera (Sony DXC-102, Japan) interfaced to a "frame-grabbing" board (Oculus 300SE, Coreco Inc., Canada) installed in an Intel 386SX-16 based personal computer (Olivetti M300, Italy). Images were then viewed with the programme Corel Photopaint (Zsoft, USA). Band gray levels were obtained by averaging five readings taken by positioning the mouse pointer on five approximately equidistant points along the middle of each visible band. Band migration was recorded by reading the image pixel position at the mid-point of each band. Using the Karkloof samples as reference, the arbitrary values of 200 and 400 were added to the gray value of slower and faster proteins respectively. Bands of dubious interpretation were coded as question marks (i.e. missing data). This procedure allowed us to construct an interval data matrix with 28 variables or characters, which was then subjected to multivariate statistical and, after appropriate coding, cladistic analyses.

Discriminant analysis was used to test the ability of the data set to separate the populations according to the chromosomally defined groups shown in figure 2.7 (page 34). The test was carried out with the Statgraphics plus 5.2 (Statistical Graphics Corp., USA) including only the 14 variables with no missing data.

Average taxonomic distances computation and cluster analysis (Unweighed Pair Group Method with Averages: UPGMA) were carried out with the NTSYS-PC 1.5 (Exeter Software, USA) programme.

Additionally, the gray value of the negative control lane, in each blot, was sampled from five points taken along each one of five equidistant lines over the length of the lane. Bands with an average gray lower than the mean, less one standard deviation, of these 25 values were considered negative or non-reacting. The range between the darkest band in each blot (lowest gray value) and the negative, in the same blot, was divided into three equal

intervals to designate strongly reactive bands, intermediate and weak bands. Therefore, from the interval data, a discrete character state matrix was produced according to the parameters listed in Table 5.1. One hundred bootstrapping replicates were performed on the cladistic matrix thus obtained (see Table 5.2, page 88), with the programme BOOT from the PHYLIP package (Felsenstein 1989).

Table 5.1 - Criteria used to define multistate characters from the two blots shown in figures 5.1(a) and 5.1(b).

| Reactivity against antiserum | Gray levelFig . 5.1(a) | Gray levelFig 5.1(b) | Migration compared to Karkloof | Character state |
|------------------------------|------------------------|----------------------|--------------------------------|-----------------|
| Strong | 45-84 | 52-91 | same | 0 |
| Medium | 85-124 | 92-130 | same | 1 |
| Weak | 125-162 | 131-169 | same | 2 |
| Negative | 163-202 | 170-209 | same | 3 |
| Strong | 45-84 | 52-91 | slower | 4 |
| Medium | 85-124 | 92-130 | slower | 5 |
| Weak | 125-162 | 131-169 | slower | 6 |
| Strong | 45-84 | 52-91 | faster | 7 |
| Medium | 85-124 | 92-130 | faster | 8 |
| Weak | 125-162 | 131-169 | faster | 9 |

◆ *Results and Discussion*

• *Immunoblot analysis*

Gradient polyacrylamide gels allowed detection and interpretation of 28 characters as opposed to 19 previously observed on continuous 12,5 % gels (see Chapter 4). As figure 5.1 (page 89) shows, considerable inter- and intra-population differences were observed in migration and reactivity to the antiserum. Significant variation was seen amongst three bands with molecular weights of 32-34 KD: in most populations three closely situated strongly cross-reactive bands were observed (characters 21-23). However, in the Port Elizabeth, Constantia and Committee's Drift samples, only one of such bands exhibited

strong cross-reactivity.

Table 5.2 - Multistate character matrix used in cladistic analysis.

| Population | Character | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|-------------------------|-----------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| | 0 | 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 |
| Karkloof 1 | 3 | 2 | 2 | 3 | 3 | 3 | 3 | 2 | 1 | 3 | 2 | 0 | ? | 0 | 1 | 2 | 1 | 2 | 1 | 1 | 0 | 0 | 0 | ? | ? | 2 | 3 | 1 |
| Karkloof 2 | 2 | 3 | 1 | 3 | 3 | 3 | 2 | 1 | 1 | 3 | 2 | 0 | ? | 0 | 1 | 2 | 2 | 3 | 2 | 2 | 0 | 0 | 0 | 1 | ? | 2 | 3 | 1 |
| Karkloof 3 | 2 | 3 | 1 | 3 | 3 | 2 | 2 | 2 | 1 | 3 | 2 | 1 | ? | 0 | 1 | 3 | 2 | 3 | 2 | 3 | 2 | 3 | 1 | 2 | 2 | 1 | 2 | 2 |
| Karkloof 4 | 3 | 3 | 3 | 2 | 3 | 2 | 1 | 0 | 1 | ? | 2 | 0 | ? | 0 | 0 | 0 | 2 | 2 | 1 | 1 | 1 | 0 | 0 | 3 | 3 | 2 | 1 | 1 |
| Karkloof 5 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 1 | 1 | 3 | 3 | 2 | ? | 3 | 1 | ? | ? | ? | 1 | 1 | 1 | 0 | 0 | 3 | 3 | 2 | 1 | 1 |
| Hogsback | 3 | 3 | 2 | 3 | 3 | 3 | 2 | 8 | ? | ? | ? | 0 | ? | 0 | 0 | 1 | 1 | 2 | 2 | 2 | 1 | 3 | 3 | 0 | 3 | 2 | 1 | 1 |
| Kamberg | 3 | 2 | 7 | 2 | 2 | 2 | 2 | 1 | 1 | 1 | 1 | 0 | ? | 1 | 0 | 1 | 1 | 2 | 2 | 2 | 2 | 1 | 0 | 1 | 3 | 2 | 1 | 1 |
| Alice | 3 | 3 | 3 | 3 | 3 | 3 | ? | ? | 2 | ? | 2 | 0 | ? | 1 | 0 | 1 | 1 | 3 | 2 | 2 | 1 | 1 | 1 | 3 | 3 | 2 | 1 | 1 |
| P. Elizabeth | 3 | 3 | 2 | 2 | 3 | 2 | 1 | 2 | 0 | 2 | 2 | 1 | ? | 0 | 0 | 0 | 2 | 2 | 1 | 2 | 1 | 0 | 0 | 3 | 3 | 1 | 1 | 1 |
| Grahamstown | 3 | 3 | 1 | 3 | 3 | 3 | 3 | 2 | 1 | 2 | 2 | 1 | ? | 1 | 0 | 1 | 2 | 3 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 2 | 1 | 1 |
| <i>O. angoniensis</i> 1 | 3 | 2 | 2 | 2 | 3 | 3 | 2 | 2 | 6 | 2 | 3 | 5 | 5 | 1 | 1 | 1 | 3 | 3 | 2 | 3 | 2 | 2 | 3 | 3 | 3 | 3 | 2 | 2 |
| <i>O. angoniensis</i> 2 | 2 | 3 | 3 | 3 | 3 | 2 | 2 | 2 | ? | ? | ? | 5 | 6 | 1 | 1 | 1 | 2 | 3 | 2 | 3 | 1 | 2 | 2 | 2 | 2 | 3 | 2 | 2 |
| Karkloof 6 | ? | 3 | 1 | 3 | 3 | 2 | 2 | 1 | 1 | 3 | 2 | 1 | 1 | 0 | 0 | 0 | 1 | 3 | 2 | 2 | 0 | 0 | 0 | 2 | 2 | 2 | 1 | 1 |
| Vergelegen | ? | 3 | 2 | 2 | 3 | 2 | 1 | 0 | 1 | 2 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 2 | 2 | 2 | 0 | 0 | 0 | 2 | 2 | 1 | 0 | 1 |
| Rietvlei 1 | ? | 3 | 0 | 2 | 2 | 2 | 0 | 0 | 0 | 2 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 2 | 2 | 2 | 0 | 0 | 0 | 2 | 2 | 2 | 1 | 1 |
| Rietvlei 2 | ? | 3 | 1 | 2 | 3 | 2 | 1 | 0 | 1 | 2 | 1 | 1 | 1 | 0 | 0 | 0 | 1 | 2 | 2 | 2 | 0 | 0 | 0 | 2 | 2 | 2 | 0 | 1 |
| Bloemfontein | ? | 3 | 1 | 3 | 3 | 2 | 1 | 0 | 2 | 2 | 2 | 1 | 1 | 0 | 0 | 0 | 1 | 2 | 2 | 2 | 0 | 0 | 0 | 2 | 2 | 2 | 0 | 1 |
| Constantia 1 | 3 | 3 | 2 | 3 | 3 | 2 | 1 | 0 | 0 | 3 | 3 | 8 | 8 | 1 | 0 | 0 | 2 | 9 | 2 | 4 | 3 | 1 | 3 | 9 | 3 | 3 | 1 | 1 |
| Constantia 2 | 3 | 3 | 2 | 3 | 3 | 3 | 1 | 0 | 1 | 3 | 3 | 8 | 8 | 1 | 0 | 0 | 2 | 9 | 2 | 5 | 3 | 2 | 3 | 9 | 3 | 3 | 2 | 1 |
| Stutterheim 1 | 3 | 2 | 1 | 3 | 3 | 2 | 2 | 2 | 0 | 2 | 2 | 1 | 1 | 0 | 0 | 0 | 1 | 2 | 2 | 2 | 0 | 0 | 0 | 1 | 2 | 2 | 0 | 1 |
| Stutterheim 2 | 3 | 3 | 1 | 3 | 3 | 3 | 2 | 2 | 0 | 2 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | ? | ? | ? | 1 | 0 | 0 | 2 | 3 | 2 | 0 | 1 |
| Comm. Drift 1 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 1 | 0 | 2 | 3 | 1 | 3 | 1 | 0 | 1 | 2 | 2 | 2 | 3 | 3 | 1 | 3 | 3 | 3 | 3 | 2 | 2 |
| Comm. Drift 2 | 3 | 3 | 3 | 2 | 2 | 2 | 2 | 1 | 0 | 2 | 3 | 1 | 2 | 1 | 0 | 1 | 2 | 2 | 2 | 3 | 3 | 1 | 3 | 3 | 3 | 3 | 2 | 2 |

In the Port Elizabeth specimen, the other two bands (22 and 23) had a lower intensity, possibly due to lower concentration of this protein in the sample (i.e. variable gene activity). In the case of Constantia, band 21 displayed a molecular weight shift while bands 22 and 23 were non-reactive. Striking variability was also observed in two bands co-migrating with the 69 KD standard (characters 12 and 13) which showed migration differences in *O. angoniensis* and Constantia.

The considerable variation in band patterns observed between and within

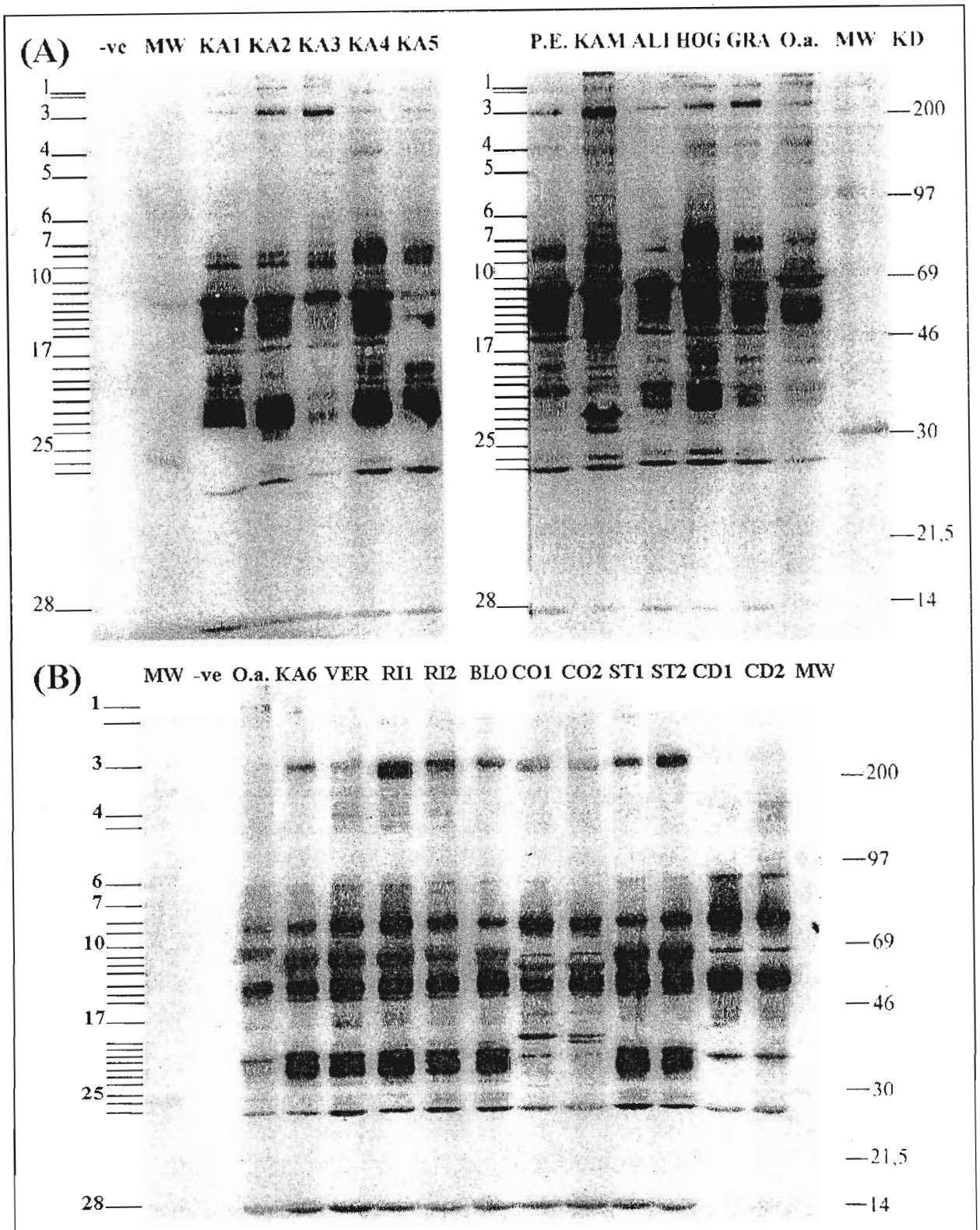


Figure 5.1 - Immunoblots of *O. irroratus* specimens studied. Lines and numbers on the left of each image indicate the bands used as characters in the statistical analyses. -ve = negative control (see text), KD = rainbow markers (Amersham, UK): 200 KD = myosin, 97.4 KD = phosphorylase b, 69 KD = albumin, 46 KD = ovalbumin, 30 KD = carbonic anhydrase, 21.5 KD = trypsin inhibitor, 14 KD = lysozime. (A): MW = molecular weight markers (see KD), KA1 - KA5 = Karkloof, P.E. = Port Elizabeth, KAM = Kamberg, ALI = Alice, HOG = Hogsback, GRA = Grahamstown, O.a. = *O. angoniensis*. (B): O.a. = *O. angoniensis*, KA6 = Karkloof, VER = Vergelegen, RI1 and RI2 = Rietvlei, BLO = Bloemfontein, CO1 and CO2 = Constantia, ST1 and ST2 = Stutterheim, CD1 and CD2 = Committees Drift.

populations indicates that this method is sufficiently sensitive to detect genetic differences at intraspecific and intrapopulation levels. Gradient gel electrophoresis enhanced resolution on the blots although some regions, such as bands 14, 15, 21, 22 and 23, remained difficult to interpret. In this respect, it was helpful to manipulate the images electronically by varying density, brightness and contrast of separate elements of each image on the computer screen, and thus increase the confidence of our interpretation. Image analysis offered the further advantage of allowing conversion of the raw information into a discrete data matrix and, consequently, the application of stricter criteria of character state assignment in constructing the cladistic matrix.

- *Statistical and cladistic analyses*

Discriminant analysis differentiated between the chromosomally defined groups of *O. irroratus*. Figure 5.2 (page 91) shows that, on the first discriminant function, there was a sharp separation of the outgroup *O. angoniensis* from the *O. irroratus* populations and that groups A, B and C could be separated from one another on the first function while group A1, carrying the tandem fusion, could be differentiated from the latter three on the second discriminant function. These results could be explained by the high positive values, for the first two functions, of variable 12 discriminant coefficients (Table 5.3) and the high negative coefficients of variable 23; these two variables also displayed a positive within-group correlation with r^2 value of 0.821. Further contributions to good discrimination were high positive standardized discriminant coefficients for variables 22 and 28 and high negative coefficients for bands 21 and 14 on functions one and two respectively (see Table 5.3, page 91).

Partitioned matrices including variables with high discriminating power, that is, the 14 variables analyzed by DA less bands 2 and 5 which had the lowest impact on the first two functions, were tested against full matrices (i.e. 28 characters) by phenetic and cladistic analyses. The resulting dendrograms are shown in figures 5.3 and 5.4 (page 91).

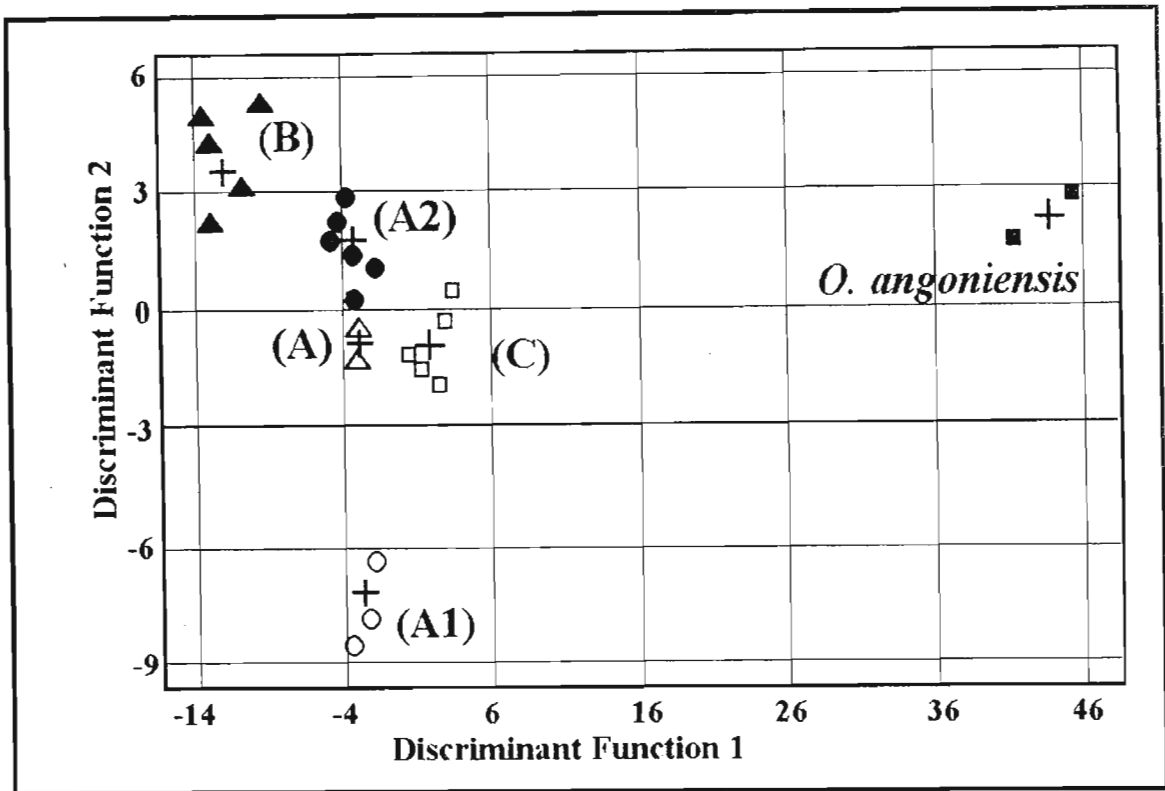


Figure 5.2 - Plot of the *O. irroratus* populations, according to cytotypes (in brackets), on the first and second discriminant functions.

Table 5.3 - Standardized discriminant function (DF) coefficients for the 14 variables which had no missing data.

| Variable | D.F. 1 | D.F. 2 | D.F. 3 | D.F. 4 |
|----------|-----------|-----------|-----------|-----------|
| 2 | - 0.82939 | 0.09981 | - 0.28245 | 0.07299 |
| 3 | - 0.26208 | - 0.83991 | 0.80962 | - 0.15870 |
| 4 | 0.11836 | 0.57963 | - 1.75993 | - 0.84966 |
| 5 | - 0.12688 | 0.22107 | 1.07893 | 1.28853 |
| 6 | - 1.29573 | - 0.26791 | 1.04167 | 0.30745 |
| 12 | 8.08942 | - 0.82927 | - 0.57672 | - 0.27215 |
| 14 | - 0.44409 | - 4.18916 | 1.38110 | - 0.09227 |
| 15 | - 1.55523 | 4.918952 | - 0.73322 | 0.96270 |
| 21 | - 3.87962 | 0.13879 | - 1.68449 | - 0.03752 |
| 22 | 2.71578 | 2.24702 | - 0.06299 | 0.56126 |
| 23 | - 5.01349 | - 1.64854 | 1.76108 | - 1.34828 |
| 26 | - 1.25512 | 4.23552 | - 0.39343 | 0.43187 |
| 27 | 0.41224 | - 3.34435 | 0.63921 | 0.18718 |
| 28 | 2.89863 | 0.85282 | 0.99146 | 0.42894 |

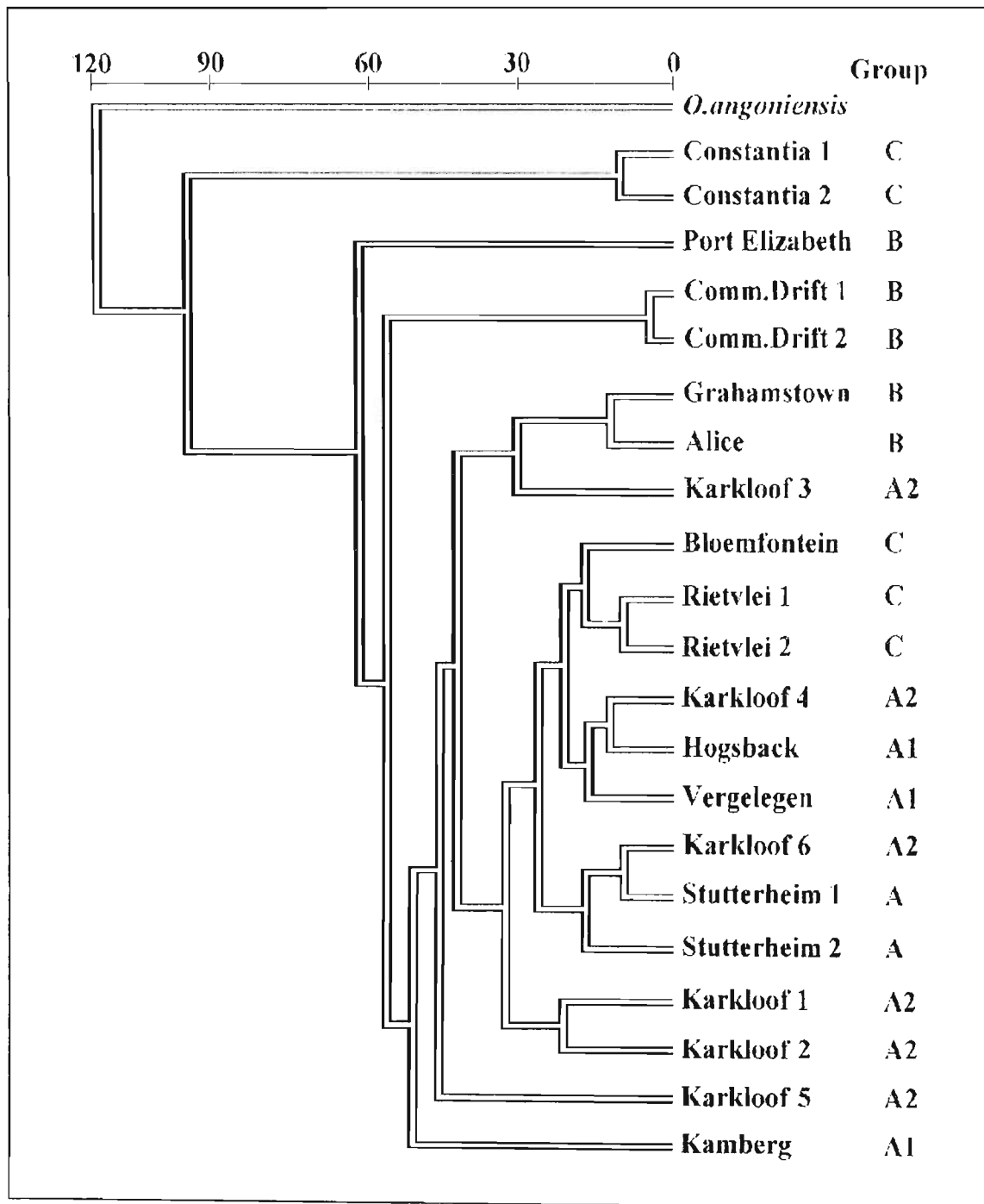


Figure 5.3 - UPGMA phenogram from full gray level matrix. Scale: taxonomic distance (Rohlf, 1988).

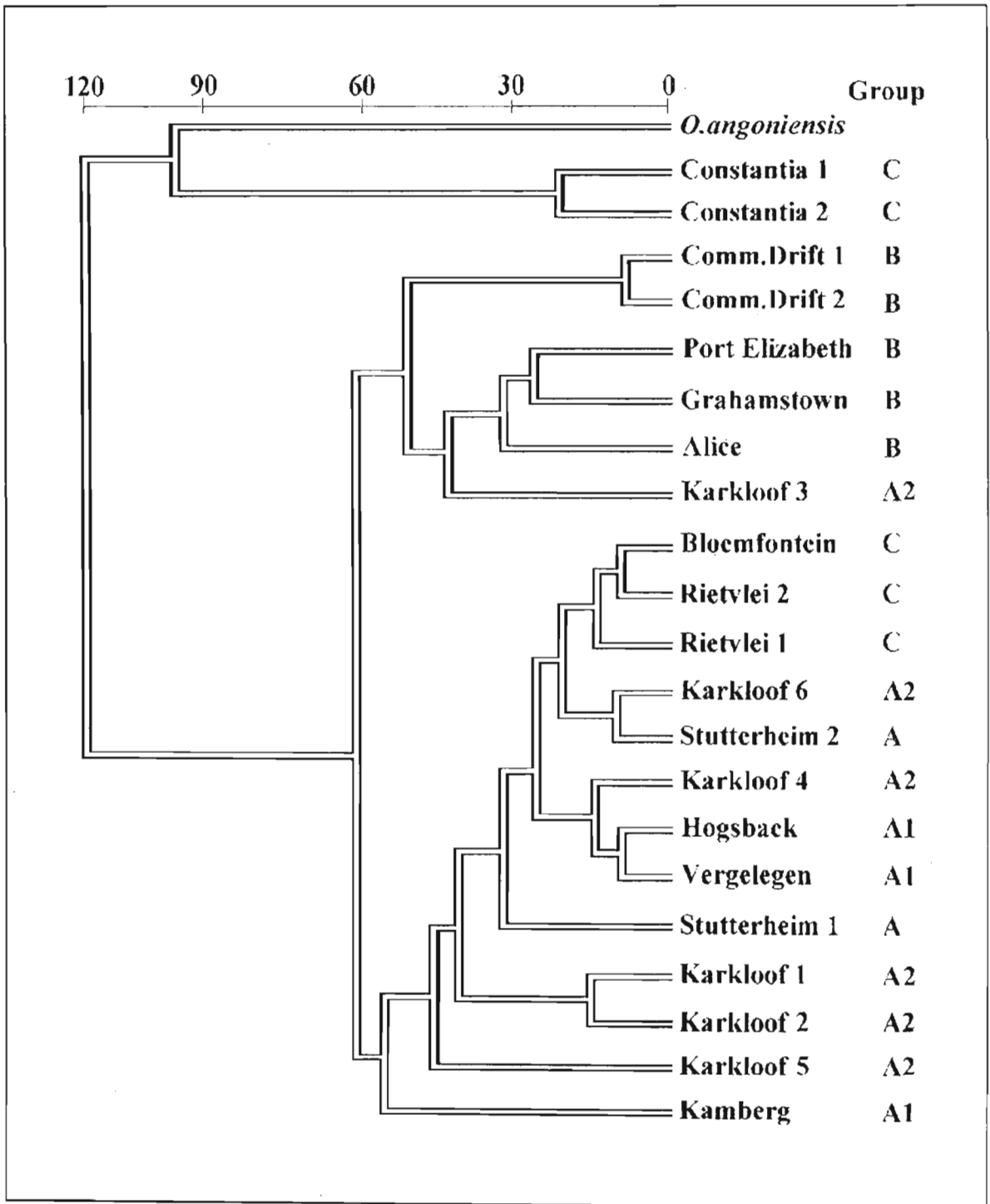


Figure 5.4 - UPGMA phenogram from partial gray level matrix which included the 12 characters with the highest positive and highest negative standardized discriminant coefficients ((3, 4, 6, 12, 14, 15, 21, 22, 23, 26, 27 and 28). Scale: taxonomic distance (Rohlf, 1988).

Poor agreement with interpretations of chromosomal data was evident in the UPGMA phenogram shown in figure 5.3 where the clustering levels were expressed in

average taxonomic distances (Rohlf 1988: 6.77); the majority of clustering levels were lower than intrapopulation distances. Analysis of the interval data matrix when reduced to 12 variables (Figure 5.4), increased the distance between some clusters and introduced topological changes, such as the appearance of a cytogenetically congruent cluster comprising all of the group B localities, albeit, also including a Karkloof specimen (group A). This emerged as a "ball cluster" (see Rohlf, 1988: 6.66) when the single-link and complete-link methods were used.

Congruence between the cladograms (Figures 5.5 and 5.6, pages 95 and 96) on the one hand, and the phenograms of Figs. 5.3 and 5.4 (pages 92 and 93) on the other, was poor. Boot-strapping estimates were generally low which indicated the presence of a number of homoplasies and the data failed to show close phylogenetic relationships between the populations belonging to the four recognized cytotypes. There were indications of an early separation of the Constantia and Committees Drift populations from all others and of a relatively close phylogenetic relationship between the Eastern Cape populations of Alice, Port Elizabeth and Grahamstown. With the exception of the position of Committees Drift, these represented similarities with the phenograms of figures 5.3 and 5.4 (pages 92 and 93). Although the Constantia individuals could have been expected, on cytogenetical grounds, to show a close relationship with those from Bloemfontein and Rietvlei, their phylogenetically isolated position was not totally unexpected as a similar separation has also been observed by Rimmer (1995) in a study of mitochondrial DNA polymorphism of restriction fragments which did not include any specimens from Committees Drift. On the other hand, the Committees Drift population was also shown by allozyme electrophoresis (Taylor *et al.* 1992), to display a similar position as that in Fig 5.5 and 5.6 although no individuals from Constantia were included in that study.

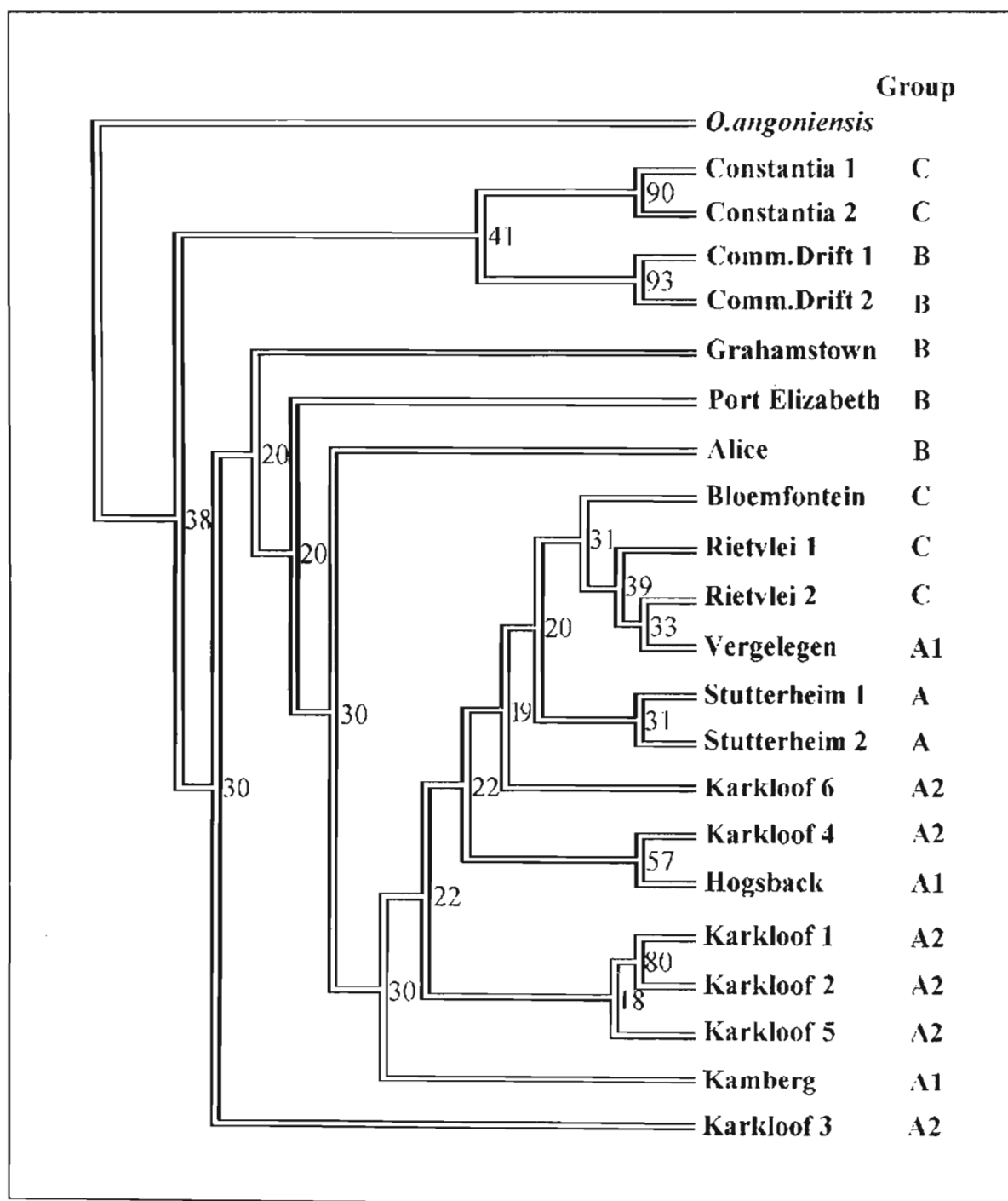


Figure 5.5 - Cladogram from full gray-level matrix. Estimates from 100 bootstrapping replicates shown at nodes.

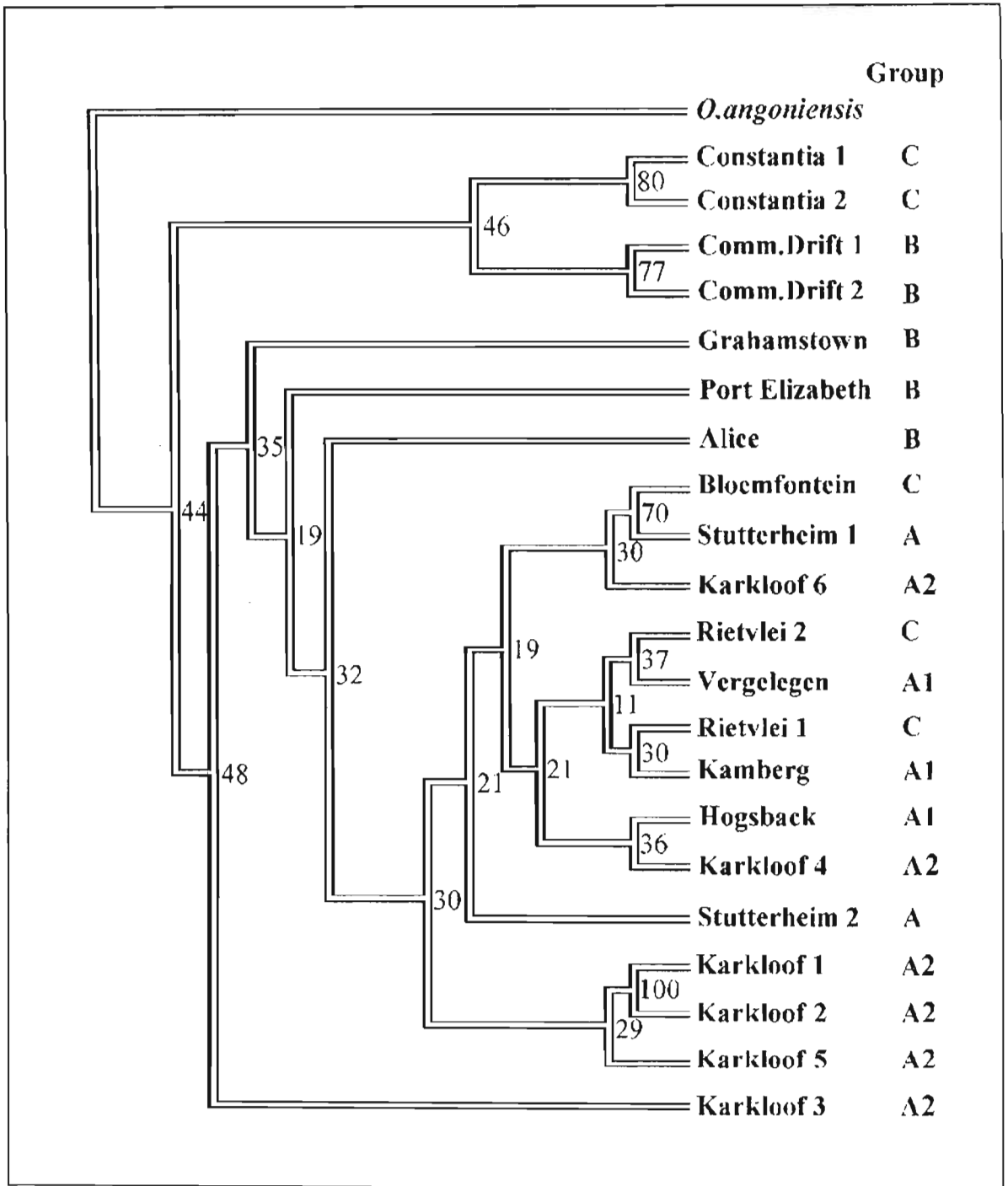


Figure 5.6. - Consensus trees from 100 boot strapping estimates from partial matrix including the 12 characters with the highest positive and negative standardized discriminant coefficients (3, 4, 6, 12, 14, 15, 21, 22, 23, 26, 27 and 28).

Use of a partial matrix limited to the 12 variables with higher discriminating power did not improve cladogram topology although it increased the confidence of some boot-

strapping estimates. Reducing the number of states in the matrix to seven, by choosing only two states of reactivity - below (state zero) and above (state one) the median gray level - and re-coding all other states accordingly, resulted in complete lack of congruence and even lower boot-strapping estimates.

- *Evolutionary implications*

The significance of the liver protein variation observed in this study remains somewhat obscure since no full agreement was found with the dendograms generated from the available cytogenetic data (Figure 2.7 page 34). Because the immunological approach has been shown to be useful in resolving inter-specific relationships, there is little doubt that it could also be applicable to intraspecific analyses. Although discriminant function could separate the various chromosomal groups from one another and from the outgroup species, phenetic analysis clustered only the Eastern Cape populations with biarmed chromosomes (group B) while cladistic analysis separated group B only partially and with low confidence. The success of discriminant analysis is not surprising since this test is expected to maximize Fisher's discriminant criterion, which can be loosely described as the ratio between sample variability (sums of squares) and variability within predefined groups (for a formal treatment see Goldstein and Dillon 1978, Jobson, 1992). On the other hand, both cladistic and phenetic analyses were applied to individual observations rather than predefined groups. It is not entirely unexpected, therefore, that outlying individuals within recognized cytotypes may readily cluster with other groups, by phenetic analysis, or show unexpected phylogenetic relationships. This is highlighted by the high degree of variability displayed, in the immunoblots, by the Karkloof specimens. By the same token, use of a reduced matrix including only the variables which maximized the discriminant criterion, produced an improvement since a distinct cluster including the B group localities was apparent in the phenogram (Figure 5.4, page 93). Complete agreement, however, between chromosomal and immunological data was not demonstrated by either phenetic or

cladistic analysis. Especially relevant to this aspect, is the situation of group A1 which carries the (7:12) tandem fusion. This chromosomal rearrangement is associated with a breakdown in reproductive success of the F1 generation from crosses between group A1 and members of other populations (Pillay 1990, Pillay *et al.* 1992). A further karyotype variant is represented by the Karkloof population, which has two extra pairs of acrocentric chromosomes of unidentified origin (see figure 2.3-C, page 25). This may correlate with the reduced fertility in matings between Karkloof and Hogsback as well as Karkloof and Committees Drift individuals, demonstrated by Pillay *et al.* (1992). As both chromosome variants present barriers to gene flow, derived characters evolved after the establishment of such barriers should generate phylogenetic relationships similar to those defined by chromosomal configuration. Thus the finding in this study of synapomorphies, causing some group A and A1 populations to associate with groups B and C, is somewhat disappointing and may suggest that the immunoblot method does not provide the resolution needed to resolve intraspecific relationships in *O. irroratus*.

To explain lack of congruence in a study of allozyme electrophoresis of 33 muroid genera, Bonhomme *et al.* (1985) have suggested, in their "homoplasy hypothesis", the occurrence of convergent events capable of producing electrophoretic parallelisms or electromorph identities. However, immunoblotting is unlikely to be sensitive to convergent characters, as discussed in Chapter 4 (page 77). Furthermore, a molecular clock estimate carried out using allozyme electrophoresis data suggested a time-span of Otomyine evolution of about 5 MY (Taylor *et al.* 1989) while Restriction Fragment Length Polymorphism (RFLP) analysis of mtDNA (Rimmer, 1995, Raubenheimer, 1994) allowed an estimate of 2 MY for the *O. angoniensis*-*O. irroratus* dichotomy. Additionally, Raubenheimer (1994: 82) placed the separation of the Kamberg population from that of Karkloof at about 0.365 MYBP. Therefore, while convergence may be a distinct possibility at intergeneric and higher taxonomic levels, we think it is highly unlikely to have played a significant role in this species, because of the relatively short evolutionary time involved.

On the other hand, homoplasies could be due to the presence of plesiomorphic character states in our data. These could have been polymorphic in a hypothetical ancestral population, according to the "ancestral polymorphism hypothesis" of Bonhomme *et al.* (1985). In this case, their inclusion in derived taxonomic units could have been accidental and their present distribution would be phylogenetically meaningless. Alternatively, the inclusion of ancient synapomorphies in derived taxa, would reflect a situation of unrestricted gene flow during early stages of development of the group, as Bonhomme *et al.* (1985) state in their "reticulate evolution hypothesis". The data presented here do not allow us to differentiate between these two possibilities. Either way, however, it seems that we are dealing with ancestral character states relative to the chromosomally defined groups. Therefore, since we discount the possibility of convergence, homoplasies in our data-set can be considered plesiomorphic characters or synapomorphies originated before the occurrence of chromosomal reproductive barriers. We take this as an indication that insufficient time has yet elapsed, since the establishment of chromosomal barriers, to allow for the fixation of such character states into synapomorphies capable of defining the known cytotypes. This supports the conclusions of Taylor *et al.* (1992) and those stated in Chapter 2 (page 42) as well as the hypothesis (Meester 1988, Meester *et al.* 1992) that chromosomal rearrangements can initiate speciation before the allele mutations usually associated with speciation, can become fixed.

◆ *Acknowledgements*

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◆ *Appendix*

All voucher specimens, with the exception of the two samples from Rietvlei (collector numbers KW769 and KW680), were deposited with the Durban Natural Science Museum where they were allocated the following accession numbers:

O. angoniensis: DM 2629 and DM 3022

O. irroratus (Alice): DM 2032; Bloemfontein: DM 3073; (Committee's Drift): DM 2577 and DM 2578; (Constantia): DM 3075 and DM 3076; (Grahamstown): DM 3082; (Hogsback): DM 2535; (Kamberg): DM 2775; (Karkloof): DM 2011, DM 2904, DM 3047, DM 3052; (Port Elizabeth): DM 3006; (Stutterheim): DM 2912 and DM 2914; (Vergelegen): DM 3031.

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CHAPTER 6

SYNTHESIS

Support for the hypothesis of sibling speciation occurring by chromosomal isolation, as outlined in Chapter 1 (page 6), can be provided if the following conditions are met:

1. Existence of negatively heterotic chromosomal differences, as discussed by White (1978), King (1993) and Capanna and Redi (1994).
2. Existence of reduced interpopulation fertility or hybrid dysgenesis as illustrated, *inter alia*, in the rock wallabies of the genus *Petrogale* (Eldridge *et al.*, 1990; Sharman *et al.*, 1990) and discussed by King (1993).
3. Low or absent genetic differentiation, between populations, as measured by methods designed to test the extent of gene product divergence (i.e. starch gel electrophoresis of isoenzymes).
4. Presence of limited genetic differentiation, as measured by more sensitive methods designed to test sequence divergence (i.e. mtDNA RFLP, or DNA sequencing). As argued in Chapter 5 (page 99), this should be taken as an indication that gene flow has become restricted in recent evolutionary times, before gene product differentiation has become apparent.
5. Absence of pre-mating behavioural barriers (i.e. modified Specific Mate Recognition Systems).
6. Proneness of the species distribution to become fragmented and, consequently, to form small isolated demes (see King, 1993).

Should evidence for all of these prerequisites be available, it may nevertheless be difficult to circumvent Carson's (1982) argument that such chromosomal rearrangements arise as a result of a species adaptive response to a new habitat. This proposition implies, albeit

from a biological species concept viewpoint only, that the colonization of a new habitat *per se* represents sufficient a barrier to interbreeding. However, in the absence of genetic differentiation (chromosomal or otherwise) and mating behaviour differences, there would be little to prevent interbreeding upon secondary contact during subsequent habitat expansion phases: hence no isolation has occurred. Also, from a species recognition concept standpoint, if the two populations still share a common fertilization system, no speciation event has occurred. Consequently, in a conceptual context where isolation mechanisms cause speciation, arguments on etiology and causality in chromosome rearrangements become secondary whereas the most relevant issue is whether chromosomal isolation takes place or not or - to give it a temporal connotation - if any form of effective isolation occurs before the establishment of chromosomal barriers. The "chromosomal isolation precedence" argument can be demonstrated by providing evidence to points 1 to 6 above.

◆ *Chromosomal variation*

• *Interspecific variation*

The cytogenetic information presented and discussed in chapter 2 documented considerable variation in the karyotype of *O. irroratus*. Such an extensive variability was not observed in the karyotypes of other congeners.

As has been shown (Chapter 2, page 23), the karyotype in *O. angoniensis* specimens from three widely separated localities, was found to be constant. Although previous work on this species (Matthey, 1964) only described unbanded chromosomes of specimens from Krugersdorp (Gauteng Region, South Africa), the correspondence in diploid and fundamental numbers and general chromosome morphology - between Matthey's and the present study - indicate that the karyotype of *O. angoniensis* is conservative over the South African area of distribution. However, since this species is reported to be present in Namibia, Botswana, central Zimbabwe, Mozambique, and sub-

Saharan Africa north of the Limpopo river (Skinner and Smithers, 1990); further cytogenetic work on specimens from these areas is required to establish whether this karyotype is consistent throughout the species distribution range.

Similarly, conservative karyotypes ($2n = 42$) were observed in the species *O. sloggetti robertsii* (Contrafatto *et al.*, 1992) and *O. unisulcatus* although in the latter, some variation in the presence of satellites on one chromosome pair was proposed (van Dyk, 1990). Both species were sampled throughout their respective distribution ranges, hence, a conclusion that they are chromosomally conservative appears justified. Two females of *O. laminatus* (from Karkloof and Umtamvuna) so far studied, displayed diploid numbers of 28 and 34. Both showed four pairs of large sub-metacentric chromosomes and eight pairs of medium and small acrocentrics but differed in the number of microchromosomes or B-chromosomes present (unpublished pers. obs.). Considering the discontinuous distribution of this species (see Skinner and Smithers, 1990), a finding of more substantial chromosomal variability would not be unexpected, although because of limited information no conclusion can be reached at this stage. From the data in hand, however, there is little doubt that *O. irroratus* is the most chromosomally variable species and the most appropriate example of chromosomal speciation within the genus.

- *Shared sequences*

Robinson and Elder (1987) found a complete lack of sequence analogy between the banding patterns of *O. irroratus* and *O. unisulcatus* chromosomes; they pointed out that such a lack of similarity could be taken as an indication of extensive chromosomal rearrangement having occurred within *O. irroratus*. Their findings were indirectly confirmed by cursory comparison of *O. irroratus* karyograms, from the present study, with those of *O. unisulcatus* prepared by van Dyk (1990); a thorough comparison was not attempted since the focus of my investigation was on the intraspecific variation in *O. irroratus*. For the same reason, comparison of *O. irroratus* chromosome banding patterns with those of *O.*

sloggetti was also not carried out. On the other hand, a search for homologies between *O. angoniensis* G-banded chromosomes and those of *O. irroratus* was considered necessary since the former species provided the most appropriate outgroup from which to root intraspecific phylogenies.

Five sequences of G-bands from the *O. angoniensis* karyotype were found to match sequences in the *O. irroratus* karyotype. A diagram of *O. irroratus* banding pattern with the coded nomenclature of the bands, modelled on the 1971 Paris report recommendations for human chromosomes (Hamerton, 1973), is shown in figure 6.1 (page 107) while the banding pattern analogies are depicted in figure 6.2 (page 107). A relatively small portion of *O. angoniensis* chromosome pair 1, which included the distal six bands, appeared to match the distal part of *O. irroratus* chromosome 1(1q26→qter). Due to the close spacing of some of these bands and the undercoiled appearance of this *O. angoniensis* autosome, the accuracy of this match was doubtful. For similar reasons the presumed homology of *O. angoniensis* chromosome 3 to most of autosome 4 of *O. irroratus* (4q12→q28) was also dubious. More confidence was placed, subjectively, in the remaining three matches. These included the five distal bands of *O. angoniensis* chromosome 5 which were similar to the 2q21→qter segment of *O. irroratus* chromosome 2; chromosome 2 of *O. angoniensis* which, with the exception of the centromeric region and the terminal dark band, matched the pattern of *O. irroratus* chromosome 3, and the whole of chromosomes 7 of *O. angoniensis* and 10 of *O. irroratus*. Taking staining intensity into account, bands 10q24 and q26 of *O. irroratus* consistently appeared inverted with respect to their corresponding bands on *O. angoniensis* chromosome 7 (see figure 6.2, page 107 and figure 2.6 page 33). This indicates a rearrangement, either in the form of a translocation or of a paracentric inversion of the 10q24→q26 portion. The hypothesis of a translocation was not testable because the small size of the segment involved, and the resolution limit of the techniques employed, made it impossible to determine its origin. Possibly, future analysis of banded prophase chromosomes may resolve this and other uncertainties of band sequence

similarities. For the moment, however, it seems more parsimonious to assume an inversion. In either case this point is not a crucial one since, for the purpose of cladistic analysis, any *O. irroratus* chromosome analogous to an *O. angoniensis* chromosome was considered an ancestral character, whether the similarity was complete or partial.

The five homologies found between the banding pattern of *O. angoniensis* and that of *O. irroratus* (see Chapter 2, page 33 and Figure 6.2, page 107) resulted from the comparison of only a portion of the two species karyotypes. *O. angoniensis* chromosomes in metaphase preparations were generally shorter than those of *O. irroratus* and many of them, such as pairs 16 to 27, showed no more than three or, at most, four distinguishable bands. Interpreting the sequence of closely spaced bands, was considered futile because the confidence in the interpretation of resulting matches would have been too low. Therefore, the final comparative analysis took into account only the first fifteen pairs of *O. angoniensis* and the first eleven pairs of *O. irroratus* chromosomes. It is, therefore possible, that further homologies may emerge should cytogenetic techniques such as high resolution G-banding, become more reliable.

- *Summary of O. irroratus variation*

To summarize the findings described and discussed in Chapter 2, five major cytotypes were apparent within the species *O. irroratus*. Most localities sampled in the Eastern Cape region of South Africa - namely Alice, Committees Drift, Grahamstown, Port Elizabeth and Tsitsikama (Robinson and Elder, 1987) - yielded specimens with a diploid chromosome number varying from 28 to 30 with autosomes 1 to 10 biarmed. The short arms of these chromosomes - with the exception of pair 6 - were both, in my study and in a previous one concerning the Tsitsikama locality (Robinson and Elder, 1987) completely heterochromatic. Numerical variability in this cytotype was due to the varying number of B-chromosomes which, for reasons of consistency with previous work on this species, were classified as autosomal pairs 8 and 9.

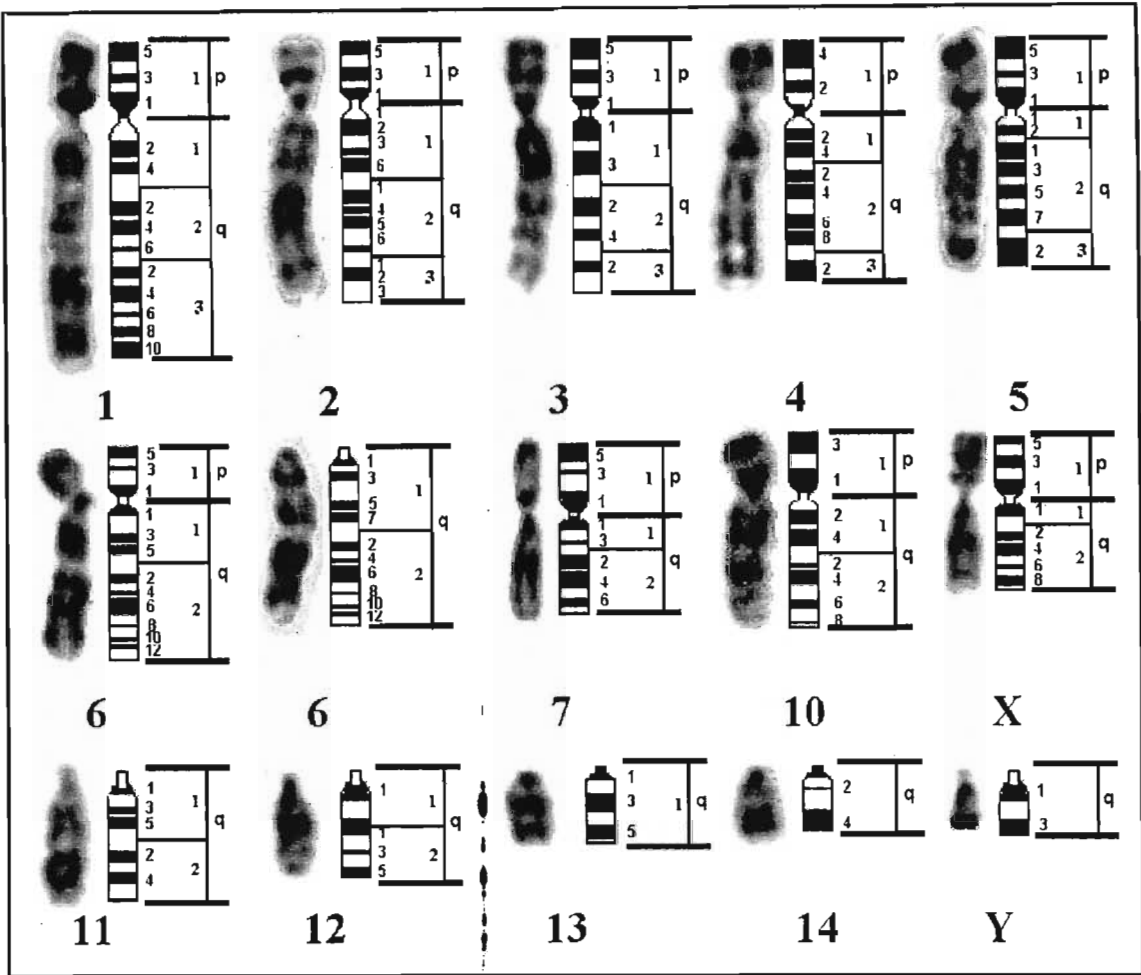


Figure 6.1 - Karyogram and ideogram of *O. irroratus* B cytotype constructed from ten chromosome sets. Polymorphic B-chromosomes (pairs 8 and 9) not included.

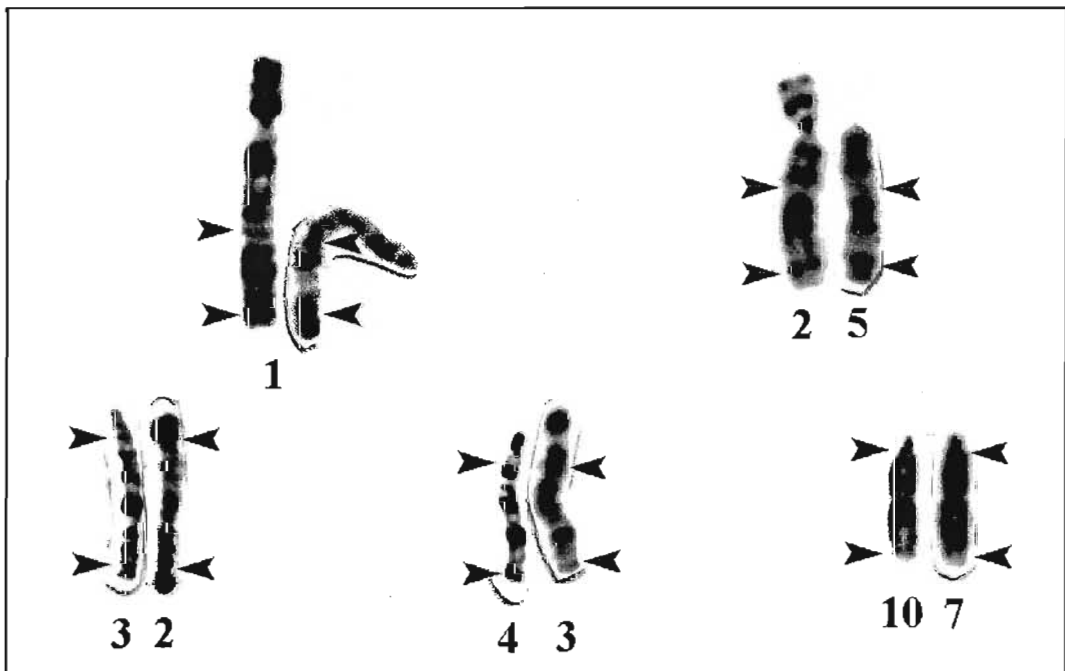


Figure 6.2 - Proposed analogies of G-banding sequence between *O. angoniensis* (right of each pair) and *O. irroratus* (left). Analogous sequences shown between arrowheads.

Moreover, at the Grahamstown locality, heterozygosity involving the presence or absence of short arms on pairs 6 and 10 was also detected. In the case of autosome 6, the short arm was not heterochromatic and was explained as being the result of a pericentric inversion. For convenience, this cytotype was labelled as Group B.

At more eastern localities, mostly in the KwaZulu/Natal region, all *O. irroratus* karyotypes were found to have almost exclusively telocentric autosomes (Group A cytotypes). These originated from Dargle, Garden Castle, Hogsback, Kamberg, Karkloof, Loteni, Stutterheim, Umgeni Valley, Umtamvuna and Vergelegen. Numerical variability here was greater than in Group B and ranged from 24 (Hogsback, Kamberg and Vergelegen) to 32 (Karkloof). Much of the variability in diploid numbers was, as in the case of Group B, due to presence or absence of varying numbers of copies of B-chromosomes in pairs 8 and 9 in different individuals from any of the populations studied. While the karyotypes from Stutterheim and Umtamvuna could be described as a B-karyotype without short arms, a significant rearrangement was observed at Garden Castle, Hogsback, Kamberg, Loteni, Vergelegen and, subsequently, at Umgeni Vlei near Kamberg (Taylor, pers. comm.). All individuals from these populations carried a tandem fusion between autosomes 7 and 12. A further numerical variant, with an extra two pairs of medium sized telocentric chromosomes and no t(7:12), was found at Dargle, Karkloof and subsequently at Nottingham Road which is 10 km from Umgeni Vlei (Taylor, Pers. Comm.).

The last cytotype (Group C), comprising the localities of Bloemfontein, Constantia, Mareepskop and Rietvlei, and later also found at Kuruman in the North-Western Region (Kumirai, Pers. Comm.), was intermediate between groups A and B as it was characterized by the presence of heterochromatic short arms on autosomes 1 to 3 (and 13 at Constantia) and euchromatic short arm on chromosome 6 (i.e. pericentric inversion). A more detailed summary of the information presented above can be seen in Table 2.3 (Chapters 2, page 24) while the localities are mapped in figure 2.1 (page 18).

Clearly both euchromatic and heterochromatic rearrangements occur in *O. irroratus*

and, in turn, give rise to numerical changes.

Amongst the euchromatic changes uncovered so far, one should include the two pairs of telocentric chromosomes, a1 and a2 unique to the A2 cytotype, the pericentric inversion of autosome 6 - typical of Groups B and C - and the 7-12 tandem fusion of Group A1 (see figure 2.6 page 33).

- *Chromosomes a1 and a2*

The significance of these two pairs is obscure because it has not been possible, so far, to confidently determine their origin. Two possibilities exist to explain their etiology: a1 and a2 are either autapomorphic, derived from other elements of the *O. irroratus* karyotype; or they are plesiomorphic chromosomes relics from a hypothetical ancestral karyotype.

The autapomorphy hypothesis implies that they were formed by (a) translocations from other *O. irroratus* chromosomes or (b) duplication or amplification of existing band sequences.

- (a) Although the banding pattern of these chromosomes may resemble that of other *O. irroratus* autosomes (i.e. pair 10), no portions appear to be missing from other chromosomes of this cytotype thus suggesting that translocations are unlikely as etiological factors. The possibility that the translocated portions are so small as to go undetected by standard cytogenetical analysis, seems implausible because the size of the chromosomes in question would require an unparsimonious number of translocated segments. Considering that the a1 and a2 chromosomes represent 11 % of the haploid autosomal set. Translocation, therefore, would require an 11 % difference in total autosomal length between the other cytotypes and the A2 cytotype excluding a1 and a2 chromosomes. However, measurements of digitized karyograms, standardized to display autosome 1 always in the same length of 144 pixel, never differed from one another by more than 1.03 %, which can be considered to be within limits of measurement error. Taken in conjunction with the

lack of obvious band sequence homologies, this rules out the possibility that chromosomes a1 and a2 were formed by translocation of existing *O. irroratus* band sequences.

- (b) Duplication of existing chromosomal portions also requires, as evidence, the detection of homologous sequences elsewhere in the same karyotype. A resemblance to the G-banding pattern of chromosome 10 and, possibly, part of autosome 2 (portion q21→q31) has been observed in some preparations. However, these observations were not consistent and did not represent convincing matches. This possibility, therefore, is not supported by clear-cut cytogenetical evidence from available material. This may mean that if duplications and/or amplifications have occurred, they must have involved several, relatively small, chromosomal portions thus reasorting existing band sequences. This kind of duplication could have been followed by interchromosomal transposition (i.e. duplicative transposition) a mechanism which has been demonstrated in *Zea mays* (McClintock, 1984) and in the genus *Drosophila* in association with Foldback (*FB*), *MR*, *I* and *P* transposable elements (Collins and Rubin, 1984; Green, 1986; John and Miklos, 1988; King, 1993). For example, *P* factors are usually stable in their original *P*-strains but their transposition is activated by their introduction, through hybridization, into *M* strains of *D. melanogaster*. Engels and Preston (1984) demonstrated that transposition of *P* factors produced chromosomal rearrangements, mostly in the form of inversions but also as multiple breaks with random rejoining.

One difficulty in extrapolating this kind of mechanism to the situation in the *O. irroratus* A2 cytotype is that, under the current proposition, transposed mobile elements are supposed to form new chromosomes; that is, a1 and a2. This in turn, should result in new cellular functions most of which, by and large, are expected to be deleterious and would thus result in the elimination of carriers from the population. However, the type of chromatin involved in such

events is most frequently made of middle-repetitive DNA sequences (see Doolittle and Sapienza, 1980) which are often, but not always, associated with heterochromatin (King, 1993) and which Orgel and Crick (1980) consider as a type of "selfish DNA". Because repetitive DNA does not generally show transcriptional activity, extranumerary chromosomes hypothetically formed by transposition of repetitive DNA, and which have reached fixation in a given organism, may be tolerated due to their lack of phenotypic effect.

There is no direct evidence indicating that transposition has occurred in *O. irroratus* and that it has played a role in the formation of the two chromosome pairs unique to the A2 cytotype. Nevertheless, the occurrence of mobile elements transposition in other organisms where it generates chromosome rearrangements and thus, raises the possibility that such a mechanism can also operate in *O. irroratus*.

On the other hand, should the a1 and a2 chromosomes be plesiomorphic, the possibility exists that they would also be found in the outgroup species, that is, analogous band sequences may be detected in the *O. angoniensis* karyotype. As discussed above, however, no strong analogies were observed, although this does not rule out a derivation of these two unique elements of the A2 cytotype from the outgroup karyotype or from that of a common ancestor. Conceivably, repeated rearrangements during the evolutionary history of the genus, could have reassorted their banding patterns. Further, a plesiomorphic hypothesis requires a mechanism to account for chromosome loss such as translocation, non-disjunction and anaphase lagging. Evidence of translocation involving these chromosomes should include demonstration of homologous band sequences in other *O. irroratus* cytotypes and increase in total autosomal length of about 11 %. No such evidence was found (see above), hence this possibility can be discounted. This, as well as suggestions of non-disjunction and/or lagging, implies that these chromosomes have been lost

altogether in the other *O. irroratus* karyotypes. It is, however, difficult to reconcile this possibility with the commonly held opinion that loss of significant euchromatic portions results in non-viable or severely deficient individuals, due to loss of vital genotypic information, as expected in any cytotypes lacking these chromosomes. This need not be the case if, as argued above, chromosomes a1 and a2 were composed mostly of non-transcribed middle-repetitive DNA. However, this appears less parsimonious than the assumption of autapomorphy since it implies two events: firstly, the formation - by duplicative transposition - of these elements in a hypothetical ancestral karyotype and, secondly, the loss of the same to generate derived *O. irroratus* cytotypes.

The more likely explanation, therefore, seems to be that chromosomes a1 and a2 are derived characters originated by duplicative transposition of non-transcribed repetitive DNA. There is at present, no direct evidence indicating that this is indeed the case and other mechanisms such as reverse transcriptase mediated transfer, gene conversion and unequal chromatid exchange are equally possible (see King, 1993). The only conclusion that can be made with the available data, is that these chromosomes have not derived from the *O. irroratus* karyotype by conventionally understood mechanisms of chromosomal rearrangements. The elucidation of this aspect, therefore, awaits further experimental work requiring the use of molecular techniques which were not planned for this study.

- *Pericentric inversion of chromosome 6*

The pericentric inversion of chromosome 6, found in specimens from several localities of the Eastern and Western Cape regions (see inset in figure 2.4-A, page 27), presented another notable vlei rat chromosome polymorphism. In its acrocentric form, this autosome was always found as homozygotic in the all-acrocentric cytotypes while its biarmed morph was homozygotic in most, but not all, animals from localities where B and C cytotypes occur. Heteromorphism was found in one case each at Alice and Constantia, and one individual from Tsitsikama was reported by Robinson and Elder (1987). This

heterozygotic condition, however, was most frequent in the Grahamstown population. There it formed a balanced polymorphism, detected in three of six individuals; two others were homozygous biarmed and the last was homozygous acrocentric. This finding may be regarded as surprising since inversions are expected to induce negative heterosis of the heterokaryotype by producing unbalanced gametes due to cross-over within the loop formed at pachytene (White, 1973 *op. cit.* King, 1993). Perhaps, the most notable example of taxa whose karyotypes differ by pericentric inversions can be found in the higher primates. Here, the human karyotype is differentiated from that of the orangutang by, amongst other rearrangements, three pericentric inversions (human chromosomes 3, 4 and 17), from that of the chimpanzee by three pericentric inversions (4,5 and 12) and from that of the gorilla by two (8 and 10) (de Grouchy *et al.*, 1973; see also Sites and Moritz, 1987). The role played by inversions in the cladogenesis within this family, however, has not been demonstrated and their heterotic effect, in general, has not been well documented. As a matter of fact, lack of inversion loop formation in synaptonemal complexes appears to be a frequent feature of mammals heterozygous for inversions. These include the sand rat (Ashley *et al.*, 1981), the mouse (Moses *et al.*, 1982), the deer mice of the *Peromyscus maniculatus* complex (Hale, 1986; Hale and Greenbaum, 1988) and humans (Saadallah and Hultén, 1986; Chandley *et al.*, 1987). In these cases, pairing occurs by synapsis of non-homologous chromosome segments (heterosynapsis). In reviewing various heterosynapsis mechanisms - such as terminalization of chiasma location, reverse pairing and synaptic adjustment - King (1993) concluded that if these mechanisms are present, pericentric inversions can reach fixation in isolated populations by stochastic processes alone. Equally, they could remain as neutral and/or balanced polymorphisms (King, 1993). Although, at the time of writing, no synaptonemal complex analysis of *O. irroratus* has been carried out and there are no available available on the breeding biology of the Grahamstown population, there is little doubt that the chromosome 6 polymorphism is consistent with the argument presented above. The proportions of heterozygotes and

homozygotes present in this population point to little, if any, negative heterosis and, hence, little involvement of this inversion in the reproductive isolation of *O. irroratus*.

A puzzling aspect of this rearrangement is the fact that the acrocentric morph is stable in the Eastern part of the *O. irroratus* range, since it was never found in its biarmed configuration in any of the acrocentric cytotypes. Three hypotheses can be proposed to explain this finding.

Firstly, the presence of a unarmed autosome 6 could be a product of introgression from the A chromosomal race. Considering the short geographic distance separating these cytotypes - less than 30 km between Stutterheim and King William's Town (Maden Dam) which is the eastern-most B cytotype population thus far uncovered (A. Kumirai, pers. comm.) - the occurrence of hybridization appears possible. This hypothesis is supported by the finding of another polymorphism, at Grahamstown, in which autosome 10 was present in both forms: acrocentric and biarmed (see figure 2.4-D page 27). That no members of pairs 1 to 5 and/or pair 7 were in the heterozygous state in the B type, may be due to limited sample sizes: further sampling in this area of the Eastern Cape is indicated in order to confirm this.

Secondly, polymorphism of the chromosome 6 inversion may in fact be a relic of past transition from acrocentric to biarmed cytotypes or *vice versa*. Depending on the direction of change, either of which has equal likelihood on cytogenetical grounds, this inversion would have been the last rearrangement following the acquisition of heterochromatic short arms by an A cytotype, or the first one preceding the loss of heterochromatic short arms in a shift from B to A cytotypes. The lack of negative heterosis of this rearrangement may have allowed the persistence of the heterokaryotype for an indefinite period after the event. Dimorphism of the heterochromatic short arm of autosome 10 could have been a concurrent event, as indicated by the presence of this balanced polymorphism in the same Grahamstown individuals polymorphic for the inversion of chromosome 6.

As illustrated in figure 6.3 (trees 1 and 2), however, this hypothesis is phylogenetically not parsimonious because it postulates a direct shift from B to A, or *vice versa*, which requires - amongst other reversals - the re-occurrence of the inversion to allow for the transition from race A to race C. No homoplasy would occur with regard to autosome 6 should the shift take place in the opposite direction, from A to B. However, extra steps would be required because autosomes 4, 5, 7 and 10 would have to lose their heterochromatic short arms to form cytotype C.

Thirdly, chromosome 6 is unstable when in the banded cytotypes and, therefore, is prone to undergo repeated inversion. If this is the case, it would appear that the position of the break-point is remarkably consistent, strongly indicating the presence of "hot spots" in this chromosome. In turn, the presence of "hot spots" can be related to the presence of transpositional activity (King, 1993). For example, Engels and Preston (1981; 1984), have documented in *Drosophila* that transposition of *P* elements causes non-randomly distributed chromosomal breakage. Almost 85 % of these break-points were located at the genomic position of *P* elements and subsequently produced rearrangements of which most were inversions. Mobile elements have also been implicated, by McClintock (1984), in the reorganization of the *Zea mays* karyotype when this is destabilized by experimentally produced breakage of chromosome 9. In view of the evidence available in the literature (see King, 1993) and the finding of this study, it may be postulated that mobile elements are present in the *O. irroratus* genome. Although such elements have not been identified thus far, a possible candidate can be observed, by cytogenetic techniques, in autosome pair 6 of the Constantia individual heterozygous for the inversion. As depicted in figure 2.5-B (page 30), when stained with Giemsa following alkaline treatment, the banded member of this pair displays a prominent magenta-red spot in the telomeric region of the short arm. This indicates chromatin homogeneity, hence sequence repetition, in this telomeric region. As can be seen in figure 2.5-A (page 30), the telomeric region of the autosome 6 short arm is sensitive to *A*/*u* I digestion thus prompting the suggestion that it may be composed of

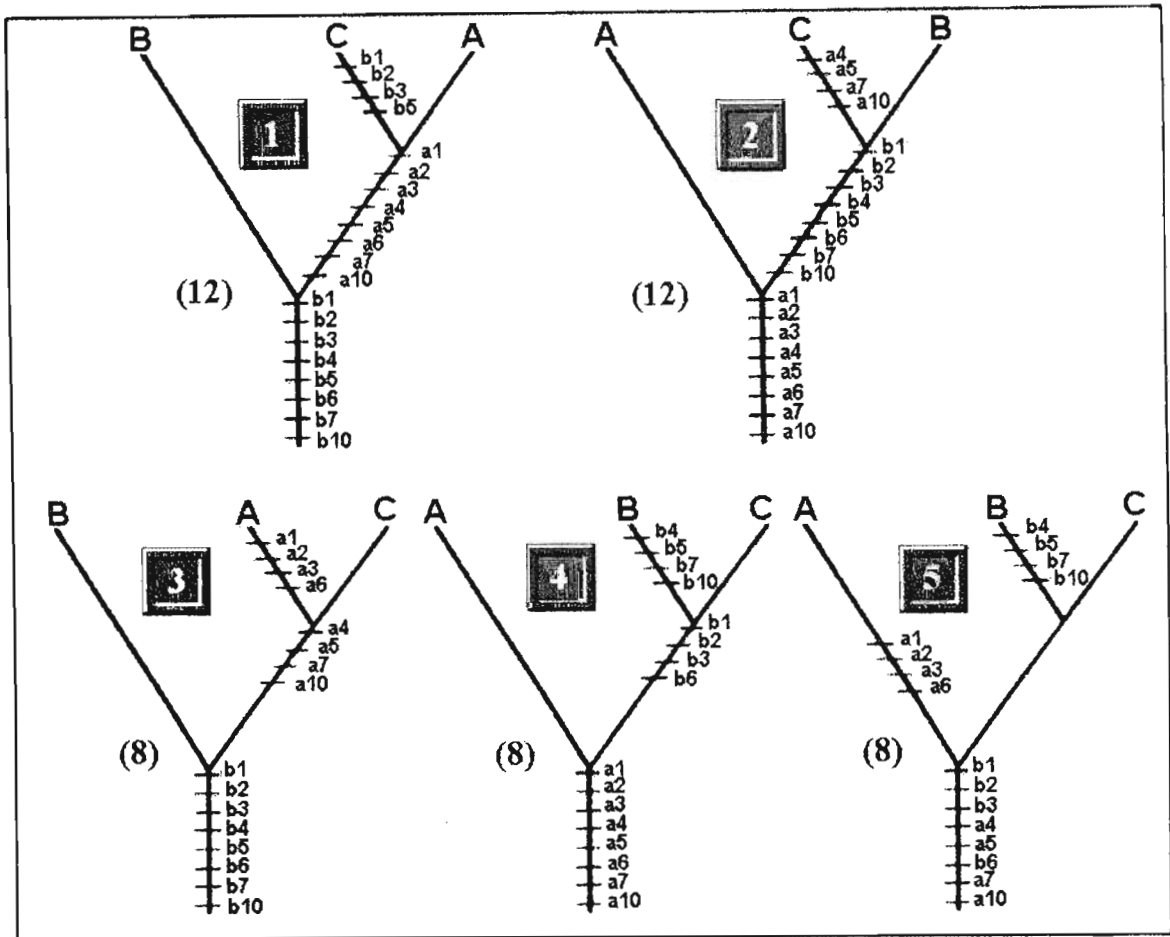


Figure 6.3 - Phylogenetic trees of *O. irroratus* cytotypes according to acrocentric (a) and biarmed (b) chromosomes with ancestral states indicated at each root (number of steps in brackets).

repeats including the *Alu I* recognition sequence (AG↑CT). The inverted homologue (see figure 2.5-B, page 30) does not show an equivalent red spot at the expected interstitial position on the long arm. This pinpoints one break-point as being located below the telomere - if the inversion proceeds from biarmed to acrocentric - or that amplification, a phenomenon for which transposons are responsible (King, 1993), of an AGCT-rich region occurs in the case of the opposite inversion.

It remains, nevertheless, difficult to explain why - after the triggering of transpositional activity and the ensuing repeated inversions - chromosome 6 dimorphisms should remain balanced at Grahamstown and have all but collapsed into monomorphism in the other populations. An intuitive answer which comes to mind readily, points to the

environment at this locality: instability of this autosome may be driven by environmental conditions. This is consistent with White's (1973) suggestion that polymorphisms are maintained by selective gradients (*op. cit.* King, 1993), a view also advocated by Shaw *et al.* (1985) and King (1982). As pointed out by King (1993), "genomic stress" seems to trigger transpositional activity and genomes could possibly become destabilized by the "bottlenecks" typical of founding populations (McClintock, 1984; Carson, 1990 *op. cit.* King, 1993). Data presented in Chapter 3 indicate that there is nothing peculiar about the climatic variables of the Grahamstown locality and that these fall squarely within the parameters of the B cytotype. Furthermore, at present there is no evidence to the effect that the animals sampled at Grahamstown originated from a recent founding event. Thus, for lack of a triggering cause - and bearing in mind William of Occam's *dictum* - the hypothesis of inversion polymorphism mediated by mobile elements should be considered, until further evidence becomes available, less likely than that of introgression by hybridization with populations of the A cytotype.

- *Tandem fusion*

The translocation of autosomes 7 and 12, typical of the A1 cytotype, was found in populations occupying the montane Drakensberg climatic region (see Chapter 3).

Meiotic malsegregation in t(7,12) heterokaryotypes is expected to generate 50 per cent aneuploid gametes (see King, 1993). The rate of failed reproduction, however, may be much higher because of the relationship between pachitene multivalents and impaired spermatogenesis (Evans, 1976; Redi and Capanna, 1988; Zuccotti *et al.*, 1995). In some cases of Robertsonian fusion in mice, for example, terminal asynapsis of trivalents and their association with the gonosomes correlates with drastically reduced sperm counts (Setterfield *et al.*, 1988). In other cases, no such a correlation has been documented although low sperm counts ranging from 0 to 50 per cent of homokaryotype counts, were recorded (Mahadevaiah *et al.*, 1990). It must be stressed, however, that these studies refer to total

sperm counts and the proportion of non-motile, slow or morphologically abnormal sperms is not known.

In conclusion, the 7/12 tandem fusion can be predicted to cause at least, but probably more than, 50 per cent reproductive failure in heterozygotic hybrids. As has been pointed out in Chapter 2 (page 39), this prediction was confirmed by breeding experiments involving individuals with A1, A2 and B karyotypes which will be discussed later (see page 121-125).

- *Heterochromatic changes*

As far as these chromosomal changes are concerned, there were two major classes: those involving gain or loss of heterochromatic short arms and those showing gain or loss of B-chromosome copies. Variation in heterochromatic short arms is not uncommon in the evolution of rodent karyotypes: it has been reported, *inter alia*, in the genera *Thomomys* (Patton and Yang, 1977; Patton and Sherwood, 1982), *Peromyscus* (Robbins and Baker, 1981), *Onychomys* (Baker *et al.*, 1979), *Uromys* (Baverstock *et al.*, 1982), *Praomys/Mastomys* (Qumsiyeh *et al.*, 1990) and the Indian pigmy mouse *Mus dunnii* (Sharma *et al.*, 1990).

Although constitutive heterochromatin has traditionally been considered to be transcriptionally inactive highly repeated DNA (Hsu, 1973), some (Fredga and Mandahl, 1973) have suggested the possibility that it may have an evolutionary function, possibly by increasing the potential for chromosomal variability. Others (Corneo, 1976; Fry and Salser, 1977) have argued for heterochromatin as an isolating mechanism because of its content of satellite DNA, which is fundamental to meiotic pairing. Hybridization studies in *Thomomys bottae* (Patton and Yang, 1977) and *Uromys caudimaculatus* (Baverstock *et al.*, 1982), however, failed to demonstrate reduced fertility in hybrids between cytotypes characterized by heterochromatic polymorphism. Furthermore, more recent reviewers (John, 1988; John and Miklos, 1979; 1988; Sharma *et al.*, 1990; King, 1993) have

concluded that the function of heterochromatin is unknown and unlikely to influence karyotype evolution.

On the other hand, Whichman *et al.* (1991) have suggested that the evolutionary function of constitutive heterochromatin may be related to its quantity and position within the karyotype. This argument implies that, rather than an intrinsic ability to cause hybrid negative heterosis, heterochromatin may influence the frequency of chromosomal rearrangements by providing "safe" break-points when interspersed within euchromatin. Thus, chromosome rearrangements would be less prone to survive in taxa with large terminal heterochromatic blocks and/or heterochromatic short arms, because breaks in euchromatic regions are more likely to disrupt coadapted or linked gene complexes or otherwise functional genes. To take this argument further, in cases where highly or middle repetitive sequences contain transposons, their consolidation within terminal heterochromatic blocks could reduce the frequency of euchromatic transpositions and concomitant disruption of transcriptionally active sequences. This might be the case in *O. irroratus* where the only potentially heterotic chromosomal rearrangements (i.e. the Robertsonian and tandem fusions illustrated in figure 2.3-D page 25) have occurred in acrocentric cytotypes which have interstitial and relatively small C-positive bands. On the other hand, only one rearrangement (polymorphic autosome 6) has been observed in the biarmed cytotypes; as discussed above, this is of low "isolating" potential and, in any event, more likely to have derived from introgression. Similarly, the congeners *O. angoniensis*, *O. sloggetti* (Contrafatto *et al.*, 1992) and *O. unisulcatus* (Robinson and Elder, 1987) display mostly small blocks of centromeric C-positive heterochromatin, and they are chromosomally conservative. Confirmation of this hypothesis, however, is unlikely to come from the analysis of rodent cytogenetic literature because of inconsistent reporting and varying quality of illustrations. Due to a lack of documented cases, therefore, influence of heterochromatin on chromosomal rearrangement frequency and, indirectly, post-zygotic isolation cannot be assumed.

B-chromosomes were originally described by Wilson (1907) in hemiptera (cited by Vujošević, 1993). They were subsequently found in both insects and plants (Östergren, 1945; Müntzing, 1958; Nur, 1966; Hewitt and John, 1967; Dover and Henderson, 1976) and can be defined as supernumerary, often partly heterochromatic, elements of the karyotype (Volobujev, 1981; John, 1988). Although B-chromosomes seem to be a common occurrence in plants, as they are recorded in more than 1300 species, they are uncommon in mammals (Vujošević and Blagojević, 1995). In 1989, Civitelli *et al.* reported that 29 rodent species were known to carry these chromosomes while Vujošević (1993), put the total of B-carrier mammal species at 34; these are probably underestimates since many reports fail to identify polymorphic extranumeraries as B-chromosomes. Because of their suspected negative effect on carriers and their tendency to accumulate in more than one copy, B-chromosomes have been considered as parasitic elements of the karyotype (i.e. neither gonosomes nor autosomes) and their presence in a karyotype seems to be due to their high transmission rates (Nur, 1967, 1977). As an alternative to this parasitic model, a heterotic model has been suggested (White, 1973) to explain why, given their meiotic and/or mitotic drive, B-chromosomes do not accumulate indefinitely in the karyotypes where they are present: low numbers of B's could be positively heterotic while high numbers could be negatively heterotic. Furthermore, B's may be parasitic in some populations, or in one sex, and heterotic in others (Lopez-Leon *et al.*, 1992). The high transmission rates, have been ascribed to meiotic drive in the form of meiotic non-disjunction (Nur, 1977) or, in cases of mosaicism, to irregular mitotic non-disjunction (Volobujev, 1981).

B-chromosomes, and heterochromatic knobs, have been shown to correlate with altered meiotic recombination rates in plants, acting as promoters of chiasma formation in some species and as depressors in others (Brandham and Bhattarai, 1977; Chang and Kikudome, 1973; Chilton and McCarthy, 1973; Nel, 1973; Rhoades, 1978). Reviews of chromosomal evolution (see John, 1988; John and Miklos, 1988; King, 1993), however,

have tended to attribute no evolutionary value to these chromosomes or to ignore them altogether. On the other hand populations of the rodent *Apodemus flavicollis*, with and without B's, have been shown to differ significantly in directional and fluctuating asymmetries, which implies reduced developmental homeostasis and, in turn, reduced heterozygosity in the presence of B-chromosomes (Blagojević and Vujošević, 1995a). Furthermore, in the same B-carrying populations of *A. flavicollis*, Blagojević and Vujošević (1995b) found marked seasonal variation in the number of individuals carrying these chromosomes which was correlated with increased population size. These authors argued that their data were consistent with high mortality rates of B-carrying juveniles under conditions of demographic stress (Vujošević and Blagojević, 1995).

While some indication of meiotic drive was apparent in the *O. irroratus* Karkloof population (see Chapter 2, page 28), there was little indication that B-chromosomes had obvious effects on population structure or individual fitness in this or other *O. irroratus* populations. As will be discussed in the next section, however, their possible contribution to negative heterosis of interpopulation hybrids cannot be excluded in view of the similarity of their heterochromatin with that of the X chromosome. Such similarities include resistance to *Alu I* digestion and identical reaction to incubation at alkaline pH (see figure 2.5, page 30) suggesting that this heterochromatin is facultative rather than constitutive. This implies that these B-chromosomes may originate from an ancestral, possibly inactivated, gonosome; their drive may, therefore, be due to likely associations with the sex chromosome bivalent during first meiotic division. Confirmation of this hypothesis requires further investigations employing molecular methods.

◆ *Reproductive biology and pre-mating behaviour*

The reproductive biology of some *O. irroratus* populations has been investigated by Pillay *et al.* (1992; 1995). In the 1992 study, these authors reported on the reproductive fitness of one population each from the A1 (Hogsback), A2 (Karkloof) and B (Committee's

Drift) cytotypes and their respective hybrids; similar information was included in the 1995 report on Karkloof and another A1 population: Kamberg. Some of the results are summarized in table 6.1. While intrapopulation matings were all successful (91/91) with an average litter size of 2.3 and fecundity of 5.8, the situation was more complex in interpopulation crosses. Here, Committee's Drift animals crossed with Hogsback specimens showed a 30 per cent reduction in mating success while Karkloof matings with Hogsback and with Committee's Drift were both 90 per cent successful but half of the matings of Karkloof and Kamberg animals failed. Average litter size were comparable to those of intrapopulation pairings, except for Kamberg and Karkloof matings, whereas average fecundity was reduced to 4.4. A significant difference in tertiary sex ratio in favour of females was recorded in the Committee's Drift x Karkloof crosses, indicating a high male preweaning mortality. Hybrids from Kamberg and Karkloof pairings were of lower body-mass and slower growth than purebred young (Pillay, 1995). A dramatic reproductive breakdown was reported for hybrids heterozygous for the tandem fusion (i.e. from Hogsback or Kamberg mated with other populations) with an overall 96 per cent failure to reproduce, either in backcrosses or hybrid crosses. This strongly implicates the 7/12 tandem fusion as a prime cause of infertility especially because reproductive breakdown in hybrids involving Karkloof and Committee's Drift was much lower (44 per cent).

A 96 percent reproductive failure appears excessive since only 50 per cent of gametes, produced by heterozygotes for a tandem fusion, should theoretically be aneuploid but may be reasonable if - as discussed earlier (pages 117-118) - one considers the likelihood that such a low euploidy may result in sperm counts inconsistent with fertility. Although this implies male sterility only, which is not indicated by the data in table 6.1, oogenesis may equally be disrupted despite the availability of the polar body to dispose of abnormal meiotic products (see King, 1993). Pillay (1990) did not observe any differences in sperm morphology between purebred and structural hybrids but there is, at present, no histological information concerning hybrid gonads, sperm counts and motility, ratio

between "normal" and "abnormal" spermatozoa and oestrus anomalies. Further work on hybrid gametogenesis is, thus, indicated to resolve this issue.

The figure of 44 per cent reduced fertility of Karkloof x Committee's Drift hybrids represents, give or take 2 per cent, the difference between the values of predicted aneuploidy and observed infertility in structural hybrids for the t(7,12). Should this be more than coincidence, it may be argued that the factors contributing to higher than expected infertility in the tandem fusion hybrids, are the same as those responsible for reduced fertility in the A2 x B (Karkloof x Committee's Drift) ones. The Karkloof a1 and a2 chromosomes cannot be implicated because they are unique to the A2 cytotype and because, due to their unaccounted origin, no prediction can be made as to their reproductive effect in structural hybrids. Hence, the causal factors must be found in features common to all the populations used in the breeding studies. To this purpose, Hogsback and Kamberg can be considered as one because, although geographically well separated, they share habitat and karyotype. Features common to these populations can be enumerated as follows.

1. They all are geographically separated and are, thus, likely to have undergone a limited amount of genetic differentiation. At present, however, there is no evidence to the effect that conspecifics, at this level of phenotypic divergence, can be reproductively isolated due to accumulation of differing gene mutations. More importantly, as will be discussed in the next section (*Allozymes and Western Blotting*, page 127), allozyme electrophoresis indicates little, if any, genetic differentiation.
2. They all can be assumed to have genetically different cytoplasmic inclusions, an assumption supported by mtDNA studies (see *Restriction Fragment Length Polymorphism*, page 0). This suggests the possibility of transposition being activated by the introduction of mobile elements into incompatible germ plasms, as considered earlier (see Sections *Chromosomes a1 and a2*, page 110 and *Pericentric inversion of chromosome 6*, page 115)

Table 6.1 - Reproductive data of some *O. irroratus* populations (modified from Pillay *et al.*, 1992). Males are indicated first first in any cross-mating pair. *: from Pillay *et al.* (1995).

| Matings | n | success | Matings | n | success |
|-------------------------|----|---------|-----------------------|----|---------|
| Intrapopulation | | | CD x Karkloof | 12 | 9 |
| Committee's Drift (CD) | 5 | 5 | Karkloof x CD | 12 | 7 |
| Hogsback | 6 | 6 | Hogsback x Karkloof | 12 | 3 |
| Karkloof | 10 | 10 | Karkloof x Hogsback | 12 | 0 |
| Karkloof * | 40 | 40 | Kamberg x Karkloof * | 24 | 0 |
| Kamberg * | 30 | 30 | Karkloof x Kamberg * | 24 | 1 |
| Interpopulation | | | Hybrid crosses within | | |
| CD x Hogsback | 10 | 7 | CD x Hogsback | 3 | 0 |
| CD x Karkloof | 10 | 9 | Hogsback x CD | 3 | 0 |
| Hogsback x Karkloof | 10 | 9 | CD x Karkloof | 3 | 1 |
| Kamberg x Karkloof | 20 | 8 | Karkloof x CD | 3 | 1 |
| Karkloof x Kamberg | 20 | 11 | Hogsback x Karkloof | 3 | 0 |
| Backcross of young from | | | Karkloof x Hogsback | 3 | 0 |
| CD x Hogsback | 12 | 0 | Kamberg x Karkloof * | 6 | 0 |
| Hogsback x CD | 12 | 0 | Karkloof x Kamberg * | 6 | 0 |

in the case of *D. melanogaster* mobile elements. Transpositional activity, in turn, could trigger multiple chromosome breaks and rearrangements.

3. They all have different karyotypes and their hybridization could result in a "chromosomal dysgenesis" of the type documented in the hybridization between biarmed and uniarmed chromosomal races of the white-footed mouse *Peromyscus leucopus* (Adkins *et al.*, 1991). Additionally, this model of recombinational breakdown is not incompatible with the previous transpositional one and, as a matter of fact, is likely to represent an overt karyological expression of it.
4. They all have B-chromosomes which, in an earlier Section (*Heterochromatic changes*, page 120) were implicated as factors influencing frequency and position of

meiotic recombinations as well as causing negative heterosis under certain conditions. Because of the similarities of *O. irroratus* B-elements (see page 121) with the X-chromosome heterochromatin, and the consequent likelihood of their meiotic associations with the gonosomal bivalent, their involvement in impaired fertility can be suspected. This hypothesis - consistent with Coyne and Orr's (1989) interpretation of Haldane's effect (see King, 1993) - predicts male inviability or sterility in F1 hybrids and is supported by Pillay's (1992) finding of higher male preweaning mortality in crosses, backcrosses and hybrid crosses involving individuals from the Committee's Drift population. Nevertheless, due to the obscure nature of B-chromosomes and the unpredictability of their behaviour, perhaps an indication of the heterogeneity of this group of chromosomes, any prediction relating to their effects on structural hybrid reproduction must be tentative.

It can be concluded, therefore, that the 7/12 tandem fusion is the major factor responsible for the post-mating isolation of the A1 from the A2 and B cytotypes and that other contributing factors are, in all likelihood, chromosomal as well. Such chromosomal factors are also the ones likely to affect reproduction in hybrids between A2 and B cytotypes. Confirmation of this hypothesis awaits further studies involving hybridization of populations from B, C and A cytotypes, which differ from one another only in the number of heterochromatic short arms.

In order to demonstrate post-mating isolation, however, it is necessary to establish if, and at which stage of the species evolution, pre-mating behaviour has played an isolating role.

Ethological pre-copulatory studies of the Committee's Drift, Hogsback and Karkloof populations (Pillay, 1990) and those of Kamberg in relation to Karkloof (Pillay, 1993) reported frequencies of videorecorded interactions at fixed daily times, between intra

and interpopulation pairs in neutral arenas. In all cases, at the beginning of a 12 days test period agonistic interactions were more frequent (80 - 100 per cent) than amicable ones, which were close to zero. Throughout the test period, agonistic interactions decreased while amicable acts increased progressively until, on day 12, they outstripped agonism by 60 to 80 percent according to the populations involved. The day when agonistic equalled amicable interactions (intersection of the two curves on a plot) might be assumed to indicate that the two individuals could recognize one another as potential mates. Thereafter agonistic behaviour frequencies never outstripped those of amicable acts.

While Hogsback and Kamberg (A1 cytotype) homotypes reached "potential recognition" in 3 and 2 days respectively, the most aggressive individuals belonged to the Committee's Drift population as they took about 5 days to reach the point of curve intersection. As may be expected from their elevated agonism and reduced amicability, interpopulation encounters involving Committee's Drift individuals reached intersection point later than other interpopulation pairs (9 days). Pairs involving Hogsback and Karkloof individuals showed no significant increase over the Karkloof homotype pairs in reaching the point of intersection. Further, amicable interaction in heterotype pairs involving Kamberg and Karkloof took considerably longer than the respective homotype pairs (day 6 *versus* day 2) to reach positive levels (Pillay, 1993). For individuals from the latter two populations, "follow-mount" (by males) and "present" (by females) acts were also recorded and were significantly less frequent in heterotype pairs than in tests involving homotypes. Pillay (1990) interpreted the Committee's Drift higher aggression levels and corresponding lower amicable frequencies, in terms of habitat carrying capacity differences: it is lower at Committee's Drift and higher in the Hogsback and KwaZulu/Natal localities. He argued that low carrying capacity, typified by scarce foraging, would tend to foster territoriality with consequent higher aggression.

Although other factors may play a role, these results are consistent with the frequency of failed interpopulation matings reported in table 6.1 (page 124) with the

exception of Committee's Drift males mated to Karkloof females where 90 per cent success was recorded. Extrapolation to the situation in the wild is difficult as demonstrated by the finding of O'Riain *et al.* (1996) in the colonial species *Heterocephalus glaber*. These authors have described, in 6 out of 48 colonies of this xenophobic mole-rat, rare individuals phenotypically well equipped for dispersal that solicit matings with non-colony members only. This suggests that colonial organisms - and by extrapolation also social ones like *O. irroratus* - may have in place mechanisms to ensure interdeme gene-flow, especially when risk of inbreeding is high. Pillay's data, nevertheless, indicate a significant breakdown of mate recognition - implying the existence of at least partial pre-mating barriers - in interactions between the Karkloof and Kamberg populations and only minor impairment in the other cases studied. The available behavioural data, therefore, make it plausible to argue that some of these minor differences represent an expression of intraspecific variation only, with no special evolutionary significance.

It is not possible, at present, to establish whether the partial pre-mating barrier present between the Karkloof and Kamberg population became established before, or after, fixation of the tandem fusion at Kamberg. However, it may be expected that, if lack of mate recognition operated as a primary reproductive barrier in this species - and thus preceded fixation of chromosomal barriers - it should be evident between chromosomally similar populations. Any other finding, could be argued to be a case of reproductive character displacement. Therefore, a decision on this issue awaits further behavioural work on populations of the same, or sufficiently similar, cytotypes such as B and C or B and A.

◆ *Allozymes and Western Blotting*

The literature concerning genetic variation as inferred by allozyme electrophoresis, in relation to chromosomal speciation has been extensively reviewed, amongst others, by White (1978) and King (1993). Both authors reached the conclusion that in all cases where chromosomal isolation had been documented, the taxa involved showed low genetic

distances and little or no genetic divergence. In the most cited cases of chromosomal isolation in rodents, *Peromyscus maniculatus* (Avise *et al.*, 1979), *Mus musculus* (Britton-Davidian *et al.*, 1989) and *Spalax ehrenbergi* (Nevo and Cleve, 1978), Nei's genetic distance and heterozygosity ranges between cytotypes were 0.04 - 0.126 ($H = 0.12 - 0.5$), 0.01 - 0.07 ($H = 0.09$) and 0.002 - 0.07 ($H = 0.016 - 0, 24$) respectively.

In an allozyme electrophoresis study of genetic variation - at 30 loci - between four Otomyine species, Taylor *et al.* (1988) observed a mean heterozygosity of 0.019 while Nei's genetic distances fell within the range of values expected for congeneric species ($D = 0.26$ to 0.81). These authors suggested the existence of two major evolutionary lineages within the subfamily: an arid adapted one, represented by the genus *Parotomys* and the species *O. unisulcatus* and a mesic adapted lineage which included *O. irroratus*. These suggestions were confirmed by the immunological study reported in Chapter 4, which included Otomyine and non-Otomyine rodents. On the other hand, an intraspecific allozyme study of the chromosomally monomorphic Otomyine species *O. unisulcatus* (van Dyk *et al.*, 1991) showed low mean values of $D = 0.03$ (0.000 to 0.069) and $H = 0.018$ (0.000 to 0.069). In a similar intraspecific investigation of 24 loci in *O. irroratus*, Taylor *et al.* (1992) reported low genetic distances ranging from 0.001 - between Stutterheim and Karkloof individuals - to 0.117 between Alice and Umkomaas (a locality geographically close to Umtamvuna). A prominent aspect of the data, when represented in the form of an UPGMA phenogram, was the presence of a deep bifurcation representing the highest clustering level and including the Committee's Drift and Umtamvuna populations. This is suggestive of an early divergence of these two populations from each other and from all the others. Although Taylor's study did not include specimens from Constantia, and the Western Blot analysis detailed in Chapter 5 did not comprise Umtamvuna, it is worth noting that a similarly deep cluster including Committee's Drift and Constantia emerged from the immunological investigation. This was congruent with the Constantia-Committee's Drift dichotomy in the corresponding cladograms and represented the most

consistent observation in the immunological study (see Chapter 5). Mean genetic distance, in the allozyme analysis, was 0.034 while mean heterozygosity for all populations was 0.071 and ranged from 0.028 (Umkomaas) to 0.130 (Kamberg). Moreover, Taylor *et al.* (1992) pointed out that cluster analysis of allozyme data failed to reveal groups consistent with cytotypes or geographic position. They concluded that the low genetic distances and relatively high heterozygosities indicated a genetic structure intermediate between that of "panmictic" (unrestricted interdeme gene-flow) and of "wrightian" populations, consistent with the hypothesis of speciation by chromosomal isolation. Furthermore, most genetic distances indicated low differentiation, comparable to that of local populations (see Ayala, 1975). These suggestions are supported by my immunological investigation and are in full agreement with others reported in the literature mentioned above. All are consistent with the idea that by the time negatively heterotic chromosome rearrangements become fixed, there has not been sufficient genetic divergence to be detected by gene-product analysis: chromosome changes are thus instrumental in establishing reproductive isolation.

Therefore, the conclusion that can be drawn from the information discussed so far, is that the *O. irroratus* A1 and, possibly, A2 cytotypes represent cases of incipient speciation through the agency of chromosomal rearrangements which have become fixed in conjunction with, but probably preceded, the onset of partial pre-mating behavioural barriers.

◆ *Mitochondrial DNA*

Because the existence of almost complete reproductive barriers in the *O. irroratus* A1 cytotype - and less drastic ones in the A2 race - has been demonstrated, it is to be expected that populations belonging to these groups would have undergone independent divergence since the establishment of these gene-flow restrictions. Conversely, allozyme electrophoresis and immunoblotting have failed to provide clear evidence of this which, in turn, can be taken as an indication that these methods do not afford sufficient resolution to

gauge divergence accurately considering the relatively short evolutionary times involved. Inspection of data produced by more sensitive methods, such as those using DNA sequences or fragment lengths, may therefore be profitable.

Arguably the most commonly employed of these methods is the size analysis of mtDNA polymorphic fragments produced by restriction enzyme digestion (RFLP). The usefulness of this method to phylogenetic studies of vertebrates has been documented in, amongst others, the genera *Peromyscus* (Awise *et al.*, 1983), *Geomys* (Baker *et al.*, 1989), *Xerobates* (Lamb *et al.*, 1989) and *Fundulus* (Gonzalez-Villaseñor and Powers, 1990). The maternal inheritance mode and lack of recombination in mtDNA allow the monitoring of recent evolutionary events such as colonizations and bottlenecks as has been shown by Riddle and Honeycutt (1990) in the grasshopper mice, *Onychomys*.

Raubenheimer (1993) investigated the mtDNA polymorphism in the three *O. irroratus* populations at Kamberg, Karkloof and Rietvlei. While modest polymorphism was detected in the outgroup *O. angoniensis* which showed three different clones among 12 individuals from Durban and Albert Falls Nature Reserve (70 km from Durban), high levels of variation were reported in *O. irroratus*. Each of the 11 individuals from Kamberg displayed a unique matrilineage while amongst the 11 specimens from Karkloof, five haplotypes were detected one of which was shared with an individual of the Kamberg population. Four clones exclusive to the Rietvlei population were seen amongst eight specimens. Additionally, intrapopulation sequence divergence at Kamberg ranged from 0.15 to 4.35 per cent which equated to the interpopulation divergence between Kamberg and Karkloof of 0.87-4.30 per cent. Raubenheimer explained this unprecedented polymorphism of the Kamberg samples in terms of restricted female mobility which coupled with high lineage retention in an older population, promotes accumulation of mutated haplotypes. Female recruitment from neighbouring demes was also considered a possible explanation. In my view, however, this finding is best explained in terms of the bi-annual grass burning regime maintained in this Nature Reserve. This would cause frequent local

extinctions of lineages, followed by recolonizations from nearby demes into areas of new growth.

Based on estimates of evolutionary rates for muroid rodent mtDNA (She *et al.*, 1990; Catzeflis *et al.*, 1992) of 5.9 ± 1.2 per cent per million years (MY), Raubenheimer (1993) calculated the divergence between *O. angoniensis* and *O. irroratus* to have occurred 2.0 ± 0.5 million year before present (MYBP). This is in agreement with estimates made by Taylor *et al.* (1989), based on an allozyme "molecular clock" calibrated according to the fossil record, which indicated speciation events within the "arid-adapted" Otomyine lineage to have occurred ca. 2.3 and 1.4 MYBP. Using the same assumed mtDNA evolutionary rate and the sequence divergence estimate of the one clone shared between the Kamberg and Karkloof populations, Raubenheimer (1993) estimated that these two populations last shared a common ancestor ca. 0.365 MYBP. The latter figure is relevant in view of the demonstrated chromosomal, and behavioural, reproductive barriers between the two populations: the mtDNA lineage in question had a low probability of being exchanged after such barriers became effective. From a cytogenetical standpoint, this permits the conclusion that the tandem fusion (7/12) was fixed at Kamberg at least during the last 0.365 MY, although the unlikely possibility that this clone may have resulted from a rare introgression event cannot be totally excluded.

Another investigation involving RFLP of *O. irroratus* mtDNA was carried out on eight populations (see figure 6.4) including all known cytotypes (Rimmer, 1994). The *O. angoniensis* - *O. irroratus* interspecific divergence estimate of approximately 2.0 MY was confirmed as were the high levels of polymorphism of the Kamberg and Karkloof populations although no shared clones were detected between any of the populations studied. Figure 6.4 depicts an UPGMA phenogram from such a study showing good separation between acrocentric and biarmed cytotypes.

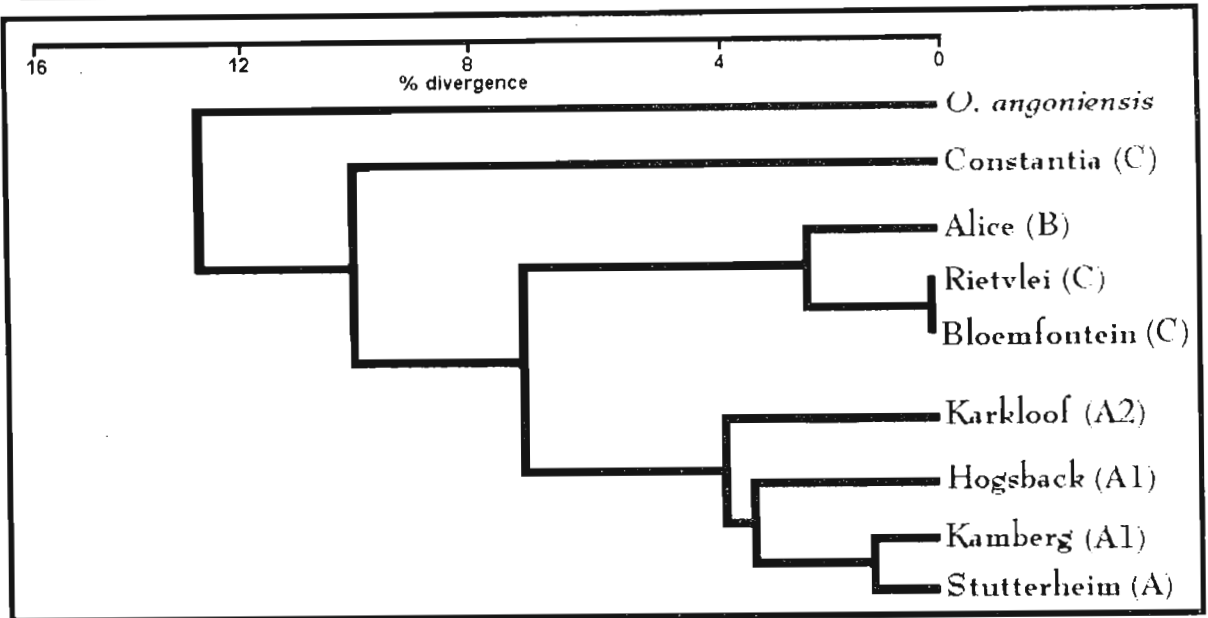


Figure 6.4 - UPGMA phenogram from RFLP data of eight *O. irroratus* populations. Cytotypes shown in brackets (redrawn from Rimmer, 1994).

While a cautious attitude is indicated in the interpretation of these results because of small sample sizes and a number of missing data points, this cladogram suggests a measure of independent divergence between "acrocentric" and "biarmed" populations. Further divergence congruent with chromosomal races, however, is not indicated beyond this level of branching. It is worth noting that the topological position of Constantia agrees with that in the dendrograms generated from immunoblotting data (see figures 5.3 to 5.6 beginning at page 92) and a similar phylogeny has been inferred from RAPD data (Dace, 1995). Moreover, the position of the populations bearing acrocentric cytotypes suggests that they are the most derived which conflicts with the assumptions, made to base the karyotype phylogeny, that evolution in this species is more likely to proceed from high (i.e. Karkloof) to low chromosome numbers and from acrocentric to biarmed cytotypes (see page 41).

◆ *Biogeographic and paleoclimatic considerations*

Molecular and serological evidence point to an early genetic divergence of the Constantia (C cytotype) and Committee's Drift (B cytotype) populations from all others.

This, in turn, implies their ancestral relationship with respect to other conspecifics and that their heterochromatic chromosomal features predated the t(7/12) typical of the A1 cytotype.

The first assumption of this scenario, that the C cytotype is the most primitive, may not necessarily be correct since the immunological data detailed in the cladograms in Chapter 5 imply that the Committee's Drift population diverged from all others at the same time as that of Constantia. The primitiveness of the Committee's Drift population is supported by the allozyme study of Taylor *et al.* (1992), where this population shows the second highest genetic distance ($D = 0.107$ from Alice), which suggests reduced gene-flow with surrounding demes. Hence, this population may represent a genetic relic of a colonization of the Fish River Valley, an event which appears to have occurred early in the species history close to 2.0 MYBP. In this regard, it is relevant to consider the disjunct *O. irroratus* population in the Eastern Highlands of Zimbabwe. Specimens from one locality north of the Chimanimani are being investigated by A. Kumirai (pers. comm.) who reports a karyotype of $2n = 28$ with at least eight pairs of heterochromatic short arms; that is, a B cytotype (Kumirai, pers. comm.). On the basis of gene-product analysis and karyotype distribution, therefore, it is likely that the most ancestral karyotype is the biarmed B type. Thus the following sequence of chromosomal changes can be postulated: early divergence of B and C populations, from an ancestral B karyotype, followed by loss of heterochromatic arms from C to form A cytotypes which would then have diverged into A1 and A2 at about 0.365 MYBP. The distribution of present *O. irroratus* cytotypes is illustrated in figure 6.5.

Correlation between cytotype distribution and bioclimatic regions of Southern Africa described in Chapter 3, has shown that B cytotypes occupy a dry portion of the sub-continent, typified by the Fish River Valley where Committee's Drift is located, subject to unpredictable rainfall. The geographic distribution of *O. irroratus* karyotypes is not readily explained by a straightforward radiation, from sub-equatorial East Africa, of diverging

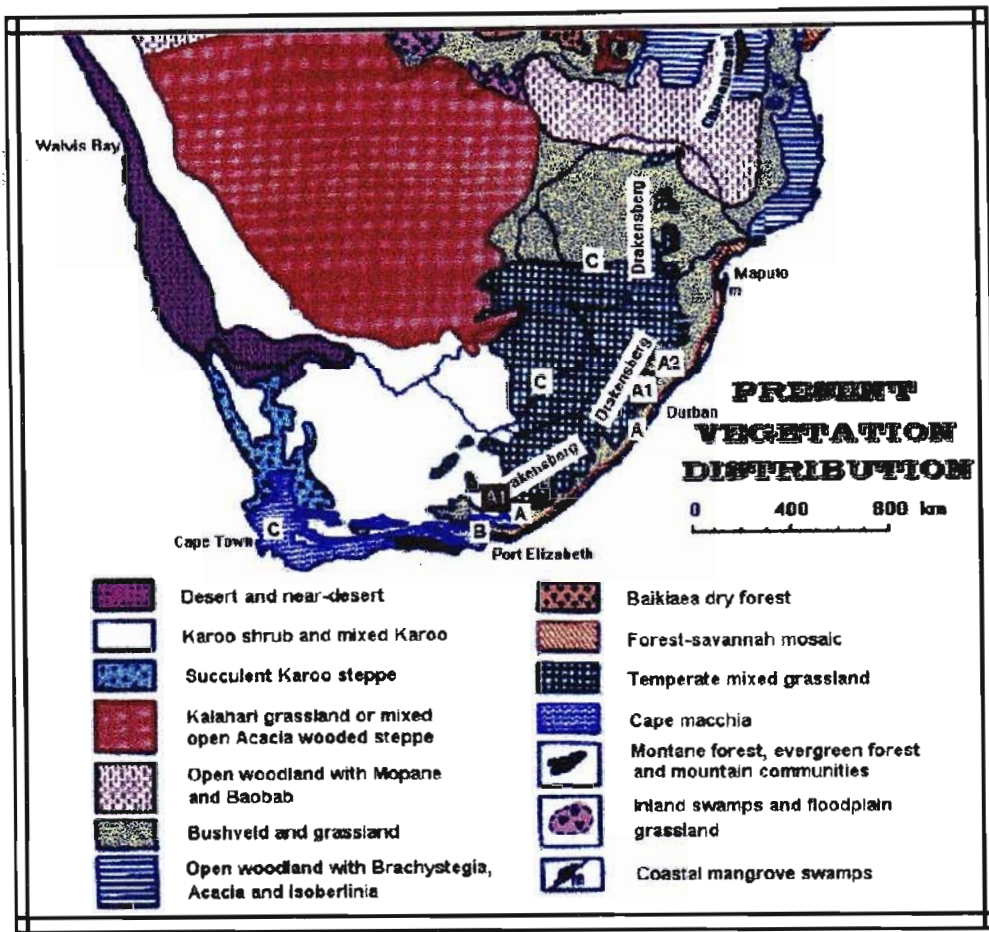


Figure 6.5 - Map of Southern Africa with *O. irroratus* cytotypes distribution (boxed letters) overimposed on present day vegetation distribution. Modified from Cooke, 1962.

cytotypes colonizing unoccupied habitats. Present distribution of these cytotypes is more consistent with a scenario positing a radiation of a single ancestral cytotypes, type B, followed by karyotype divergence and local extinctions caused by habitat fragmentation of the ancestral distribution range. This suggestion is consistent with the hypothesis of "turnover-pulse" as proposed by Vrba (1985).

The 2 MYR evolutionary history of *O. irroratus* occurred during the Plio-Pleistocene, a period marked by 21 glacial-interglacial fluctuations in the northern hemisphere (van Donk, 1976 cited by Masters, 1988) with a mean duration of 0.1 MY each (Kukla, 1985). These oscillations also occurred in Antarctica and had a strong influence on the climate of the African continent (Emiliani, 1955; Brain, 1981); they

correlated with alternating periods of high and low rainfall, or pluvials and interpluvials. According to Masters (1988), European glacial periods apparently correspond to African interpluvials and *vice versa*. In illustrating the meteorological mechanisms responsible for these phenomena, Lawes (1990) linked pluvials - which he called hyperthermals or interglacials - to southward shifts of the intertropical convergence zone (ITCZ) and rainfall belts with consequent decrease in south-easterly trade winds. These shifts seem to have coincided with southward movements of the Antarctic Polar Front (APF) and the Subtropical Convergence (STC) which mark the poleward and subtropical limits, respectively, of the subpolar gyre waters (see Howard, 1985). The STC lies at about 40° S and between the 10° and 14°C isotherms in the southern hemisphere winter and between the 14° and 18°C isotherms in summer (Howard, 1985). Pliocene and Pleistocene northward movements of APF and STC may have corresponded to major glaciations in Antarctica such as the Queen Maud glaciation (2.4 to 1.8 MYBP) and a considerable cooling event dated at 1.3-1.0 MYBP (Harwood, 1985).

Vogel (1985) suggested, on the basis of oxygen isotope measurements of groundwaters and stalagmites from the southern Cape Province, that the last interpluvial or glacial maximum (18 000 YBP) showed a decline in mean annual temperature of 5-6°C. Analysis of microfauna and plant remains, from the same area, suggest that the last glacial maximum was drier than present times. Therefore, it is likely that rainfall and temperature, at least at higher altitudes, would have had a major impact on vegetation distribution during Pleistocene fluctuations. Figure 6.6 illustrates projected vegetation distribution maps inferred by Cooke (1962), in accordance with rainfall figures at 140-150 % and 60 % of present levels. According to present day climate (see figure 6.5, page 134), *O. irroratus* distribution is compatible with temperate and bushveld grasslands, open woodlands and with Cape macchia vegetation (fynbos). Therefore, ample scope must have existed for habitat expansion, along a corridor of grasslands and bushveld leading from the

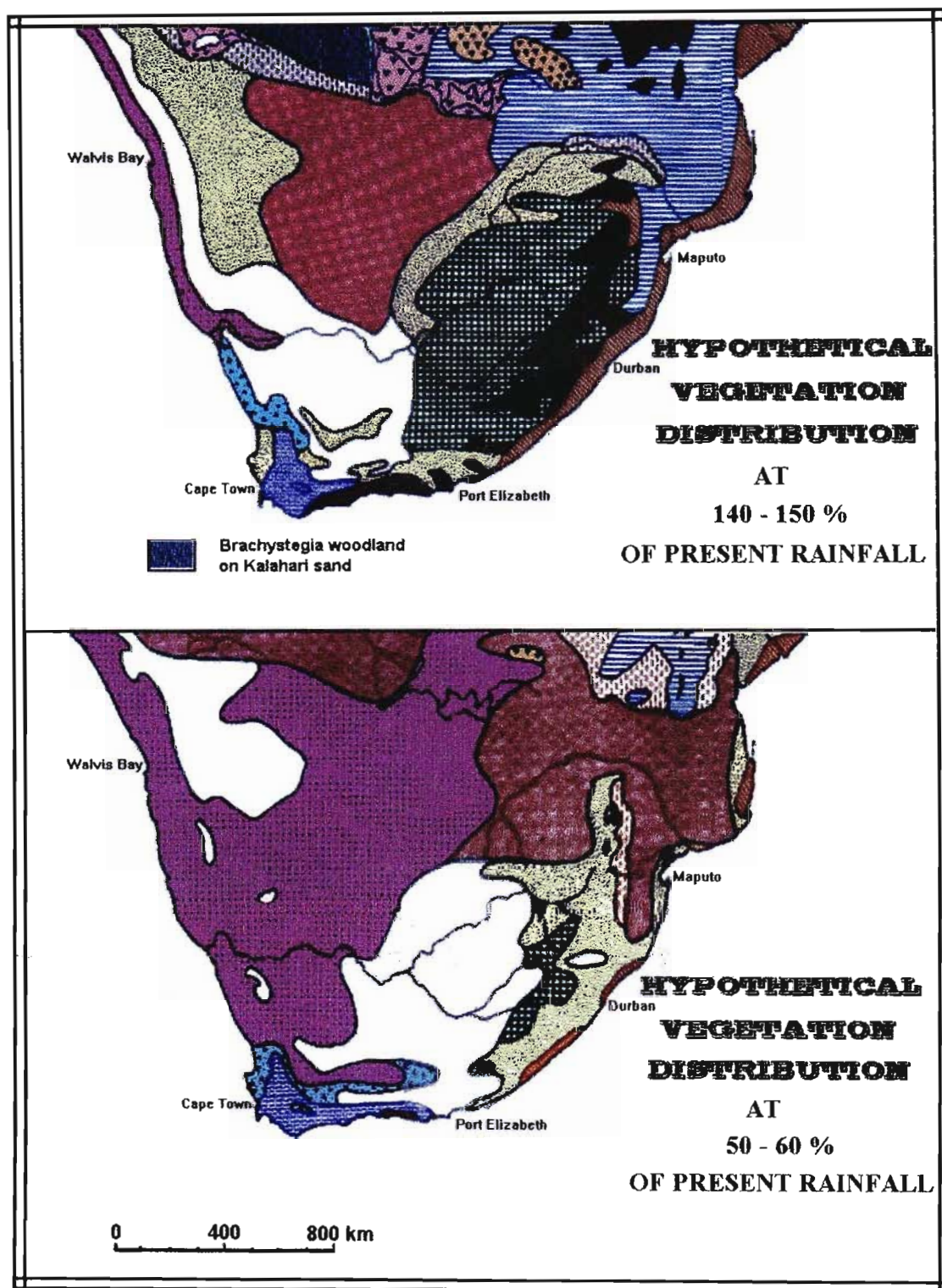


Figure 6.6 - Inferred vegetation distribution during Pleistocene pluvial fluctuations (modified from Cooke, 1962). Key to vegetation as in figure 6.5

Chimanimani mountains to the South-Western Cape (see figure 6.6-A). On the other hand, figure 6.6-B shows that during interpluvials, the species distribution would have been divided into at least three parts by the unsuitable Kalahari steppes in the north-east and the Karroo shrub in the south. Since these maps only represent inferences of likely vegetation distribution under the stated conditions, and because of minor climate fluctuations during interpluvials (see Howard, 1985), it is possible to visualize further fragmentation of the *O.irroratus* habitat into smaller pockets, possibly mountain valley refugia, depending on local conditions and climate harshness. In all likelihood, therefore, transient habitat barriers were formed during the species' evolutionary history. These might not be effective because their lability during Pleistocene pluvial phases, or fluctuations during interpluvials, would have allowed secondary contact between isolated populations (i.e. parapatric contact), with consequent re-establishment of gene-flow. Hence, no speciation event would have occurred, under these conditions, in the absence of negatively heterotic chromosomal changes. This seems to have been the case with the B and C chromosomal races and may be supported by the finding of what are likely vestiges of hybridization between A and B cytotypes at Grahamstown.

In any event, given that biochemically the Constantia and Committee's Drift populations seem the first to have diverged from their conspecifics, it is not unfeasible to propose that the fixation of the C karyotype occurred in a small isolated deme in the South-Western Cape while the remainder of the region was occupied by the more primitive B type. This was followed by the spread, during pluvial conditions, of the C type from the south-western region of South Africa to the Highveld in the north-east. As mentioned earlier, subsequent divergence of the A types from C can be estimated, on the basis of the mtDNA molecular clock, to have occurred at ca 1.25 MYBP: this is synchronous with a cooling phase in the Antarctica having taken place 1.3-1.0 MYBP (Harwood, 1985). The divergence between the Karkloof and Kamberg populations, at ca 0.365 MYBP, seems to coincide with a phase of falling sea-surface temperature, indicative of interpluvial climate

patterns, at ca 370 MYBP (see Howard, 1985).

Attempts to use present data in order to reconstruct the exact sequence of events leading to the present cytotype distribution would be highly speculative. However, it is not unfounded to postulate, at least for the A1 cytotype, a speciation event mediated by the formation of small isolated demes under interpluvial climatic conditions. Chromosomal rearrangements would have been instrumental in the reproductive isolation ensuing from secondary contact during pluvial conditions. Although this implies allopatry, it is not in conflict with a parapatric situation required to satisfy a model of stasipatric speciation as postulated by White (1978). Temperate grasslands, favoured by A1 populations, and the bushveld occupied by the other A cytotypes appear to be equally suitable habitats to *O. irroratus* and, as the maps in figure 6.6 (page 136) suggest, retreat of temperate grasslands during interpluvials was accompanied by expansion of bushveld grasslands. It is thus unlikely that the A1 populations were physically separated for long from the other A cytotypes during Pleistocene fluctuations.

◆ *Conclusions*

The paleoclimatic aspects just discussed support, in my opinion, the suggestion made by Robinson and Elder (1987) that habitat specificity and population size played a crucial role in the isolation and speciation of *O. irroratus*. Fixation of heterotic chromosome rearrangements is a likely occurrence in small isolated demes (see King, 1993) and such a "Wrightian" population structure is more likely to be the hallmark of species whose distribution is restricted by habitat specificity.

The late J.A. "Waldo" Meester matured his hypothesis of sibling speciation (see Chapter 1) also as a result of discussions with colleagues and friends, including myself, and based it essentially on established methods of classic taxonomy. Although he saw the necessity of going beyond classic taxonomic tools to karyology and allozyme analysis (Meester, 1988), the potential of more recent molecular techniques such as RFLP and

RAPD was, perhaps, not fully appreciated at that time. I believe that the studies reported here highlight the significance of interdisciplinary approaches in testing the occurrence of chromosomal speciation. It is, therefore, essential to integrate experimental evidence from not only morphological, karyological and biochemical studies, but also from molecular, behavioural and ecological ones. If this approach is adopted, the best opportunities of finding evidence of chromosomal isolation are found, not between already recognized sibling species (see Meester 1988) but, in cryptic species within formerly recognized monomorphic taxa.

Earlier discussions show that the conditions for sibling chromosomal speciation, as set out at the opening of this chapter, are met in at least some populations of the *O. irroratus* complex. Such conditions are largely met in the A1 cytotype populations where, notwithstanding possible contributions from other chromosomal factors (i.e. B-chromosomes), prediction of negatively heterotic effects by the autosomes 7 and 12 tandem fusion are confirmed by the demonstration of reproductive breakdown in F1 hybrids. Impaired pre-mating recognition has been documented but is not complete and has not been demonstrated to precede the t(7,12) fixation. Thus it can be argued that the available behavioural data represent an example of reproductive character displacement. Biochemical data argue in favour of open gene-flow and lack of genic divergence. Although habitat preference may seem to be a factor in maintaining separation of A1 populations from other cytotypes, boundaries between habitats are not accompanied by physical barriers so that the existence of contact zones cannot be excluded. I conclude that the A1 cytotype populations represent an incipient sibling species where chromosomal rearrangements are instrumental in producing post-zygotic isolation.

Similar conclusions cannot be reached with regard to the A2 populations because the heterotic potential of its chromosome variants (pairs a1 and a2) cannot be predicted, from the information in hand. A measure of reproductive impairment in F1 hybrids with the B type population from Committee's Drift has been detected experimentally but the role

of chromosome rearrangements in this, although likely, has not been substantiated. A possible role for behavioural mechanisms cannot be excluded.

Chromosomal difference between cytotypes A, B and C is mainly represented by gain or loss of heterochromatic short arms and their participation in negative heterosis is not indicated. Although a possible role of B-chromosomes cannot be ruled out, my conclusion is that these cytotypes are highly unlikely to be undergoing chromosomal isolation. A measure of geographic structuring is indicated by allozyme, immunoblotting and mtDNA data which, although indicative of a conventional allopatric speciation mode, is not sufficient to indicate incipient speciation. Some reproductive impairment has been shown in one B type population but lack of breeding data concerning the other B and C populations precludes a conclusion as to its role as an isolation mechanism. Further studies on breeding biology and pre-mating behavioural are required to allow any firm conclusion on this issue.

In conclusion, it can be stated that chromosomal speciation has occurred within the *O. irroratus* species complex in the group of populations occupying the Drakensberg montane habitat, but this is not the only speciation mechanism possible in this species.

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