POPULATION GENETICS OF THE STRIPED-MOUSE, *RHABDOMYS PUMILIO* (SPARRMAN, 1784)

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TO MY PARENTS

for caring

PREFACE

This study was carried out between 1993 and 1998 in the Department of Biology at the University of Natal, Durban. The project was supervised by Dr. Glen Campbell and Dr. Peter Taylor.

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.

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I would also like to thank Neil Burgess for providing the map on the distribution of *Rhabdomys pumilio*.

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ABSTRACT

The striped-mouse, *Rhabdomys pumilio*, is widely distributed throughout southern Africa within a variety of habitats and rainfall regimes. It is found at sea level in the Eastern and Western Cape regions and at altitudes above 2700 m in the Drakensberg mountains. The attraction of *R.pumilio* to cultivated land and crops has resulted in extensive damage to plants and cultivated crops.

A study of the genetic variation between populations of *R.pumilio* from different regions of southern Africa was undertaken by protein electrophoresis and randomly amplified polymorphic DNA using the polymerase chain reaction (PCR-RAPD). A cytogenetic study was also undertaken.

The mean heterozygosity (H=0.074) for *R.pumilio* was more than twice that estimated for mammals (H=0.036) while the mean percent polymorphism (P=16.1%) was only slightly higher than the mean percent polymorphism obtained for mammals (P=14.7%). The highest heterozygosities were recorded in the Potchefstroom (H=0.145) and Zimbabwe (H=0.118) samples and the lowest mean heterozygosity was recorded in the peninsular Western Cape (H=0.032). A mean F_{st} value of 0.459 was obtained, suggesting a

high degree of genetic differentiation between the samples of R.pumilio but the negative F_{is} (-0.01) value emphasized that R.pumilio retained an outbreeding population structure. The similarity coefficient between the samples of R.pumilio using PCR-RAPD's ranged between 0.471 and 0.853 and substantiated the argument for genetic divergence between the samples of R.pumilio. An isolation by distance model for the population genetic structure of R.pumilio was supported by the allozymes (r=0.58, p<0.001) and PCR-RAPD's (0.75, p<0.001). Temperature and rainfall also had an influence on the allelic frequency distribution of certain loci of R.pumilio.

Rogers (1972) genetic similarity varied between 0.796 and 0.988 while the values for Nei's (1978) unbiased genetic distance varied between 0.000 and 0.189 for the different samples of R.pumilio. Subgrouping of the KwaZulu-Natal samples, the peninsular Western Cape and Eastern Cape samples of R.pumilio was evident with the allozymes. With the PCR-RAPD's the Zimbabwe sample showed the least similarity to the other samples with a KwaZulu-Natal/Potchefstroom subgroup separating from the less well defined Eastern Cape and Western Cape subgroup.

Cytogenetic studies of specimens of R.pumilio from some of the localities in southern Africa revealed a chromosomal number

of 2n=48, while the Potchefstroom and Zimbabwe specimens displayed a chromosomal number of 2n=46. Homology in G-and C-banding was recorded.

The allozymes, PCR-RAPD's and chromosomal studies suggested subspecies status for the Zimbabwe population of *R.pumilio*. The Potchefstroom sample displayed a greater genetic similarity to the remaining South African samples of *R.pumilio* than the Zimbabwe samples and therefore could not be considered for subspecies status. Although the South African samples of *R.pumilio* displayed a certain degree of genetic divergence, it was insufficient to warrant subspecies status although evolution in this direction was suggested.

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CHAPTER 1

INTRODUCTION

Distribution and economic importance

The striped-mouse, *Rhabdomys pumilio*, is found throughout southern Africa (De Graaff, 1981) except for the eastern tropical corridor (Northern Zululand and the lowveld of the Kruger National Park (Coetzee, 1970). It is found at sea level in the Eastern and Western Cape regions and at altitudes above 2700 m in the Drakensberg mountains (Skinner and Smithers, 1990). It is also found in low rainfall areas such as the Kalahari Gemsbok National Park where the mean annual rainfall is about 200 mm per annum and in areas such as Port St. Johns in the Eastern Cape where the rainfall is above 1000 mm per annum.

Rhabdomys pumilio occupies a wide variety of habitat types. It prefers grasslands but is also found in bushy vlei country, dry river beds and the edges of forests (Brooks, 1974) but it has also been observed kilometers into forests (P.Taylor, per. comm.). This species has a continuous distribution along much of its range but the savanna vegetation (Rutherford and Westfall, 1986) separates the more northerly populations in Zimbabwe from the South African populations. *R.pumilio* has been recorded in

central Angola, eastern Botswana, western and central Kenya, northern Malawi, Mozambique, Namibia, Tanzania, eastern Uganda, parts of southern Zaire, north-eastern Zambia and the eastern parts of Zimbabwe (De Graaff, 1981; Skinner and Smithers, 1990).

Rhabdomys pumilio is of economic importance to man because of its abundance, wide distribution and granivorous feeding habits. David (1979) estimated 200 mice per hectare in a peak year on the Cape Flats. The attraction of *R.pumilio* to cultivated land and crops has resulted in extensive damage to plants (De Graaff, 1981). Serious damage to wheat and maize crops by *R.pumilio* has been recorded in east Africa (Delany, 1972), whereas in Zimbabwe this species was reported as harvesting seeds for human consumption (Choate, 1971).

Species biology

Rhabdomys pumilio is easily recognised by the four black and three white strips that extend from the occipital region of the head to the base of the tail (De Graaff, 1981; Skinner and Smithers, 1990). Roberts (1951) described its colour as speckled buffy on a dark-grey background with whitish underparts and a dark-brown tail. Specimens from the eastern sub-region are darker in colour than those from the western sub-region (Skinner and Smithers, 1990). Davis (1962) noted that the western semi-desert

forms tended to have longer tails than the eastern representatives and Coetzee (1970) suggested that the mean annual rainfall and temperature could be the determining factors.

Using tail length as a taxonomic feature, Roberts (1951) listed twenty subspecies. De Graaff (1981) questioned the validity of these described forms. He contended that there may be a valid eastern subspecies and a western subspecies but concluded that "very little can be added until the entire species is revisited". Misonne (1974) considered all these forms as conspecifics under *R.pumilio*. Meester *et al.* (1986) retained seven of the twenty subspecies listed by Roberts (1951). These were *R.p.pumilio* (Sparrman, 1784) from the south-western and southern Cape Province extending to the Free State; *R.p.bechuanae* (Thomas, 1983) from Namibia; *R.p.dilectus* (De Winton, 1897) from western Lesotho, northern KwaZulu-Natal and eastern Zimbabwe; *R.p.cinereus* (Thomas and Schwann, 1904) from the Northern Cape and North-West Province; *R.p.griquae* (Wroughton, 1905) from parts of the Northern Cape Province, central Botswana, southern Namibia, North-West Province and Gauteng; *R.p.intermedius* (Wroughton, 1905) from the central and Eastern Cape Province and *R.p.fourei* (Roberts, 1946) from northern Namibia. Recently in Angola, Crawford-Cabral (1998) recognised *R.p.angolae* and *R.p.bechuanae* as good subspecies based on morphological

measurements.

Rationale for the study and the techniques used

Because numerous ecological and behavioural studies have been carried out with R.pumilio (Choate, 1972; Brooks, 1974; Johnson, 1980; Perrin, 1980; Willan and Meester, 1989), a population genetic study was deemed necessary. Furthermore, the application of genetic techniques to the problems of identifying individual populations of rodents, although recognised as being important, has had very limited application in southern Africa compared to other parts of the world (Taylor, in press). This study therefore attempts to provide genetic information for R.pumilio which together with morphological data will enable taxonomists to produce a more stable and objective classification within this species. Since this study introduces genetic analyses of the striped-mouse for the first time, different techniques were used to sample the genome of R.pumilio to improve the confidence level in the assessment of genomic diversity in R.pumilio. Another reason for the multidisciplinary approach is that only a minute portion of the genome is sampled by the different genetic techniques and therefore it is essential to compare the genetic diversity obtained by the different techniques.

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Genetic variation between and within species can be measured by protein and DNA assays. Protein (allozymes) variation as a reflection of genetic variation is determined by using starch-gel electrophoresis. A number of techniques exist for the direct measurement of DNA variation. These include DNA-DNA hybridization, restriction analyses, and sequencing of mitochondrial and nuclear DNA. In the present study, the techniques used included the measurement of protein variation (allozymes) and the measurement of sequence polymorphism of DNA by the random amplification of polymorphic nuclear DNA involving the polymerase chain reaction (PCR-RAPD).

The intrinsic value of protein electrophoretic analyses lies in the detection of allelic differences between and within populations of a species. A shortcoming of electrophoresis is that only structural genes coding for soluble proteins can be studied and not all allelic variations are detected by this method (Nei, 1971). However, an important aspect of electrophoresis is that it does provide a universally recognised standard approach that can be used in understanding population genetic structure and population dynamics (Grant, 1989). The large number of electrophoretic studies carried out by, inter alia, Hunt and Selander (1973), Avise and Aquandro (1982), Thorpe (1982), Wake *et al.* (1986), Hafner *et al.* (1987), Nevo *et al.*

(1987), Apfelbaum and Reig (1989), Taylor *et al.* (1992), Nevo *et al.* (1995), Ruedi *et al.* (1996) and Wójcik *et al.* (1996), makes comparative studies between species possible.

The PCR-RAPD method for measuring nuclear DNA variation was used because it is regarded as a powerful technique for measuring genetic diversity in populations of the same species (Welsh and McClelland, 1990; Williams *et al.*, 1990). Although Van de Zande and Bijlsma (1995) demonstrated that RAPD markers were very efficient in the identification of *Drosophila* species, they conceded that this method was limited to sibling species in assessments of phylogenetic relationships and that reliable measures of genetic distances cannot be obtained. However, the advantages of RAPD's are that no prior knowledge of the genome sequence is required, a small amount of DNA is required and a large number of samples can be analysed simultaneously (Welsh and McClelland, 1990; Williams *et al.*, 1990). But in this study the number of tissue samples used was limited because of the high cost of the chemicals used in PCR-RAPD techniques, particularly the enzyme, Taq polymerase. In an attempt to obtain representative samples for the PCR-RAPD study, tissue samples from a locality in each of the provinces in South Africa and Vumba in Zimbabwe from which specimens had been collected, were used.

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A cytogenetic study of some of the populations of *R.pumilio* was undertaken since the evidence indicated that evolutionary processes run relatively independently at the karyotypic and genotypic levels in other species (Qumsiyeh and Chesser, 1988; Bogdanowicz and Owen, 1992; Dannelid, 1994; Wójcik and Wójcik, 1994; Nevo *et al.*, 1995; Zima *et al.*, 1996). The cytogenetic study involved a chromosomal count, G- and C-banding, and silver nitrate banding for nucleolar-organizing regions (NOR's).

Correspondence between G-banding and genetic homology has been demonstrated by numerous studies (Yates *et al.*, 1979; Elder, 1980; Viegas-Pequignot *et al.*, 1983; Baker *et al.*, 1987; Searle, 1988; Contrafatto *et al.*, 1992; Volobouev *et al.*, 1996). C-bands are usually equated with constitutive heterochromatin and differences in quantity, position and type of heterochromatin between related species have been documented (Yosida, 1975; Contrafatto *et al.*, 1992). The nucleolar-organizing regions are the chromosomal sites of the genes for ribosomal RNA and there is good evidence for heteromorphism of the nucleolar regions within closely related species (Sumner, 1972).

To appreciate the genetic data, the species concept has to be reviewed.

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The species concept

With regard to sexually reproducing organisms, several definitions of a species have been advanced (King, 1993; Avise, 1994) of which the Biological Species Concept (Mayr, 1942) is the most popular. It defined species as "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups". The key issue is reproductive isolation.

Geographic isolation is considered the most common mode of speciation among sexually reproducing organisms and can be divided into two main stages (Ayala, 1975). During the first stage, genetic differentiation occurred mostly as a result of adaptations to different environments. Although this initial period involves only a slight amount of genetic variation and is a reversible process, it is a prerequisite for speciation. The second stage occurs when sufficiently differentiated populations come into geographic contact and natural selection results in the development of reproductive isolation between the populations so that two species ultimately emerge. According to Mayr (1963) and Dobzhansky (1970) natural selection favoured the development of pre-zygotic reproductive isolation among genetically differentiated populations when they exchange genes by hybridization. Similarly, Meester (1988) stated that reproductive

isolation was reinforced either behaviourally or by some other pre-mating isolating mechanism because of the energetic cost of failed reproduction. However, Paterson (1978) strongly disagreed with the speciation by reinforcement model and presented evidence for divergence in the specific mate recognition system (SMRS) as the only mechanism for speciation.

Allopatric populations in the first stage of the speciation process are considered as subspecies. Semispecies are populations in the second stage of the speciation process. Ayala (1975) showed that the average genetic distance between the semispecies of *Drosophila paulistorum* was not significantly different from the average genetic distance between subspecies and concluded that little additional genetic differentiation occurred during the second stage of geographic speciation. While accepting that sexual isolation may come about by changing only a few genes that affected courtship and mating behaviour, Ayala did not rule out the possibility that a substantial fraction of the genes may have evolved at this stage that were not studied by electrophoresis. The genes studied by electrophoresis code mostly for enzymes involved in cell metabolism and probably do not affect courtship and mating behaviour. Therefore, in the present study, a distinction was only made between species and subspecies.

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Aims

The aims of the study were (1) to measure the extent of genetic variation between and within populations of *R.pumilio*. (2) to measure the correlation between genetic variation in different populations of *R.pumilio* and factors such as temperature, rainfall, altitude, latitude and longitude (3) to test the applicability of the isolation by distance model (Wright, 1943) to the presumed panmictic population structure of *R.pumilio*, based on the opinions of Demastes *et al.* (1996) and Patton *et al.* (1996) that in the absence of physical barriers to gene flow, geographic genetic differentiation is expected to exhibit an isolation by distance relationship (4) to test the applicability of the niche-width variation hypothesis (Van Valen, 1965; Nevo, 1990; Lavie *et al.*, 1993) to the population genetic structure of *R.pumilio*, based on the findings of *Nevo* (1978) who showed that among the vertebrates, habitat specialists have a mean heterozygosity of 0.037 whereas habitat generalist, which are found in a variety of environments, have a mean value of 0.071 (5) to examine the importance of behavioural factors in the genetic structure of a species (Chesser, 1983; Lidicker and Patton, 1987; Pope, 1992; White and Svendsen, 1992; Van Staaden, 1995; Van Staaden *et al.*, 1996) and to emphasize the importance of an interdisciplinary approach to the study of population

genetics (6) to cytogenetically survey different populations of
R.pumilio to document karyotypic monomorphism or polymorphism (7)
if karyotypic polymorphism occurred, the potential for
reproductive isolation had to be investigated.

The fixation of chromosomal variants in populations
peripheral to the main species distribution (peripatric) has been
shown, for example, in the house mouse, *Mus domesticus* (Capanna,
1982) and the Israeli mole rat superspecies, *Spalax ehrenbergi*
(Nevo, 1985 and 1991) and is predicted to occur under a
peripatric model of chromosomal speciation (Mayr, 1982; Nevo,
1985 and 1991). The role of chromosomal change in the speciation
process remains elusive and disputed, with some authors arguing
in favour of (White, 1978; Meester, 1988; King, 1993; Capanna and
Redi, 1994) whereas others argue against (Carson, 1982; Patton
and Sherwood, 1983; Paterson, 1985; Vrba, 1985) its role in
speciation. One of the requirements postulated by King (1993) for
establishing a relationship between chromosomal change and
speciation was that the derived chromosomal changes result in an
effective post-zygotic reproductive barrier. To determine whether
this has resulted, cross-breeding experiments which include F_2
back-crosses have to be undertaken in the laboratory so that "all
ramifications of recombinational effects on the genome which are
only realized in the F_2 generation" are determined (King, 1993).

Therefore, if *R.pumilio* displays any chromosomal variation, cross-breeding experiments will have to be done between the chromosomal variants to determine its role in speciation. Such breeding experiments will not be possible in the present study as it is not within the scope of the biochemical genetic survey being undertaken, but if necessary a few preliminary breeding trials will be conducted. Similarly, sufficient allelic differences based on electrophoretic data could indicate a reproductive barrier resulting in speciation. Richardson *et al.* (1986) suggested that allopatric populations with differences at more than 20% of their loci based on electrophoretic data alone can be regarded as separate species. Therefore, if sufficient genetic variation exists between populations of *R.pumilio*, the question of speciation will have to be examined.

Hypotheses

Rhabdomys pumilio is widely distributed throughout southern Africa with high population densities in a variety of habitats. It is found at sea level in the Eastern and Western Cape regions and at altitudes above 2700 m in the Drakensberg mountains (Skinner and Smithers, 1990). Its range also extends from arid regions (mean annual precipitation about 200 mm) in the Kalahari Gemsbok National Park to high rainfall regions (mean annual

precipitation above 1000 mm) in areas such as Port St. Johns in the Eastern Cape. Specific (allelic) adaptations might be favoured in each of these habitats. However, *R.pumilio* has excellent colonizing abilities, high vagility and continuous distribution over much of its range and this will tend to dampen genetic divergence between populations. Therefore, high heterozygosities (measure of genetic diversity) and polymorphisms (multiple alleles of a gene) were expected in populations of *R.pumilio* in different localities. The local genetic differentiation (F_{st}) value was expected to be moderately positive but at the same time the (F_{is}) value was expected to be close to zero (indicative of an outbreeding population). The mathematical formula for the relationship between the different F-statistics is: $(1-F_{it}) = (1-F_{is})(1-F_{st})$

where F_{it} and F_{is} are the fixation indices of individuals relative to the total population and its subpopulations respectively. F_{st} measures the amount of genetic differentiation among subpopulations. Genetic divergence was expected in the Zimbabwe population of *R.pumilio* because of its geographic isolation from the South African populations by savanna vegetation. The presumed panmictic population structure of *R.pumilio* suggests chromosomal conservatism because small isolated demes are required for fixation of polymorphic chromosomes (King, 1993).

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In summary, the following three studies were conducted to independently sample the genome and determine the genetic diversity in *R.pumilio*.

(1) Allozyme study - measurement of protein variation as an expression of genetic variation (Chapter 2).

(2) Randomly amplified polymorphic studies of nuclear DNA involving the polymerase chain reaction (PCR-RAPD) - measurement of DNA polymorphism (Chapter 3).

(3) Cytogenetic study - Chromosomal count and G- and C-banding, and staining the nucleolar-organizing regions of the chromosomes of some of the specimens of *R.pumilio* (Chapter 4).

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CHAPTER 2

GENETIC VARIATION - ALLOZYMES

INTRODUCTION

Amongst the techniques used to measure genetic variation between and within species are DNA and protein assays. These include DNA-DNA hybridization, restriction analyses, sequencing of mitochondrial and nuclear DNA, immunology and starch-gel electrophoresis. Although Ayala (1975) maintained that gel electrophoresis was the most cost effective method when comparing closely related species, Mayr (1970) regarded the phenetic approach of electrophoresis to systematics as meaningless and emphasized "the total system of developmental interaction" as being important. The high degree of similarity between conspecific populations has often made it difficult for biochemical systematists to identify subspecies (Avise *et al*, 1974a; Sage *et al*., 1986; Hartl *et al*., 1990; Kitchner *et al*., 1994; Wójcik and Wójcik, 1994; Ruedi *et al*., 1996). Another shortcoming of electrophoresis is that only structural genes coding for soluble proteins can be studied and not all allelic

variations are detected by this method (Nei, 1971). On the other hand it must be emphasized that electrophoresis not only has provided support for morphological data (Avise, 1975; Grobler and Van der Bank, 1995; Raman and Perrin, 1997) but also has revealed relationships that were lacking in previous classifications (Johnson and Selander, 1971; Avise *et al.*, 1974a and b). It has also been useful in cases where morphology alone appeared insufficient to resolve the classification of a species (Nevo *et al.*, 1987; Van Dyk *et al.*, 1991).

The important aspect of electrophoresis is that it does provide a relative measure that can be used in understanding population genetic structure and population dynamics (Grant, 1989). The importance of diagnostic loci within species is well documented (Adams *et al.*, 1982; Robbins *et al.*, 1983; Adams *et al.*, 1987; Gill *et al.*, 1987; Janecek *et al.*, 1992; Meester *et al.*, 1992; Macholán *et al.*, 1994; Ruedi, 1996; Ruedi *et al.*, 1996; Wójcik *et al.*, 1996; Raman and Perrin, 1997). The heterozygosity and F-statistics as measures of population genetic diversity provides important information about the population structure (Chesser, 1983; Taylor *et al.*, 1989, 1990, 1992; Lavie *et al.*, 1993; McCracken *et al.*, 1994; Wójcik *et al.*, 1996).

A comparative allozyme study of different samples of *R.pumilio* in various regions of southern Africa was undertaken

to describe the population genetic structure of this species. Heterozygosity, percent polymorphism, mean number of alleles per locus, F-statistics, and genetic similarities and distances between samples of *R.pumilio* were determined. The test of the applicability of the isolation by distance model (Wright, 1943), the niche-width variation hypothesis (Van Valen, 1965) and the influence of temperature, rainfall, altitude, latitude and longitude on the spatial genetic variation in *R.pumilio* was also investigated.

Materials and methods

Rhabdomys pumilio specimens were live-trapped from different regions of southern Africa (Table 2.1) using Sherman-type (Titian Productions, Cape Town) and PVC traps (Willan, 1979). Randomised sampling was carried out by having 3 or 4 trap-lines (depending on the size of the area) running in different directions. Each trap-line consisted of 50 traps placed successively in pairs, at intervals of about one meter. Completely randomised sampling was not possible in the Kalahari Gemsbok National Park because of the danger posed by wild animals and the removal of traps at night by wild animals. Another problem was the limitation on the number of specimens allowed to be collected in certain nature reserves and also very few specimens of *R.pumilio* were trapped in some

other localities. The problem of small sample size is a frequent problem in these types of studies and resulted in various mathematical and computer applications being made available to reduce the effects of this problem. In this study, Levene's (1949) correction for small sample size, Nei's (1978) unbiased genetic distance and the computer program, Genepop, (version 3.1b) (Raymond and Rousset, 1995) was used to minimise the errors associated with small sample sizes.

The Malawi and KwaZulu-Natal specimens were collected in 1992, whereas the remaining samples were collected between December 1995 and January 1996. The electrophoretic work was done two months later. The distribution and sampling sites for *R.pumilio* are illustrated in Figure 2.1. The animals were sacrificed in the field and standard measurements recorded. The liver, heart and kidneys were removed and stored in liquid nitrogen (-190^0C). Voucher specimens were deposited in the Durban Natural Science Museum and Transvaal Museum (Appendix 16).

The tissues were transferred to the ultrafreeze (Nuaire) to be stored at a temperature of -83^0C in the Biology Department at the University of Natal, Durban.

Starch-gel electrophoresis (Murphy *et al.*, 1990) was carried out using a discontinuous and two continuous buffers (Appendix 1). The continuous buffers were those of Whitt (1970) and Markert

and Faulhaber (1965), while the discontinous buffer of Ridgway *et al.* (1970) was used (Table 2.2). Initially the Whitt (1970) buffer was used and if problems such as poor resolution was encountered when staining for a particular enzyme, the process was repeated using the Markert and Faulhaber (1965) buffer. If this was unsuccessful a final attempt was made with the buffer of Ridgway *et al.* (1970).

The BIOSYS-1 computer program (Swofford and Selander, 1981) was used to determine the allele frequency and calculate the mean heterozygosity (measure of genetic diversity) for each sample. Avise (1994) defined the population heterozygosity as the mean percentage of loci heterozygous per individual. It has been shown by Gorman and Renzi (1979) and Nei (1978) that estimates of genetic distances (measure of genetic dissimilarity between populations) were independent of sample sizes provided that the estimated genetic distances were large and the average heterozygosities low. Since high heterozygosities and small genetic distances between populations were expected between the different populations of *R.pumilio* because of the presumed panmictic population genetic structure of *R.pumilio*, Levene's (1949) correction for small sample size was introduced. The Genepop computer program (version 3.1b) (Raymond and Rousset, 1995) was also used as it minimised the errors associated with

small sample size. Genepop was also used for the exact tests for Hardy-Weinberg proportions, contingency analysis of allelic distribution across samples, the effective number of migrants (Nm) (Slatkin, 1985) and the F-statistics (Weir and Cockerham, 1984). The mathematical formula for the F-statistics is:

$$(1-F_{it}) = (1-F_{is})(1-F_{st})$$

where F_{it} and F_{is} are the fixation indices of individuals relative to the total population and its subpopulations respectively. F_{st} measures the amount of genetic differentiation among subpopulations.

Rogers (1972) measure of genetic similarity and Nei's (1978) unbiased genetic distance were calculated for each sample pair. Cluster analyses were performed using the unweighted pair group method with arithmetic averages (UPGMA) (Sneath and Sokal, 1973). The DA distances (Nei *et al.*, 1983) measured by the DISPAN computer program (Ota, 1993) were similar to Nei's (1978) unbiased genetic distances obtained by the BIOSYS-1 computer program and was used for bootstrapping. However, due regard was taken of the comments made by Efron (1982), Felsenstein (1985) and Hillis and Bull (1993). Efron (1982) and Felsenstein (1985) maintained that bootstrapping placed confidence intervals on phylogenies whereas Hillis and Bull (1993) argued that boostrapping measured the precision in producing the same

clusters and does not necessarily measure the confidence of capturing the "true" topology.

The correlation coefficient between Nei's (1978) unbiased genetic distance and the geographical distance between sample pairs was also calculated (Zar, 1984) (Appendix 3). The Mantel test (Mantel, 1967) was used to determine the relationship between the local genetic differentiation (F_{st}) values and the geographical distance between sample pairs. The spatial autocorrelation analysis program (SAAP), version 4.3 (Wartenberg, 1989) was used to test the strength of association between frequencies of alleles in adjacent localities (Sokal and Oden, 1979; Cliff and Ord, 1981). A strong positive autocorrelation is indicative of allele frequencies in nearby subpopulations being similar whereas a strong negative autocorrelation is obtained in widely separated subpopulations with dissimilar allele frequencies (Grant, 1993). The "A" allele was taken as a measure of the allelic frequency at loci with two alleles whereas at loci with 3 alleles, the frequency of all 3 alleles was selected for analyses. Five distance classes of 243, 461, 840, 1107 and 4652 km, containing 46, 46, 46, 46 and 47 sample pairs respectively was used.

Simple correlations between various environmental factors, such as temperature and rainfall, altitude, latitude and

longitude indicated significant correlations between these variables. Therefore, multiple regression analyses was done to determine independently the effects of temperature, rainfall, altitude, latitude and longitude on the allelic frequencies and heterozygosities of samples of *R.pumilio* in different localities. Since, only the South African weather data were available (WB40, 1986) (Appendix 4), the Zimbabwe and Malawi samples were excluded from these analyses.

Table 2.1 The sampling sites and number of specimens (N)
          of *Rhabdomys pumilio* analysed from each location.

Sampling sites	Geographical co-ordinates	Number of specimens (N)
KwaZulu-Natal		
1. Kamberg	29°24'S, 29°40'E	15
2. Linwood	29°33'S, 30°05'E	14
3. Boschoek	29°21'S, 30°06'E	3
4. Fort Nottingham	29°25'S, 29°55'E	12
5. Karkloof	29°21'S, 30°13'E	8
6. Midmar Dam	29°30'S, 30°12'E	5
7. Good Hope	29°39'S, 29°58'E	6
8. Cathedral Peak	28°55'S, 29°15'E	20
9. Van Reenen	28°22'S, 29°24'E	9
Eastern Cape		
10. Groendal	33°40'S, 25°28'E	10
11. King William's Town	32°53'S, 27°24'E	7
12. Umtata	31°35'S, 28°47'E	13
Western Cape		
13. Beaufort West	32°18'S, 22°36'E	14
14. Cape Point	34°18'S, 18°26'E	3
15. Cedarberg	32°21'S, 19°10'E	6
16. Paarl	33°45'S, 18°58'E	13
17. Swartberg	33°13'S, 22°03'E	7
18. Wellington	33°39'S, 19°00'E	7
Free State		
19. Bloemfontein	29°07'S, 26°14'E	7
Northern Cape		
20. Kalahari Gemsbok National Park	25°30'S, 20°30'E	7
Gauteng		
21. Potchefstroom	26°42'S, 27°06'E	9
Zimbabwe		
22. Inyanga	18°12'S, 32°40'E	5
Vumba	18°55'S, 32°40'E	13
Malawi		
23. Ngika National Park Chelinda	10°34'S, 33°48'E	4
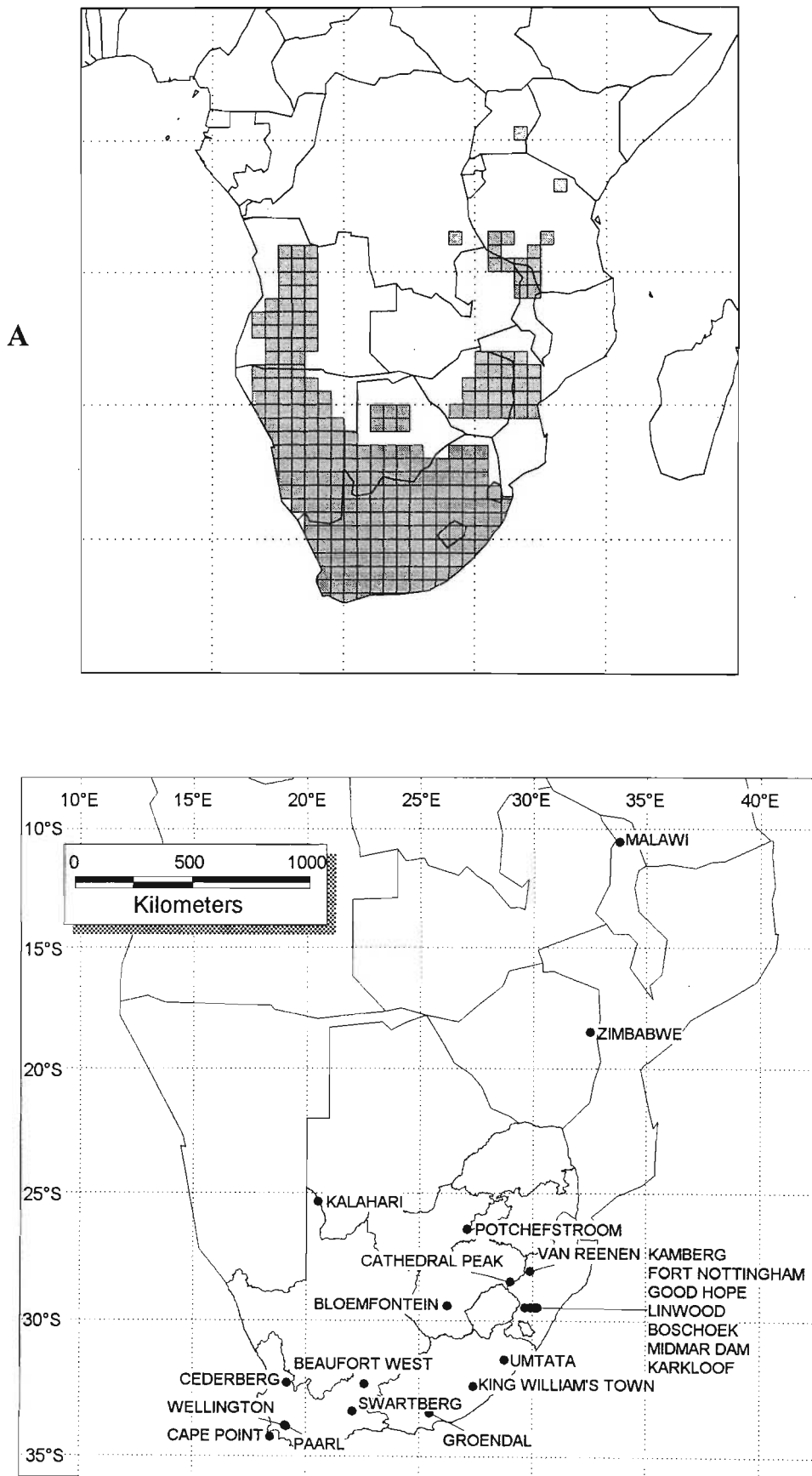
Figure 2.1 Maps showing (A) the distribution of *Rhabdomys pumilio* (courtesy of Neil Burgess) and (B) the sampling sites.

Table 2.2 The proteins examined and buffers used for the different loci in the *Rhabdomys pumilio* samples.

Proteins	Enzyme commission number	Locus	Buffers
Adenosine deaminase	3.5.4.4	*Ada*	2
Albumin	-------	*Alb*	3
Aspartate aminotransferase-1	2.6.1.1	*Aat-1*	2
Aspartate aminotransferase-2	2.6.1.1	*Aat-2*	2
Creatine kinase	2.7.3.2	*Ck*	1
Esterase-1	3.1.1.1	*Est-1*	2
Esterase-2	3.1.1.1	*Est-2*	2
Glucose-6-phosphate dehydrogenase	1.1.1.49	*G6pdh*	2
Glucose phosphate isomerase	5.3.1.9	*Gpi*	2
Glycerol-3-phosphate dehydrogenase	1.1.1.8	*G3pdh*	2
Haemoglobin-1	-------	*Hb-1*	2
Haemoglobin-2	-------	*Hb-2*	2
Isocitrate dehydrogenase	1.1.1.42	*Icdh*	1
Lactate dehydrogenase-1	1.1.1.27	*Ldh-1*	1
Lactate dehydrogenase-2	1.1.1.27	*Ldh-2*	1
Malate dehydrogenase-1	1.1.1.37	*Mdh-1*	2
Malate dehydrogenase-2	1.1.1.37	*Mdh-2*	2
Malic enzyme	1.1.1.40	*Mal*	3
Nucleoside phosphorylase	2.4.2.1	*Np*	1
Peptidase-1	3.4.11	*Pep-1*	2
Peptidase-2	3.4.11	*Pep-2*	2
Phosphoglucomutase	2.7.5.1	*Pgm*	1
6-phosphogluconate dehydrogenase	1.1.1.44	*Pgdh*	3
Sorbitol dehydrogenase	1.1.1.14	*Sdh*	1
Superoxide dismutase	1.15.1.1	*Sod*	2
Xanthine dehydrogenase	1.2.1.37	*Xdh*	1

Buffers:
 1. Whitt, 1970 (continuous).
 2. Markert and Faulhaber, 1965 (continuous).
 3. Ridgway *et al.*, 1970 (discontinuous).
(Appendix 1)

 (For continuous and discontinuous buffers, refer Grant, 1989)

Results

Eleven of the 26 loci studied were monomorphic (Table 2.3). These were Aspartate aminotransferase-1 (*Aat-1*), Aspartate aminotransferase-2 (*Aat-2*), Albumin (*Alb*), Lactate dehydrogenase-1 (*Ldh-1*), Lactate dehydrogenase-2 (*Ldh-2*), Malic enzyme (*Mal*), Malate dehydrogenase-1 (*Mdh-1*), Malate dehydrogenase-2 (*Mdh-2*), Peptidase-1 (*Pep-1*), Sorbitol dehydrogenase (*Sdh*) and Xanthine dehydrogenase (*Xdh*). Fifteen polymorphic loci were identified and scored (Table 2.3)(Appendix 2). These included Adenosine deaminase (*Ada*), Creatine kinase (*Ck*), Esterase-1 (*Est-1*), Esterase-2 (*Est-2*), Glucose 6-phosphate dehydrogenase (*G6pdh*), Glucose phosphate isomerase (*Gpi*), Glucose 3-phosphate dehydrogenase (*G3pdh*), Haemoglobin-1 (*Hb-1*), Haemoglobin-2 (*Hb-2*), Isocitrate dehydrogenase (*Icdh*), Nucleoside phosphorylase (*Np*), Peptidase-2 (*Pep-2*), 6-phosphogluconate dehydrogenase (*Pgdh*), Phosphoglucomutase (*Pgm*) and Superoxide dismutase (*Sod*). Although Fructose 1,6-biphosphatase was identified as being polymorphic, it was difficult to score. This enzyme was subsequently omitted from further analyses.

The "B" allele of the *Hb-2* locus was presumed to be a "null" allele because it was not expressed in *R.pumilio*. Further breeding studies and biochemical analyses are necessary to verify that the "B" allele is a "null" allele. This locus was only

expressed in the four Malawi animals, in a few specimens from Gauteng (0.111) and most of the animals from the Western Cape excluding Beaufort West, Cedarberg and Swartberg. It was not expressed in the Free State, KwaZulu-Natal, Northern Cape and Zimbabwe samples. The *G6pdh* locus was polymorphic only for the Zimbabwe sample and its sex linkage was taken into account when recording the genotype.

Using contingency analysis (Genepop, version 3.1b), the allelic distribution across all the samples was significant ($p<0.05$) for 13 of the 15 polymorphic loci. Only the *Gpi* and *Icdh* loci were not significant. Significant deviation from Hardy-Weinberg proportions was obtained at the *Est-2* locus of the Karkloof, Potchefstroom, Umtata, Van Reenen and Zimbabwe samples; the *Hb-1* locus in the Linwood sample; the *Hb-2* locus in the Paarl and Wellington samples and the *Np* locus in the Beaufort West sample (Table 2.4). Some difficulty was experienced with accurately scoring the *Est-2* locus because of some overlapping bands and this could account for the deviation from Hardy-Weinberg proportions. The high F_{st} values obtained for *Hb-1* (0.60), *Hb-2* (0.80), *Np* (0.54) and *Sod* (0.99) indicated localized differentiation of these loci (Table 2.6).

The mean heterozygosity per locus according to the direct count method ranged between 0.022 and 0.145 with the overall mean

being 0.074 (Table 2.5). Because of the problem of small sample size from some localities, the unbiased estimate of the mean heterozygosity is also presented and ranged between 0.036-0.135 with an overall mean of 0.073. The mean heterozygosities (direct count) for the peninsular Western Cape were low, with the Cape Point, Paarl and Wellington samples at 0.038, 0.036 and 0.022 respectively, However, the unbiased estimate of the mean heterozygosity for the same regions were 0.044, 0.054 and 0.036 respectively. Cedarberg and Swartberg in the Western Cape compared favourably with samples from other regions at 0.064 and 0.071 respectively (direct count). Potchefstroom and Zimbabwe expressed fairly high mean heterozygosities of 0.145 and 0.118 respectively (direct count). Using the Kruskal-Wallis Anova test to compare the unbiased estimate of the mean heterozygosities of the KwaZulu-Natal subgroup, the peninsular Western Cape subgroup and the Eastern Cape subgroup of *R.pumilio* showed a significant difference between the KwaZulu-Natal and peninsular Western Cape samples (p=0.013), and between the Eastern Cape and Western Cape samples (p=0.049). No significant difference was obtained between the KwaZulu-Natal and the Eastern Cape samples of *R.pumilio* (p=0.78).

The mean number of alleles per locus ranged between 1.08 and 1.31 with an overall mean of 1.20 (Table 2.5). The percent

polymorphic loci ranged between 7.69% and 30.77% with a mean of 16.05% (Table 2.5).

A mean F_{is} (fixation index of individuals relative to the subpopulation) value of -0.010, a mean F_{st} (genetic differentiation among subpopulations) value of 0.459 and a mean F_{it} (fixation index of individuals relative to the total population) value of 0.453 for the 15 polymorphic loci was obtained (Table 2.6). The mean number of inter-deme migrants per generation was 0.179 (Table 2.6).

Rogers (1972) genetic similarities for the different samples ranged between 0.796 and 0.988 and Nei's (1978) unbiased genetic distances were between 0.000 and 0.189 (Table 2.7). The UPGMA phenogram shows subgrouping of the samples of *R.pumilio* from various localities (Figure 2.2). The Bloemfontein, Kalahari and KwaZulu-Natal samples of *R.pumilio* separated from the Eastern and Western Cape samples with the Zimbabwe sample as an outlier. Bootstrap analyses of DA distances confirmed the above subgrouping but with the Zimbabwe sample as an outlier of the Bloemfontein, Kalahari and KwaZulu-Natal complex (Figure 2.3).

A correlation coefficient of 0.54 (p<0.001) was obtained between Nei's (1978) unbiased genetic distances and the geographical distances between sample pairs of *R.pumilio* (Figure 2.4). The Mantel test indicated a significant (p<0.001)

relationship between the F_{st} values and the geographical distances between different localities of *R.pumilio*. The spatial autocorrelation between allele frequency and the 5 distance classes indicated 8 significant ($p<0.05$) positive autocorrelation values in the first distance class (243 km) whereas the remaining four distance classes (461, 840, 1107 and 4652 km) showed 6, 2, 5 and 8 significant ($p<0.05$) negative autocorrelations respectively. Classes 4 and 5 also each showed 2 significantly positive autocorrelations (Table 2.8).

The South African weather data up to 1984 were available from the Department of Environmental Affairs (WB40, 1986) (Appendix 4). The allelic frequency and heterozygosity values for the different samples of *R.pumilio* were extracted from Table 2.3 and 2.5 and is presented in Appendix 5. The multi-regression analyses of the weather data, altitude, latitude and longitude versus the allelic frequency and heterozygosity of the different samples of *R.pumilio* are presented in Table 2.9. The heterozygosity and allele frequencies of *Ck*, *Est-1*, *Est-2*(A), *G3pdh*, *Hb-1*, *Hb-2*, *Np*(A), *Np*(B), *Np*(C), *Pep-2* and *Sod* alleles showed a significant ($p<0.05$) relationship with some of the independent variables. Heterozygosity, *Ck*, *Est-1*, *G3pdh*, *Np*(A), *Np*(B), *Np*(C) and *Sod* were more affected by temperature and to a lesser extent by rainfall. The allelic frequencies of the *Est-*

2(A), *Hb-2* and *Pep-2* appeared to be more or less equally affected by temperature and rainfall whereas the *Hb-1* locus was only significantly affected by temperature. The heterozygosity and the various allele frequency showed very little correlation with altitude and the geographical co-ordinates. The effects of temperature and rainfall on allelic distribution does require further investigation but is not within the scope of the present study. The situation may be much more complex for a species such as *R.pumilio* which lives underground and feeds in the early morning hours and late afternoon and thus is not directly exposed to the air temperatures that were measured. The humidity in the micro-environment of *R.pumilio* also has to be determined. This whole question of the effects of temperature and rainfall on the allelic distribution in *R.pumilio* is a full scale study.

Table 2.3 The allelic frequencies of the polymorphic loci in the
samples of *Rhabdomys pumilio* from different localities.
(A, B and C are alleles of decreasing mobilities
respectively).

	1	2	3	4	5	6	7	8
	Kamberg		Boschoek		Karkloof		Good Hope	
		Limwood		Fort Nottingham		Midmar Dam		Cathedral Peak

Adenosine deaminase (Ada)

	1	2	3	4	5	6	7	8
A	0.500	0.429	0.333	0.500	0.187	0.625	0.583	0.441
B	0.500	0.571	0.667	0.500	0.813	0.375	0.417	0.559

Creatine kinase (Ck)

A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Esterase-1 (Est-1)

A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Esterase-2 (Est-2)

A	0.500	0.429	0.500	0.583	0.375	0.125	0.667	0.525
B	0.167	0.429	0.500	0.000	0.375	0.250	0.250	0.125
C	0.333	0.142	0.000	0.417	0.250	0.625	0.083	0.350

Glucose 6-phosphate dehydrogenase (G6pdh)

A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Glucose-phosphate isomerase (Gpi)

A	0.967	1.000	1.000	1.000	1.000	1.000	1.000	0.950
B	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.050

Glycerol 3-phosphate dehydrogenase (G3pdh)

A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Haemoglobin-1 (Hb-1)

A	0.967	0.857	1.000	0.875	0.688	0.900	0.917	0.725
B	0.033	0.143	0.000	0.125	0.312	0.100	0.083	0.275

Haemoglobin-2 (Hb-2)

A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
B	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

Isocitrate dehydrogenase (Icdh)

A	0.967	1.000	1.000	1.000	0.937	1.000	1.000	1.000
B	0.033	0.000	0.000	0.000	0.063	0.000	0.000	0.000

Nucleoside phosphorylase (Np)

A	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.475
B	0.967	1.000	1.000	1.000	1.000	1.000	1.000	0.525
C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Peptidase-2 (Pep-2)

A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

6-phosphogluconate dehydrogenase (Pgdh)

A	0.733	0.615	0.833	0.727	0.250	0.400	0.750	0.316
B	0.267	0.385	0.167	0.273	0.750	0.600	0.250	0.684

Phosphoglucomutase (Pgm)

A	1.000	1.000	1.000	1.000	1.000	1.000	0.833	1.000
B	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.000

Superoxide dismutase (Sod)

A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table 2.3   continued
      The allelic frequencies of the polymorphic loci in the
      samples of *Rhabdomys pumilio* from different localities.
      (A, B and C are alleles of decreasing mobilities
      respectively).

	9	10	11	12	13	14	15	16
	Van Reenen	Groendal	King William's Town	Umtata	Beaufort West	Cape Point	Cedarberg	Paarl

Adenosine deaminase (Ada)

	9	10	11	12	13	14	15	16
A	0.444	0.000	0.000	0.192	0.036	0.333	0.000	0.000
B	0.556	1.000	1.000	0.808	0.964	0.667	1.000	1.000

Creatine kinase (Ck)

A	0.000	0.000	0.000	0.269	0.036	0.000	0.000	0.000
B	1.000	1.000	1.000	0.731	0.964	1.000	1.000	1.000

Esterase-1 (Est-1)

A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Esterase-2 (Est-2)

A	0.444	0.400	0.800	0.417	0.536	1.000	0.250	0.800
B	0.222	0.400	0.200	0.417	0.179	0.000	0.417	0.100
C	0.333	0.200	0.000	0.167	0.286	0.000	0.333	0.100

Glucose 6-phosphate dehydrogenase (G6pdh)

A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Glucose-phosphate isomerase (Gpi)

A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Glycerol-3-phosphate dehydrogenase (G3pdh)

A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Haemoglobin-1 (Hb-1)

A	0.722	0.400	1.000	1.000	0.000	1.000	1.000	1.000
B	0.278	0.600	0.000	0.000	1.000	0.000	0.000	0.000

Haemoglobin-2 (Hb-2)

A	0.000	0.000	0.000	0.000	0.000	1.000	0.000	0.846
B	1.000	1.000	1.000	1.000	1.000	0.000	1.000	0.154

Isocitrate dehydrogenase (Icdh)

A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Nucleoside phosphorylase (Np)

A	0.611	0.450	0.571	0.615	0.071	0.000	0.500	0.308
B	0.389	0.000	0.000	0.077	0.000	0.000	0.000	0.000
C	0.000	0.550	0.429	0.308	0.929	1.000	0.500	0.692

Peptidase-2 (Pep-2)

A	1.000	1.000	0.857	1.000	1.000	1.000	1.000	1.000
B	0.000	0.000	0.143	0.000	0.000	0.000	0.000	0.000

6-phosphogluconate dehydrogenase (Pgdh)

A	0.357	0.600	0.286	0.462	0.679	0.500	0.917	0.808
B	0.643	0.400	0.714	0.538	0.321	0.500	0.083	0.192

Phosphoglucomutase (Pgm)

A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Superoxide dismutase (Sod)

A	1.000	0.000	0.000	0.000	0.036	0.000	0.000	0.000
B	0.000	1.000	1.000	1.000	0.964	1.000	1.000	1.000

Table 2.3 continued
The allelic frequencies of the polymorphic loci in the
samples of *Rhabdomys pumilio* from different localities.
(A, B and C are alleles of decreasing mobilities
respectively).

	17 Swartberg	18 Wellington	19 Bloemfontein	20 Kalahari	21 Potchefstroom	22 Zimbabwe	23 Malawi
Adenosine deaminase (Ada)							
A	0.000	0.000	0.571	0.143	0.222	0.778	0.000
B	1.000	1.000	0.429	0.857	0.778	0.222	1.000
Creatine kinase (Ck)							
A	0.000	0.000	0.714	0.000	0.333	0.444	0.000
B	1.000	1.000	0.286	1.000	0.667	0.556	1.000
Esterase-1 (Est-1)							
A	1.000	1.000	1.000	1.000	0.667	1.000	1.000
B	0.000	0.000	0.000	0.000	0.333	0.000	0.000
Esterase-2 (Est-2)							
A	0.500	0.929	0.786	0.429	0.500	0.556	0.500
B	0.071	0.000	0.214	0.429	0.389	0.361	0.500
C	0.429	0.071	0.000	0.143	0.111	0.083	0.000
Glucose 6-phosphate dehydrogenase (G6pdh)							
A	1.000	1.000	1.000	1.000	1.000	0.444	1.000
B	0.000	0.000	0.000	0.000	0.000	0.556	0.000
Glucose-phosphate isomerase (Gpi)							
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Glycerol-3-phosphate dehydrogenase (G3pdh)							
A	0.857	1.000	1.000	1.000	1.000	1.000	1.000
B	0.143	0.000	0.000	0.000	0.000	0.000	0.000
Haemoglobin-1 (Hb-1)							
A	0.643	1.000	0.000	0.000	0.222	0.111	0.000
B	0.357	0.000	1.000	1.000	0.778	0.889	1.000
Haemoglobin-2 (Hb-2)							
A	0.000	0.714	0.000	0.000	0.111	0.000	1.000
B	1.000	0.286	1.000	1.000	0.889	1.000	0.000
Isocitrate dehydrogenase (Icdh)							
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Nucleoside phosphorylase (Np)							
A	0.571	0.214	0.286	1.000	0.667	0.528	0.625
B	0.000	0.000	0.000	0.000	0.000	0.472	0.000
C	0.429	0.786	0.714	0.000	0.333	0.000	0.375
Peptidase-2 (Pep-2)							
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000
6-phosphogluconate dehydrogenase (Pgdh)							
A	1.000	1.000	1.000	0.429	0.500	0.444	0.750
B	0.000	0.000	0.000	0.571	0.500	0.556	0.250
Phosphoglucomutase (Pgm)							
A	1.000	1.000	1.000	1.000	1.000	1.000	1.000
B	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Superoxide dismutase (Sod)							
A	0.000	0.000	1.000	1.000	0.000	0.000	0.000
B	1.000	1.000	0.000	0.000	1.000	1.000	1.000

Table 2.4 Localities with significant Chi-square results for deviation from Hardy-Weinberg proportions in the samples of *Rhabdomys pumilio* from different localities.

Locality	Locus	Probability
Linwood		
	Hb-1	0.004
Karkloof		
	Est-2	0.007
Umtata		
	Est-2	0.014
Van Reenen		
	Est-2	0.026
Beaufort West		
	Np	0.037
Paarl		
	Hb-2	0.005
Wellington		
	Hb-2	0.021
Potchefstroom		
	Est-2	0.024
Zimbabwe		
	Est-2	0.005

Table 2.5 Comparison of the mean (±se) heterozygosity (het.),
mean number (±se) of alleles per locus and percent
polymorphism in the samples of *Rhabdomys pumilio*
from different localities.

Locality	Mean het. (unbiased estimate)	Mean het. (direct count)	Mean number of alleles per locus	% loci poly- morphic (0.95)
KwaZulu-Natal				
Kamberg	0.070 (0.033)	0.077 (0.039)	1.31 (0.11)	11.54
Linwood	0.073 (0.036)	0.068 (0.039)	1.19 (0.10)	15.38
Boschoek	0.056 (0.032)	0.077 (0.046)	1.12 (0.06)	11.54
Fort Nottingham	0.064 (0.032)	0.088 (0.045)	1.15 (0.07)	15.38
Karkloof	0.077 (0.036)	0.072 (0.033)	1.23 (0.10)	19.23
Midmar Dam	0.072 (0.036)	0.042 (0.021)	1.19 (0.10)	15.38
Good Hope	0.075 (0.033)	0.058 (0.029)	1.23 (0.10)	19.23
Cathedral Peak	0.099 (0.039)	0.098 (0.041)	1.27 (0.10)	23.08
Van Reenen	0.101 (0.042)	0.113 (0.052)	1.23 (0.10)	19.23
Eastern Cape				
Groendal	0.085 (0.040)	0.073 (0.038)	1.19 (0.10)	15.38
King Will- iam's Town	0.061 (0.030)	0.070 (0.034)	1.15 (0.07)	15.38
Umtata	0.094 (0.040)	0.093 (0.042)	1.27 (0.12)	19.23
Western Cape				
Beaufort West	0.055 (0.029)	0.058 (0.035)	1.27 (0.10)	11.54
Cape Point	0.044 (0.030)	0.038 (0.028)	1.08 (0.05)	7.69

Table 2.5 continued
Comparison of the mean (±se) heterozygosity (het.),
mean number (±se) of alleles per locus and percent
polymorphism in the samples of *Rhabdomys pumilio*
from different localities.

Locality	Mean het. (unbiased estimate)	Mean het. (direct count)	Mean number of alleles per locus	% loci poly-morphic (0.95)
Western Cape				
Cedarberg	0.055 (0.034)	0.064 (0.040)	1.15 (0.09)	11.54
Paarl	0.054 (0.026)	0.036 (0.021)	1.19 (0.10)	15.38
Swartberg	0.073 (0.036)	0.071 (0.035)	1.19 (0.10)	15.38
Wellington	0.036 (0.022)	0.022 (0.017)	1.12 (0.06)	11.54
Free State				
Bloemfontein	0.068 (0.032)	0.071 (0.035)	1.15 (0.07)	15.38
Northern Cape				
Kalahari	0.056 (0.033)	0.055 (0.032)	1.15 (0.09)	11.54
Gauteng				
Potchefstroom	0.135 (0.042)	0.145 (0.055)	1.31 (0.11)	30.77
Zimbabwe				
Inyanga/ Vumba	0.122 (0.042)	0.118 (0.044)	1.31 (0.11)	26.92
Malawi				
Chelinda	0.059 (0.033)	0.087 (0.050)	1.12 (0.06)	11.54
Mean (±se)	0.073 (0.005)	0.074 (0.006)	1.20 (0.014)	16.05 (1.13)

Table 2.6   Summary of the F-statistics for the different loci
            (Weir and Cockerham, 1984) and the mean number of
            migrants (Nm) for the different samples of
            Rhabdomys pumilio.

Locus	$F_{(is)}$	$F_{(it)}$	$F_{(st)}$	Nm
Ada	-0.046	0.245	0.279	
Ck	0.229	0.515	0.371	
Est-1	0.056	0.336	0.297	
Est-2	-0.140	-0.055	0.075	
Hb-1	0.325	0.731	0.601	
G3pdh	-0.112	0.003	0.103	
G6pdh	-0.778	0.204	0.553	
Gpi	-0.010	-0.005	0.015	
Hb-2	1.000	1.000	0.799	
Icdh	0.006	-0.003	-0.008	
Np	-0.049	0.518	0.541	
Pep-2	-0.112	0.003	0.103	
Pgdh	-0.075	0.106	0.169	
Pgm	1.000	1.000	0.071	
Sod	0.019	0.991	0.991	
Mean	-0.010	0.453	0.459	0.179

$F_{(is)}$: inbreeding coefficient
$F_{(it)}$: overall fixation index
$F_{(st)}$: local genetic differentiation

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Table 2.7   Matrix of similarity and distance coefficients between the
            samples of *Rhabdomys pumilio* from different localities.

            Above diagonal: Rogers (1972) genetic similarity.
            Below diagonal: Nei's (1978) unbiased genetic distance.

Locality	1	2	3	4	5	6	7	8
1 Kamberg	*****	0.976	0.972	0.987	0.948	0.963	0.975	0.952
2 Linwood	0.001	*****	0.978	0.978	0.964	0.966	0.972	0.952
3 Boschoek	0.000	0.000	*****	0.967	0.949	0.947	0.969	0.931
4 Fort Nottingham	0.000	0.004	0.005	*****	0.947	0.966	0.976	0.952
5 Karkloof	0.015	0.006	0.013	0.015	*****	0.954	0.938	0.955
6 Midmar Dam	0.005	0.005	0.014	0.006	0.008	*****	0.957	0.949
7 Good Hope	0.000	0.000	0.000	0.000	0.017	0.011	*****	0.935
8 Cathedral Peak	0.016	0.014	0.023	0.015	0.011	0.012	0.019	*****
9 Van Reenen	0.018	0.016	0.024	0.019	0.014	0.015	0.022	0.000
10 Groendal	0.099	0.090	0.092	0.099	0.082	0.106	0.102	0.072
11 King William's Town	0.094	0.090	0.089	0.098	0.083	0.106	0.097	0.066
12 Umtata	0.080	0.076	0.075	0.085	0.078	0.085	0.084	0.060
13 Beaufort West	0.128	0.120	0.127	0.122	0.108	0.137	0.130	0.103
14 Cape Point	0.136	0.139	0.136	0.136	0.146	0.155	0.134	0.129
15 Cedarberg	0.084	0.085	0.075	0.090	0.096	0.100	0.092	0.081
16 Paarl	0.119	0.122	0.112	0.121	0.132	0.146	0.122	0.116
17 Swartberg	0.089	0.094	0.087	0.089	0.101	0.110	0.097	0.083
18 Wellington	0.116	0.123	0.108	0.117	0.139	0.152	0.118	0.122
19 Bloemfontein	0.099	0.096	0.098	0.094	0.109	0.119	0.091	0.085
20 Kalahari	0.089	0.075	0.087	0.087	0.060	0.088	0.089	0.038
21 Potchefstroom	0.115	0.105	0.112	0.114	0.097	0.119	0.115	0.077
22 Zimbabwe	0.118	0.109	0.124	0.115	0.109	0.113	0.111	0.091
23 Malawi	0.174	0.160	0.162	0.173	0.152	0.189	0.173	0.140

Table 2.7   continued
        Matrix of similarity and distance coefficients between the
        samples of *Rhabdomys pumilio* from different localities.

        Above diagonal: Rogers (1972) genetic similarity.
        Below diagonal: Nei's (1978) unbiased genetic distance.

Locality	9	10	11	12	13	14	15	16
1 Kamberg	0.947	0.873	0.871	0.886	0.864	0.849	0.890	0.859
2 Linwood	0.953	0.892	0.875	0.899	0.866	0.851	0.888	0.952
3 Boschoek	0.931	0.877	0.877	0.895	0.857	0.853	0.901	0.867
4 Fort Nottingham	0.947	0.872	0.868	0.879	0.865	0.849	0.882	0.857
5 Karkloof	0.954	0.892	0.885	0.894	0.866	0.834	0.877	0.837
6 Midmar Dam	0.949	0.863	0.865	0.882	0.842	0.836	0.871	0.828
7 Good Hope	0.933	0.865	0.868	0.876	0.852	0.844	0.876	0.855
8 Cathedral Peak	0.988	0.889	0.895	0.898	0.869	0.850	0.878	0.846
9 Van Reenen	*****	0.898	0.896	0.908	0.872	0.850	0.887	0.849
10 Groendal	0.065	*****	0.941	0.944	0.955	0.884	0.957	0.917
11 King William's Town	0.062	0.021	*****	0.953	0.907	0.905	0.949	0.928
12 Umtata	0.054	0.018	0.007	*****	0.906	0.899	0.952	0.909
13 Beaufort West	0.100	0.011	0.060	0.061	*****	0.884	0.922	0.902
14 Cape Point	0.129	0.077	0.058	0.070	0.092	*****	0.888	0.951
15 Cedarberg	0.073	0.015	0.022	0.011	0.051	0.076	*****	0.937
16 Paarl	0.112	0.050	0.042	0.047	0.076	0.009	0.038	*****
17 Swartberg	0.073	0.009	0.029	0.023	0.030	0.008	0.005	0.041
18 Wellington	0.119	0.051	0.046	0.052	0.071	0.015	0.035	0.001
19 Bloemfontein	0.079	0.095	0.147	0.123	0.077	0.167	0.131	0.155
20 Kalahari	0.028	0.060	0.096	0.091	0.078	0.184	0.104	0.149
21 Potchefstroom	0.069	0.009	0.037	0.026	0.025	0.101	0.042	0.078
22 Zimbabwe	0.087	0.062	0.092	0.068	0.074	0.147	0.103	0.140
23 Malawi	0.131	0.046	0.093	0.090	0.054	0.068	0.084	0.047

Table 2.7 continued
Matrix of similarity and distance coefficients between the samples of *Rhabdomys pumilio* from different localities.

Above diagonal: Rogers (1972) genetic similarity.
Below diagonal: Nei's (1978) unbiased genetic distance.

Locality	17	18	19	20	21	22	23
1 Kamberg	0.876	0.852	0.874	0.889	0.844	0.838	0.818
2 Linwood	0.871	0.844	0.873	0.910	0.863	0.850	0.830
3 Boschoek	0.873	0.862	0.873	0.895	0.851	0.832	0.835
4 Fort Nottingham	0.881	0.851	0.877	0.888	0.844	0.838	0.818
5 Karkloof	0.872	0.830	0.852	0.921	0.866	0.841	0.826
6 Midmar Dam	0.853	0.821	0.854	0.891	0.839	0.846	0.796
7 Good Hope	0.862	0.847	0.882	0.882	0.839	0.842	0.817
8 Cathedral Peak	0.886	0.838	0.875	0.924	0.872	0.868	0.828
9 Van Reenen	0.890	0.839	0.878	0.935	0.881	0.872	0.837
10 Groendal	0.954	0.907	0.862	0.911	0.943	0.889	0.927
11 King William's Town	0.933	0.919	0.829	0.878	0.908	0.860	0.886
12 Umtata	0.926	0.899	0.844	0.895	0.947	0.896	0.886
13 Beaufort West	0.929	0.899	0.885	0.904	0.921	0.881	0.922
14 Cape Point	0.870	0.946	0.810	0.816	0.859	0.821	0.896
15 Cedarberg	0.963	0.936	0.844	0.872	0.904	0.850	0.900
16 Paarl	0.919	0.979	0.829	0.830	0.866	0.818	0.927
17 Swartberg	*****	0.924	0.856	0.875	0.909	0.855	0.903
18 Wellington	0.035	*****	0.836	0.820	0.856	0.808	0.907
19 Bloemfontein	0.103	0.143	*****	0.895	0.869	0.864	0.840
20 Kalahari	0.084	0.157	0.064	*****	0.902	0.871	0.886
21 Potchefstroom	0.029	0.081	0.076	0.054	*****	0.916	0.903
22 Zimbabwe	0.085	0.145	0.092	0.090	0.036	*****	0.856
23 Malawi	0.065	0.058	0.129	0.091	0.052	0.101	*****

Cophenetic Correlation = 0.852

Genetic Distance

0.15   0.10   0.05   0.00
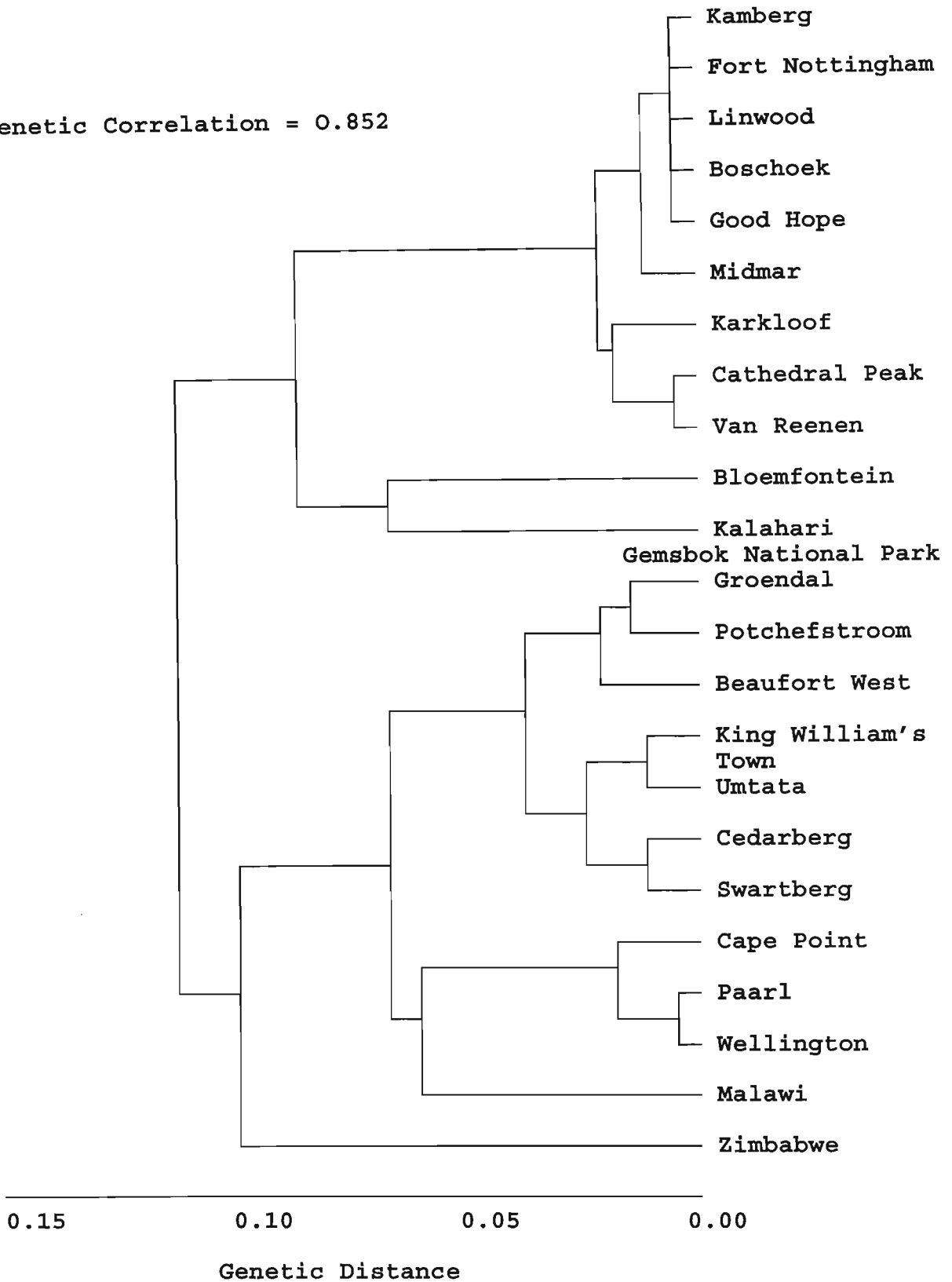
Figure 2.2   Unweighted pair group method with arithmetic averages
              (UPGMA) phenogram using Nei's (1978) unbiased genetic
              distance between the samples of *Rhabdomys pumilio* from
              different localities.

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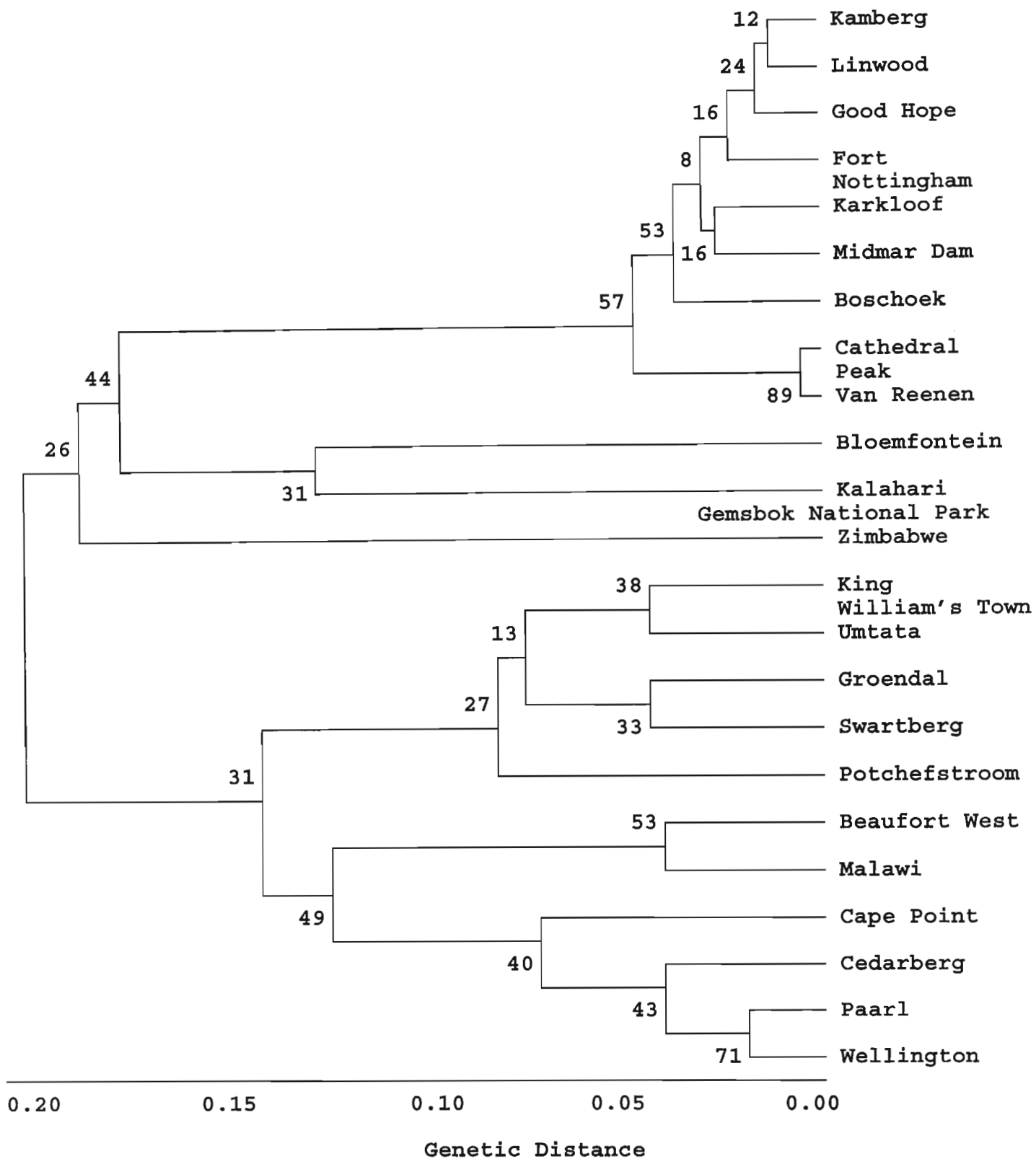
Figure 2.3   Unweighted pair group method with arithmetic averages
(UPGMA) phenogram showing bootstrapping (Dispan computer
programme; Ota, 1993) using the genetic distance, DA
(Nei *et al.*, 1993) between the samples of *Rhabdomys*
pumilio from different localities.
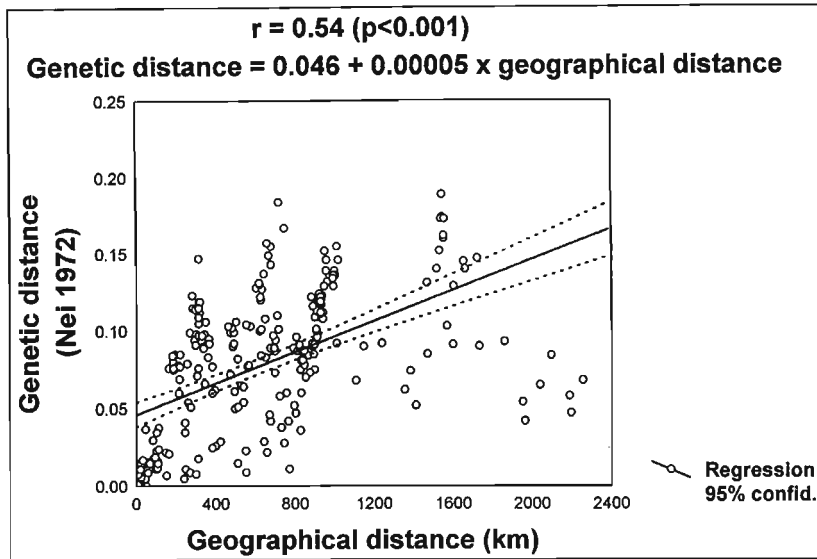
Figure 2.4   Graph showing the relationship between the geographical distances (km) and Nei's (1978) unbiased genetic distances between the different samples of *Rhabdomys pumilio*.

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Table 2.8 Spatial autocorrelation (Moran's I values) between heterozygosity (het.), allelic frequencies and geographical distances (km) between the different populations of *R.pumilio* in various localities.

Variable	Distance classes (km)					Probability (Bonferroni approximation)
	243	461	840	1107	4652	
Het.	0.12	_0.15_	-0.06	_-0.27_	-0.18	0.088
Ada	_0.46_	0.13	-0.20	_-0.29_	_-0.33_	0.000
Ck	_0.17_	_-0.30_	-0.06	-0.06	0.01	0.036
Est-1	0.05	_-0.18_	-0.07	-0.04	0.00	0.139
Est-2 (A)	0.08	-0.07	-0.05	_0.25_	_-0.43_	0.000
Est-2 (B)	-0.06	0.12	0.00	-0.16	-0.13	0.392
Est-2 (C)	0.09	_-0.32_	-0.01	_0.22_	_-0.21_	0.034
G6pdh	0.05	0.05	0.05	0.05	_-0.42_	0.001
Gpi	-0.03	-0.06	-0.07	-0.05	-0.03	1.000
G3pdh	0.02	-0.07	-0.07	-0.16	0.03	0.437
Hb-1	_0.27_	_-0.34_	0.00	_-0.32_	_0.15_	0.010
Hb-2	_0.48_	-0.02	0.03	-0.16	_-0.55_	0.000
Icdh	-0.04	-0.05	-0.04	-0.02	-0.08	1.000
Np (A)	_0.25_	_-0.36_	0.13	_-0.40_	_0.14_	0.003
Np (B)	_1.05_	_-0.39_	-0.12	_-0.25_	_-0.52_	0.000
Np (C)	_0.84_	0.05	_-0.35_	-0.02	_-0.74_	0.000
Pep-2	0.00	-0.11	-0.16	0.00	0.03	0.448
Pgdh	0.06	0.00	-0.06	-0.20	-0.04	0.425
Pgm	-0.16	-0.02	0.00	-0.02	-0.04	0.393
Sod	_0.96_	-0.10	_-0.33_	-0.22	_-0.54_	0.000
Average	0.23	-0.10	-0.07	-0.11	-0.19	

Underlined = Significant at p<0.05.

Table 2.9 Multiple regression analyses between heterozygosity (het.), various temperature and rainfall parameters, altitude and geographical co-ordinates and allele frequencies of the different polymorphic loci in samples of *R.pumilio* in different localities. Values for significant (p<0.05) regression coefficients are included.

	Het.	Ada	Ck	Est-1	Est-2 (A)	Est-2 (B)	Est-2 (C)
$F_{15,5}$	<u>10.58</u>	3.13	<u>70.00</u>	<u>64.50</u>	<u>59.50</u>	2.38	0.97
R^2	0.97	0.90	0.99	0.99	0.99	0.88	0.74
A	-20.0	----	-27.0	24.7	9.5	----	----
B	11.9	----	17.5	23.0	-5.8	----	----
C	12.5	----	13.5	-14.0	----	----	----
D	----	----	----	8.3	----	----	----
E	----	----	3.8	-4.3	7.2	----	----
F	----	----	----	-8.1	-7.2	----	----
G	----	----	----	6.6	9.7	----	----
H	6.9	----	5.9	-7.2	-5.1	----	----
I	----	----	3.9	4.2	1.4	----	----
J	2.4	----	-9.8	4.0	3.7	----	----
K	----	----	1.5	----	-2.3	----	----
L	----	----	-3.5	4.3	----	----	----
M	----	----	----	----	----	----	----
N	----	----	----	-1.8	2.1	----	----
O	----	----	2.6	-3.7	-2.1	----	----

UNDERLINED = Significant at p<0.05

A : Mean annual temperature (^{0}C)
B : Mean monthly maximum temperature (^{0}C)
C : Mean monthly minimum temperature (^{0}C)
D : Absolute maximum temperature (^{0}C)
E : Absolute minimum temperature (^{0}C)
F : Mean annual precipitation (mm)
G : Minimum annual precipitation (mm)
H : Maximal annual precipitation (mm)
I : Maximum precipitation in 24 hours (mm)
J : Highest maximum monthly precipitation (mm)
K : Months with potentially zero rainfall (mm)
L : Average days with greater than 10 mm rainfall
M : Altitude (m)
N : Latitude
O : Longitude

Table 2.9 continued
        Multiple regression analyses between
        heterozygosity (het.), various temperature and
        rainfall parameters, altitude and geographical
        co-ordinates and allele frequencies of the
        different polymorphic loci in samples of *R.pumilio*
        in different localities. Values for significant
        (p<0.05) regression coefficients are included.

	Gpi	G3pdh	Hb-1	Hb-2	Icdh	Np (A)	Np (B)
$F_{15,5}$	0.31	<u>80.85</u>	<u>955.00</u>	<u>3935.60</u>	0.10	<u>82.3</u>	<u>94.00</u>
R^2	0.49	0.90	0.97	0.99	0.22	0.99	0.99
A	----	-48.0	22.9	-2.8	----	----	12.4
B	----	25.0	12.0	-2.8	----	-5.6	6.5
C	----	34.5	-14.0	1.3	----	----	-8.6
D	----	3.0	----	-0.8	----	4.0	----
E	----	-7.2	----	1.6	----	-4.0	----
F	----	-6.5	----	-0.9	----	-9.8	5.3
G	----	17.3	----	-2.1	----	7.8	-4.6
H	----	3.7	----	2.1	----	----	2.9
I	----	----	----	-1.1	----	1.4	1.2
J	----	0.9	----	-0.6	----	0.9	----
K	----	3.5	----	0.6	----	-1.3	----
L	----	10.0	----	2.9	----	----	----
M	----	----	----	----	----	----	----
N	----	2.4	----	0.4	----	-1.6	-0.6
O	----	----	----	0.5	----	-2.1	----

UNDERLINED = Significant at p<0.05

A : Mean annual temperature (°C)
B : Mean monthly maximum temperature (°C)
C : Mean monthly minimum temperature (°C)
D : Absolute maximum temperature (°C)
E : Absolute minimum temperature (°C)
F : Mean annual precipitation (mm)
G : Minimum annual precipitation (mm)
H : Maximal annual precipitation (mm)
I : Maximum precipitation in 24 hours (mm)
J : Highest maximum monthly precipitation (mm)
K : Months with potentially zero rainfall (mm)
L : Average days with greater than 10 mm rainfall
M : Altitude (m)
N : Latitude
O : Longitude

Table 2.9 continued
        Multiple regression analyses between
        heterozygosity (het.), various temperature and
        rainfall parameters, altitude and geographical
        co-ordinates and allele frequencies of the
        different polymorphic loci in samples of *R.pumilio*
        in different localities. Values for significant
        (p<0.05) regression coefficients are included.

	Np (C)	Pep-2	Pgdh	Pgm	Sod
$F_{15,5}$	198.40	356.00	1.53	0.14	167.9
R^2	0.99	0.99	0.82	0.30	0.99
A	-19.0	-10.0	----	----	6.7
B	12.9	6.8	----	----	-7.5
C	11.6	----	----	----	-4.0
D	2.3	----	----	----	3.7
E	----	7.6	----	----	2.3
F	----	8.7	----	----	-3.5
G	----	-12.0	----	----	----
H	4.6	4.9	----	----	-3.5
I	-2.8	-1.4	----	----	2.9
J	-0.6	-3.9	----	----	1.6
K	1.5	2.3	----	----	-0.6
L	-3.5	----	----	----	2.0
M	----	----	----	----	----
N	2.1	-2.0	----	----	-1.6
O	1.7	----	----	----	-2.4

UNDERLINED = Significant at p<0.05

A : Mean annual temperature (^{0}C)
B : Mean monthly maximum temperature (^{0}C)
C : Mean monthly minimum temperature (^{0}C)
D : Absolute maximum temperature (^{0}C)
E : Absolute minimum temperature (^{0}C)
F : Mean annual precipitation (mm)
G : Minimum annual precipitation (mm)
H : Maximal annual precipitation (mm)
I : Maximum precipitation in 24 hours (mm)
J : Highest maximum monthly precipitation (mm)
K : Months with potentially zero rainfall (mm)
L : Average days with greater than 10 mm rainfall
M : Altitude (m)
N : Latitude
O : Longitude

Discussion

Allelic variation

Haemoglobin (*Hb*), nucleoside phosphorylase (*Np*), superoxide dismutase (*Sod*) and to a lesser extent creatine kinase (*Ck*) were the only loci to provide a geographic pattern of allelic variation. The localities in which the "A" allele of *Hb-1* locus was fixed were King William's Town and Umtata in the Eastern Cape, and Cape Point, Cedarberg, Paarl and Wellington in the Western Cape. The localities in which the "B" allele of the *Hb-1* locus was fixed were Beaufort West in the Western Cape, Bloemfontein in the Free State, Kalahari in the Northern Cape and Chelinda in Malawi. The allelic frequency distribution of this locus was only affected by the mean annual temperature and the mean monthly maximum and minimum temperatures. Rainfall appeared to have no effect (Table 2.9). The significant Bonferroni approximation for the spatial autocorrelation of the *Hb-1* allele (Table 2.8) and the high F_{st} (0.60) value (Table 2.6) supported the geographic allelic frequency distribution of this locus. The "A" allele for the *Hb-2* locus appeared only in the peninsular region of the Western Cape, Gauteng and Malawi. The "B" allele for this locus was presumed to be a "null" allele as it was not expressed in samples of *R.pumilio*. The geographic allelic distribution of this locus was significantly affected by

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temperature, rainfall latitude and longitude (Table 2.9). Similar to the *Hb-1* locus, the significant Bonferroni approximation for the spatial autocorrelation of the *Hb-2* allele (Table 2.8) and the high F_{st} (0.80) value (Table 2.6), supported the geographic allelic frequency distribution of this locus. The *Np* locus was fixed for the "A" allele in the Kalahari and the "C" allele in Cape Point. Temperature, rainfall, latitude and longitude affected the allelic frequency distribution of this locus (Table 2.9). The significant Bonferroni approximation for the spatial autocorrelations of the "A" and "B" alleles of the *Np* locus (Table 2.8) and the high F_{st} (0.54) value (Table 2.6) supported the geographic allelic frequency distribution of this locus. The "A" allele for *Sod* was fixed in Bloemfontein, Kalahari and KwaZulu-Natal while the Eastern and Western Cape localities were fixed for the "B" allele. Potchefstroom, Zimbabwe and Malawi were also fixed for the "B" allele for *Sod*. Temperature, rainfall, latitude and longitude affected the allelic frequency distribution of this locus (Table 2.9). The significant Bonferroni approximation for the spatial autocorrelation of the *Sod* allele (Table 2.8) and the very high F_{st} (0.99) value (Table 2.6) supported the geographic allelic frequency distribution of this locus. The *Ck* locus was monomorphic for the "B" allele in KwaZulu-Natal, the Western Cape and Northern Cape. The "A" allele

was expressed in Beaufort West, Bloemfontein and Umtata. The allelic frequency distribution of this locus was significantly affected by temperature, rainfall and longitude (Table 2.9). The significant Bonferroni approximation for the spatial autocorrelation of the allelic frequency for this locus (Table 2.8) and the relatively high F_{st} (0.37) value (Table 2.6) supported the geographic allele frequency distribution of this locus.

Although the *Est-2* (A) allelic frequency did display a significant Bonferroni approximation for spatial autocorrelation (Table 2.8) and was also affected by temperature, rainfall, latitude and longitude (Table 2.9), it did not display geographic variation. This was supported by the low F_{st} (0.075) value for this locus (Table 2.6).

Heterozygosity and polymorphism

As predicted, the mean heterozygosity (0.073) for *R.pumilio* was high compared to the mammalian mean (0.036) and vertebrate mean (0.049) obtained by Nevo (1978) and Wooten and Smith (1985) respectively. The high percent polymorphism and high mean number of alleles per locus predicted for *R.pumilio* did not materialise. The mean polymorphism (0.161) obtained for *R.pumilio* was only slightly higher than the mean polymorphism for mammals (0.147)

and lower than that for vertebrates (0.173) (Nevo, 1978). However the Potchefstroom and Zimbabwe samples produced polymorphisms of 0.308 and 0.268 respectively. *R.pumilio* is a generalist and a higher polymorphism was expected. The high heterozygosity (H) of some of the samples of *R.pumilio*, for example the Cathedral Peak (H=0.099) sample, suggested some genetic exchange across the Drakensberg mountains between the Free State and KwaZulu-Natal populations.

F-statistics

A low mean F_{is} and F_{it} and slightly positive F_{st} was predicted for *R.pumilio* because of its presumed pannmictic population structure. However, a high F_{it} value of 0.459 was obtained indicating a high fixation index of individuals relative to the total population. This was supported by the low effective number of migrants (Nm=0.179) which indicated a low level of gene flow (Loxterman *et al.*, 1998) and the high F_{st} value of 0.460 which according to Hartl (1988), Hogan *et al.* (1993), McCracken *et al.* (1994), Peppers *et al.* (1996) and Loxterman *et al.* (1998) is indicative of a high level of genetic differentiation among the sampled regions. Swart and Ferguson (1977) did not attach any importance to the statistically significant large F_{it} values obtained in their study of the black rhinoceros as they assumed

that the large F_{it} value was mostly the result of the large F_{st} value observed ($(1-F_{it}) = (1-F_{is})(1-F_{st})$. Therefore in the present study, the F_{is} and F_{st} values will be the main focus of attention. Although a mean F_{is} value of -0.01 indicated little inbreeding within samples of R.pumilio, Chesser (1991) and De Jong et al. (1994) showed that for socially structured populations a negative F_{is} value also indicated a complicated substructure (refer Chapter 5).

Population genetic structure

The extremes in population structure are the panmictic (outcrossed) and Wrightian (sub-divided) (Templeton, 1980). Panmictic populations are characterised by high heterozygosities, low F_{st}, low F_{is} and low F_{it} values, while Wrightian populations are characterised by low heterozygosities, high F_{st}, high negative F_{is} and high F_{it} values. The high F_{st} (0.459), the negative F_{is} (-0.01) and the high F_{it} (0.453) values obtained for R.pumilio suggests that it fits in somewhere between the panmictic and Wrightian population structures. This is further supported by the variation in heterozygosity (0.022-0.145) between the different samples.

Demastes et al. (1996) and Patton et al. (1996) were of the opinion that in the absence of physical barriers to gene flow,

geographical differentiation is expected to exhibit an isolation by distance relationship. The correlation coefficient of 0.54 (p<0.001) between the genetic and geographical distances between the different sample pairs and the significant Mantel test (p<0.001) between the F_{st} values and the geographical distances between the different sample pairs supports an isolation by distance relationship in *R.pumilio*. However, the strength of the relationship between the genetic and geographical distances was not very strong (r^2 =0.29). Furthermore, although the isolation by distance model is supported by the spatial autocorrelation results (Table 2.8) because of the positive autocorrelation within small distances and the negative autocorrelation between larger distances, only half of the alleles analysed supported the isolation by distance model. Therefore, besides the isolation by distance model, other factors must also be contributing to the geographic genetic differentiation between populations of *R.pumilio*.

Temperature and rainfall were found to be significantly correlated to the allelic frequency distribution of more than half of the polymorphic loci (Table 2.9) and therefore contributes to the geographic genetic differentiation in *R.pumilio*. A literature survey suggests very little work has been done on the role of temperature and rainfall on the geographic.

allelic distribution and it is important that further work be done in this direction. This is a full scale study and falls outside the scope of the present study.

Little evidence for the niche-width variation hypothesis (Van Valen, 1965; Johnson and Selander, 1971) was provided for by the population genetic structure of *R.pumilio*. Nevo (1978) showed that among the vertebrates, habitat specialists have a mean heterozygosity of 0.037, while habitat generalist, which are found in a variety of environments, have a mean value of 0.071. Similar results were obtained by Lavie *et al*. (1993) and Wójcik *et al*. (1996). According to Nevo *et al*. (1984), Nevo (1990) and Nevo *et al*. (1990) narrow-niche subterranean species also displayed significantly lower heterozygosities. The increased genetic variation (H=0.073) of *R.pumilio* may be regarded as an adaptive strategy for greater population fitness in a variety of environments. But if this argument is extended to the KwaZulu-Natal (Drakensberg and midlands) sample of *R.pumilio* which experience the most stable climate (i.e. narrow-niche), it should have a low heterozygosity, but in fact the heterozygosity displayed is similar to the overall mean heterozygosity (0.074). Nevo *et al*. (1995) further noted that when subterranean mammals ranged towards stressful environments, genetic diversity increased. A similar argument for *R.pumilio* is not applicable to

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samples from arid regions such as Bloemfontein (H=0.068), Beaufort West (H=0.055) and Kalahari (H=0.056) which had lower heterozygosities than the overall mean heterozygosity (H=0.073).

Athough temperature and rainfall, and the isolation by distance model contributed towards the geographic genetic differentiation, the importance of breeding tactics and social patterns in population genetic structure must also be considered. This aspect will be examined in Chapter 5.

Comparison of genetic similarities and distances

The prediction of high genetic similarities between samples of *R.pumilio* was substantiated by the experimental evidence. But Rogers (1972) genetic similarity values (0.796-0.988) represented a much wider range compared to other rodents. Work done on local populations of a species or subspecies from different animal groups recorded Rogers (1972) genetic similarity values ranging between 0.933 and 0.990 (Johnson and Selander, 1971; Patton *et al.*, 1972; Rogers, 1972; Hunt and Selander, 1973; Calhoun *et al.*, 1988).

The prediction of low genetic distances between samples of *R.pumilio* was also substantiated by the experimental evidence but represented a wider range when compared to studies with other rodents. Using Nei's (1978) unbiased genetic distance, the values

recorded for *R.pumilio* ranged between 0.000-0.189 wheres values

recorded for some other rodents ranged between 0.01 and 0.08

(Sage *et al.*, 1986, Gill *et al.*, 1987, Taylor *et al.*, 1992). This

does provide support for the argument that genetic

diversification has occurred in *R.pumilio*.

Cluster analysis

Based on cluster analysis of Nei's (1978) unbiased genetic

distance, the KwaZulu-Natal sample is a subgroup with the

Kalahari and Bloemfontein samples as outliers (Figure 2.2).

Cluster analyses of DA genetic distances (Nei *et al.*, 1983) with

bootstrapping (Figure 2.3) shows the association of the Kalahari

and Bloemfontein samples to be relatively weak. It is possible

that these two populations are becoming isolated from each other

and tending towards separate subgroups. Figure 2.2 also shows a

peninsular Western Cape sample with the Beaufort West, Cedarberg

and Swartberg being associated with the Eastern Cape subgroup.

However, cluster analyses of Nei's *et al.* (1983) DA genetic

distance with bootstrapping (Figure 2.3) grouped the Cedarberg

sample with the Western Cape samples. Further allozymic studies

of samples of *R.pumilio* from other areas of the Northern Cape is

essential for determining the status of the Cedarberg population.

Comparison of subgroups

Meester *et al.* (1986) retained seven of the twenty subspecies listed by Roberts (1951). Two of these subspecies are from Namibia and cannot be considered here because of lack of samples. Meester *et al.* (1986) identified an Eastern Cape subspecies, *R.p.intermedius* (Wroughton,1905) from the central and eastern Cape, a Western Cape subspecies, *R.p.pumilio* (Sparrman, 1784) from the south-western and southern Cape towards the Free State and a northern KwaZulu-Natal population extending to eastern Zimbabwe (*R.p.dilectus*, De Winton, 1897). The present study supports this to a certain extent in that it identifies an Eastern Cape and and peninsular Western Cape subgroup. However, although samples are lacking from northern KwaZulu-Natal, the sample from the midlands of KwaZulu-Natal was considered a distinct subgroup with the Zimbabwe sample as an outlier and probably a distinct subgroup. The two other subspecies identified by Meester *et al.* (1986) were *R.p.cinereus* (Thomas and Schwann, 1904) from the Northern Cape Province and *R.p.griquae* (Wroughton, 1905) from parts of the Northern Cape Province, central Botswana, southern Namibia and the south western area of the former Transvaal. It appears that the Kalahari and Potchefstroom samples correlate with the range of these two subspecies, with the Kalahari population forming part of the former subspecies and the

Potchefstroom population forming part of the latter subspecies. More samples are required from the Northern Cape, North Western Cape, Gauteng, Mpumalanga and Northern Province to address the issue of the latter two subspecies and to determine the boundaries of the different subgroups. Also more localities need to be sampled from Malawi and Zimbabwe before their subgrouping can be confirmed.

Further studies

A further study of the genetic variation in *R.pumilio* was undertaken to measure genetic variation at the nuclear level. The method used was the random amplification of polymorphic nuclear DNA using the polymerase chain reaction (PCR-RAPD). This is a powerful recent technique which has been used to compare the genomes of closely related species and to determine the extent of genetic divergence (Bowditch *et al.*, 1993).

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CHAPTER 3

GENETIC VARIATION - PCR-RAPD

Introduction

Techniques used in DNA studies involve DNA-DNA hybridization, restriction endonuclease analysis and sequencing. The limitation of DNA-DNA hybridization studies is that a large amount of intraspecific polymorphism can be problematic in the estimation of phylogenetic relationships between closely related species (Werman *et al.*, 1990), whereas Dowling *et al.* (1990) argued that restriction studies should be confined to very closely related sequences because of the difficulties presented by length variation. Although nucleic acid sequencing can be used to study virtually any systematic problem, it is not necessarily the best approach to a particular problem (Hillis *et al.*, 1990). A powerful recent technique for revealing sequence polymorphisms, the Randomly Amplified Polymorphic DNA technique (RAPD) using the Polymerase Chain Reaction (PCR) (Welsh and McClelland, 1990; Williams *et al.*, 1990) has been used to compare the genomes of closely related species and to determine the extent of genetic

divergence (Bowditch *et al.*, 1993). This technique has also been used in mapping studies, species identification and genome fingerprinting (Arnold *et al.*, 1991; Welsh *et al.*, 1991; Wilde *et al.*, 1992; Bardakci and Skinbinski, 1994; Tamate *et al.*, 1995).

Several problems are associated with the PCR-RAPD method. Amongst these are the reproducibility of weakly amplified bands, the type of polymerase used in the reaction (Schierwater and Ender, 1993), the concentration of the primer and template (Muralidharan and Wakeland, 1993) and the acceptance of certain assumptions before any statistics can be applied to RAPD data (Clark and Lanigan, 1993; Weissing and Velterop, 1993). Although Van de Zande and Bijlsma (1995) demonstrated that RAPD markers were very efficient in the identification of *Drosophila* species, they conceded that this method was limited to sibling species in assessments of phylogenetic relationships and that reliable measures of genetic distances cannot be obtained. However, the advantages of RAPD's far outweigh the disadvantages, in that no prior knowledge of the genome sequence is required, a small amount of DNA is required and a large number of samples can be analysed simultaneously (Welsh and McClelland, 1990; Williams *et al.*, 1990). However, in this study, the high cost of the chemicals used in the PCR-RAPD study limited the number of tissue

samples that could be analysed. Because of the small sample size (N=5) from each of 9 localities, statistically valid conclusions could not be made and this study was therefore used primarily as a support base for the allozyme data.

The present study involved the use of the PCR-RAPD technique to compare the genetic similarity between selected samples of *R.pumilio* from some of the provinces in South Africa and Zimbabwe.

Materials and methods

Rhabdomys pumilio specimens were live-trapped from different regions of southern Africa using Sherman-type (Titian Productions, Cape Town) and PVC traps (Willan, 1979). Randomised sampling was carried out in all the different localities except the Kalahari Gemsbok National Park (refer Chapter 2). The animals were sacrificed in the field and standard measurements recorded. The liver, heart and kidneys were removed and stored in liquid nitrogen. The voucher specimens were deposited in the Durban Natural Science Museum and Transvaal Museum (Appendix 17).

The distribution of *R.pumilio* and sampling sites for the present study are illustrated in Figure 3.1. The geographical co-ordinates of the sampled localities are presented in Table 3.1, with KwaZulu-Natal being represented by the Fort Nottingham

sample and the Zimbabwe sample in the Vumba area. In an attempt to obtain a representative sample, 5 tissue samples were used from a locality in each of the provinces in South Africa in which specimens had been collected and from Vumba in Zimbabwe. Three localities were selected from the Western Cape (Paarl, Swartberg and Beaufort West) because of the variation in the haemoglobin (*Hb*) loci detected in this province in the allozyme study (Chapter 2).

DNA was prepared from the liver tissue according to the method described by Hillis *et al.* (1990) (Appendix 6). Proteinase K was excluded from this procedure as it was found to be unnecessary for the isolation of DNA. A brief summary of the PCR-RAPD technique and the preparation of the master mix is presented in Appendix 7. Five primers (Operon Technologies, Inc.) were used in this study and these were primer numbers 5, 6, 7, 9 and 10 (Table 3.2). All 20 primers (Operon Technologies series A) were tested and 5 primers were selected based on resolution and nunber of bands. The optimal magnesium concentration for the different primers was estimated to be 5 micromoles, with lower concentrations resulting in fewer bands being amplified and a higher concentration resulting in no amplification. The Stoffel fragment of Taq polymerase was chosen as the amplification enzyme because it was reported to be magnesium tolerant and generated

a high number of amplification fragments (Bishop, 1995). Because of the high cost of the enzyme, Taq polymerase, the minimum amount that could be used to produce repeatable PCR-RAPD profiles had to be determined. The number of units of Taq polymerase was tested between 2 and 3 units per reaction and the optimum was found to be 2.5 units. The DNA concentration was optimized by varying it between 10 and 50 ng per reaction. Consistent results were obtained between 10 and 30 ng of DNA per reaction. Amplifications were performed according to that originally recommended by Williams *et al.* (1990) but with slight modifications. For the first cycle in the thermal cycler (ESU Programmable Temperature Cycler), denaturation, annealing and extention was 94°C for three minutes, 39°C for two minutes and 72°C for three minutes respectively. Denaturation time was decreased by one minute for the following forty cycles, with the extension time being increased by four minutes in the final cycle. The products were run on a 5% vertical polyacramide gel and thereafter stained according to the non-ammoniacal silver staining method (Ausubel *et al.*, 1992) (Appendix 8). Fragments were scored as 0 and 1 depending on whether they were absent or present. The index of similarity (simple-match coefficient) between individuals was calculated using the NTSYS-PC computer programme (Rohlf, 1989).

Table 3.1 The sampling sites and geographical co-ordinates for specimens of *Rhabdomys pumilio*. N = sample size.

Sampling site	Geographical co-ordinates	N
KwaZulu-Natal		
1. Fort Nottingham	29°25'S, 29°55'E	5
Eastern Cape		
2. Groendal	33°40'S, 25°28'E	5
Western Cape		
3. Beaufort West	32°18'S, 22°36'E	5
4. Paarl	33°45'S, 18°58'E	5
5. Swartberg	33°13'S, 22°03'E	5
Free State		
6. Bloemfontein	29°07'S, 26°14'E	5
Northern Cape		
7. Kalahari Gemsbok National Park	25°30'S, 20°30'E	5
Gauteng		
8. Potchefstroom	26°42'S, 27°06'E	5
Zimbabwe		
9. Vumba	18°55'S, 32°40'E	5

Table 3.2 Operon codes and sequence of the primers used for DNA amplification in the samples of *Rhabdomys pumilio*.

Primer number	Primer code	5' to 3'	Molecular weight
5	OPA-05	AGGGGTCTTG	3090
6	OPA-06	GGTCCCTGAC	2995
7	OPA-07	GAAACGGGTG	3108
9	OPA-09	GGGTAACGCC	3044
10	OPA-10	GTGATCGCAG	3059
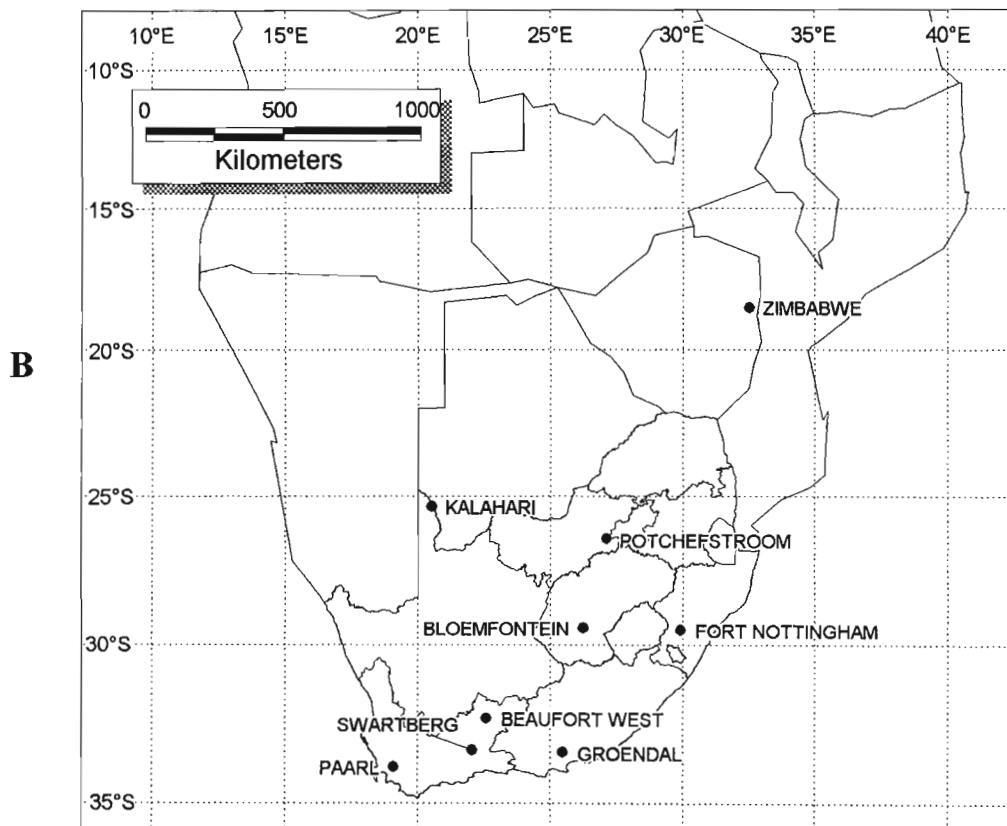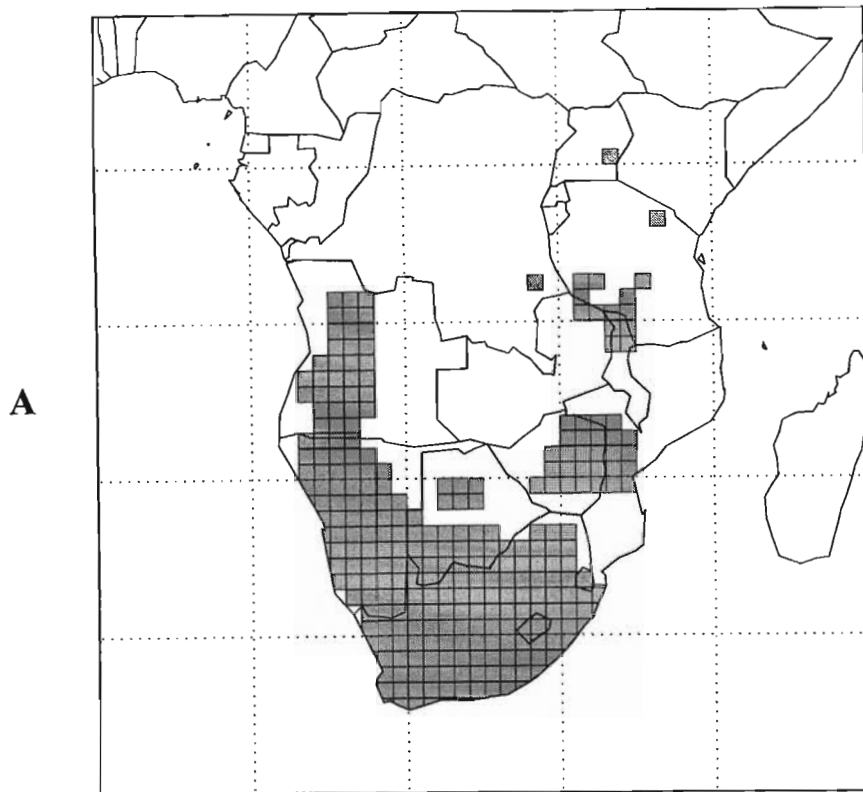
Figure 3.1 Maps showing (A) the distribution of *Rhabdomys pumilio* (courtesy of Neil Burgess) and (B) the sampling sites.

Results

The number of bands scored with primer number five, six, seven, nine and ten were fourteen, sixteen, twelve, sixteen and nineteen respectively. The percentpolymorphism between the samples from different localities ranged between 41.6% and 50.6% with a mean of 47.1% (Table 3.3). Because of the small sample size (N=5), error could be introduced when determining the percent polymorphism. The reproducibility of the bands and presence of polymorphism in certain specimens of *R.pumilio* is illustrated in Figure 3.2. The clustering of the individuals from the different samples using the combined results of the five primers is illustrated in Figure 3.3.

Using primer number 5, the similarity ranged between 0.400 and 0.999. The lowest similarity coefficient was obtained between the Paarl and Zimbabwe samples (0.40) while the similarity between the other sample pairs ranged between 0.500 and 0.999 (Table 3.4). The phenogram (Figure 3.4a) shows the Zimbabwe and Kalahari samples as outliers.

Using primer number 6, the similarity ranged between 0.267 and 0.999, but if the Zimbabwe sample was excluded, the similarity ranged between 0.600 and 0.999 (Table 3.4). The phenogram (Figure 3.4b) shows the Zimbabwe sample as an outlier.

Using primer number 7, the similarity ranged between 0.429

85

and 0.999 (Table 3.4). The Zimbabwe and Kalahari samples were grouped together and associated with the Potchefstroom and KwaZulu-Natal samples (Figure 3.5a).

Using primer number 9, the similarity ranged between 0.250 and 0.833 (Table 3.4). The phenogram shows KwaZulu-Natal as an outlier sample (Figure 3.5b).

Using primer number 10, the similarity ranged between 0.353 and 0.882, but if the Zimbabwe sample was excluded the similarity ranged between 0.471 and 0.882 (Table 3.4). The Zimbabwe and the Kalahari samples are outliers to the remaining samples (Figure 3.6a).

The combining of the results for the 5 primers used, showed a similarity range of 0.471-0.853 (Table 3.4). The phenogram (Figure 3.6b) shows the Zimbabwe and Kalahari samples as outliers to the remaining samples.

A significant (p<0.001) correlation coefficient between the genetic dissimilarities (inverse of simple match) and geographic distances (Appendix 9) between the samples of *R.pumilio* was obtained with primer numbers 5 (r=0.75), 6 (r=0.60) and 10 (r=0.72) (Figures 3.7 and 3.9a) whereas primer number 7 (r=0.26) and primer number 9 (r=0.18) had probabilities of 0.12 and 0.29 respectively (Figure 3.8). With the combining of the results of the 5 primers, the correlation coefficient between the genetic

divergence and geographic distances between the samples was 0.75

(p<0.001) (Figure 3.9b).
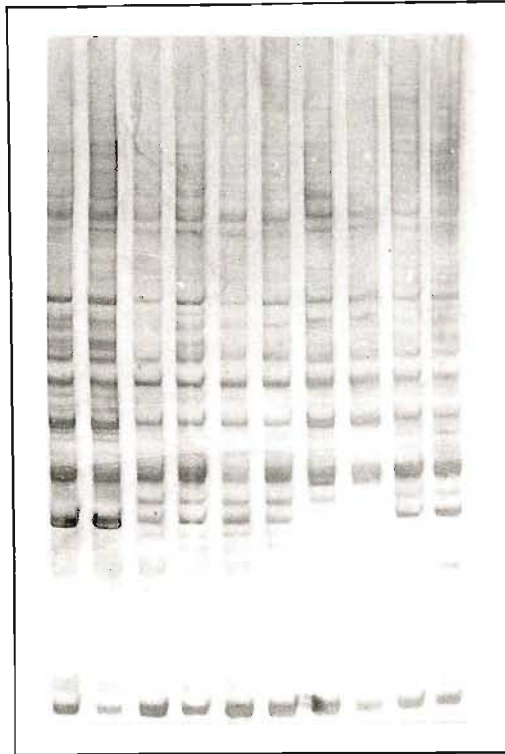
Figure 3.2   Photograph showing the reproducibility of
bands and the presence of monomorphic and
polymorphic bands.

Table 3.3   Percent polymorphism of samples of
Rhabdomys pumilio.

Locality	Polymorphism (%)
Beaufort West	41.6
Bloemfontein	49.1
Groendal	48.0
Kalahari	42.9
KwaZulu-Natal	48.0
Paarl	43.9
Potchefstroom	50.6
Swartberg	43.9
Zimbabwe	45.5
Mean	47.1
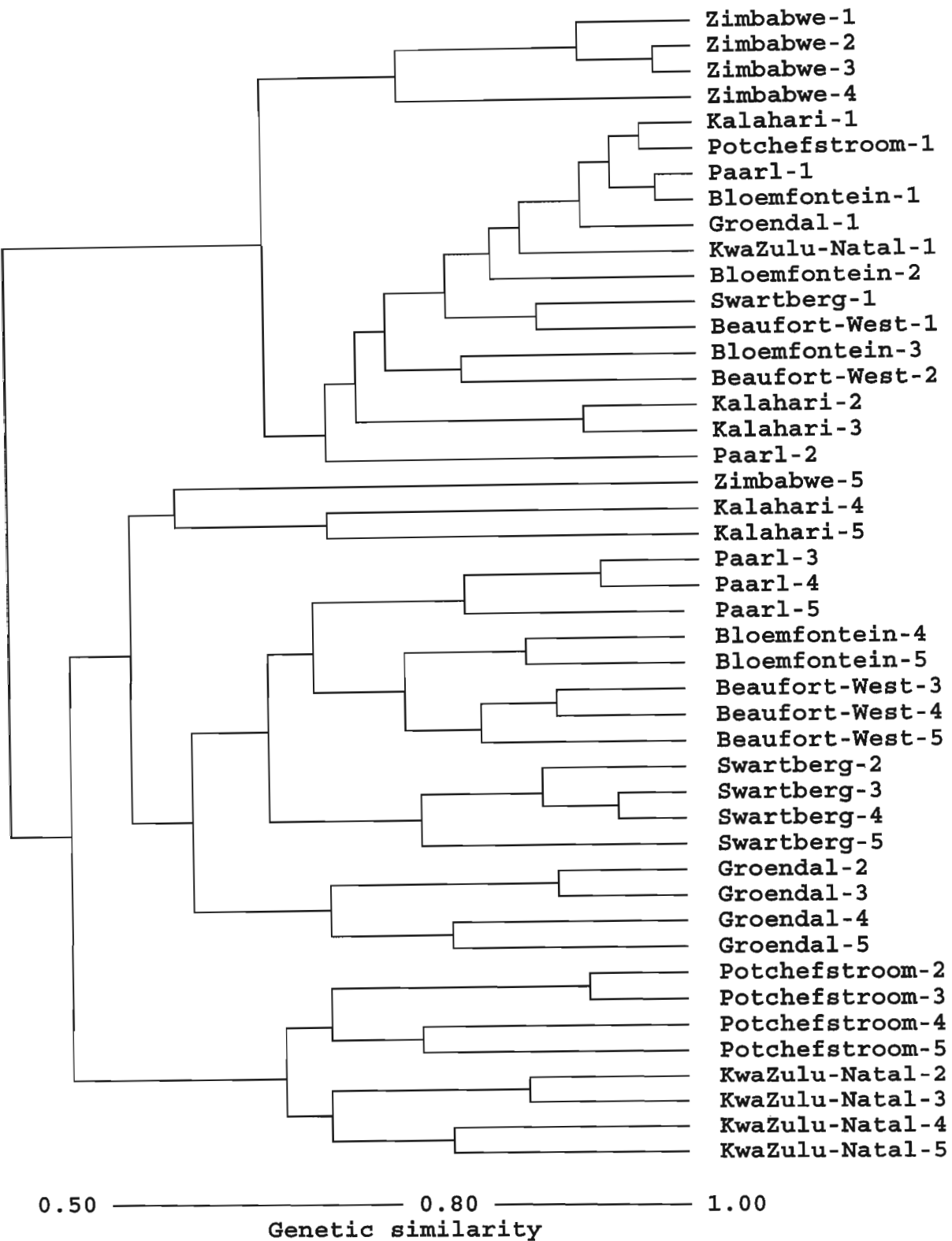
Figure 3.3   Phenogram showing the similarity betweeen individuals of *Rhabdomys pumilio* using the Ntsys-pc computer programme (Rohlf, 1989).

Table 3.4 Matrix of similarity between the different samples of *Rhabdomys pumilio* using various primers.

Above diagonal: primer 5
Below diagonal: primer 6

Locality of sample	1	2	3	4	5	6	7	8	9
1. KwaZulu-Natal	-----	0.600	0.600	0.700	0.800	0.900	0.500	0.900	0.700
2. Groendal	0.800	-----	0.999	0.900	0.800	0.700	0.700	0.700	0.500
3. Beaufort West	0.667	0.600	-----	0.900	0.800	0.700	0.700	0.700	0.500
4. Paarl	0.667	0.600	0.867	-----	0.900	0.800	0.600	0.800	0.400
5. Swartberg	0.600	0.667	0.800	0.933	-----	0.900	0.700	0.900	0.500
6. Bloemfontein	0.667	0.600	0.867	0.999	0.933	-----	0.600	0.999	0.600
7. Kalahari	0.667	0.600	0.733	0.733	0.667	0.733	-----	0.600	0.600
8. Potchefstroom	0.867	0.933	0.667	0.667	0.600	0.667	0.667	-----	0.600
9. Zimbabwe	0.333	0.267	0.400	0.533	0.467	0.533	0.667	0.333	-----

Above diagonal: primer 7
Below diagonal: primer 9

Locality of sample	1	2	3	4	5	6	7	8	9
1. KwaZulu-Natal	-----	0.714	0.643	0.714	0.571	0.714	0.571	0.857	0.714
2. Groendal	0.333	-----	0.929	0.857	0.857	0.857	0.571	0.571	0.714
3. Beaufort West	0.333	0.417	-----	0.786	0.786	0.786	0.643	0.500	0.643
4. Paarl	0.500	0.417	0.500	-----	0.857	0.999	0.571	0.571	0.714
5. Swartberg	0.333	0.417	0.667	0.417	-----	0.857	0.429	0.429	0.571
6. Bloemfontein	0.417	0.583	0.667	0.833	0.583	-----	0.571	0.571	0.714
7. Kalahari	0.500	0.583	0.500	0.667	0.417	0.750	-----	0.714	0.714
8. Potchefstroom	0.333	0.667	0.250	0.333	0.333	0.417	0.667	-----	0.571
9. Zimbabwe	0.250	0.500	0.500	0.333	0.250	0.500	0.500	0.500	-----

Above diagonal: primer 10
Below diagonal: combined primers

Locality of sample	1	2	3	4	5	6	7	8	9
1. KwaZulu-Natal	-----	0.647	0.647	0.471	0.588	0.706	0.529	0.706	0.412
2. Groendal	0.618	-----	0.882	0.824	0.824	0.700	0.588	0.824	0.529
3. Beaufort West	0.588	0.779	-----	0.706	0.706	0.706	0.471	0.706	0.412
4. Paarl	0.603	0.735	0.750	-----	0.765	0.647	0.647	0.765	0.412
5. Swartberg	0.574	0.735	0.750	0.779	-----	0.647	0.647	0.882	0.529
6. Bloemfontein	0.676	0.706	0.750	0.853	0.779	-----	0.529	0.647	0.529
7. Kalahari	0.559	0.618	0.603	0.647	0.574	0.632	-----	0.647	0.353
8. Potchefstroom	0.735	0.735	0.574	0.632	0.632	0.647	0.662	-----	0.470
9. Zimbabwe	0.471	0.515	0.484	0.485	0.471	0.574	0.559	0.485	-----
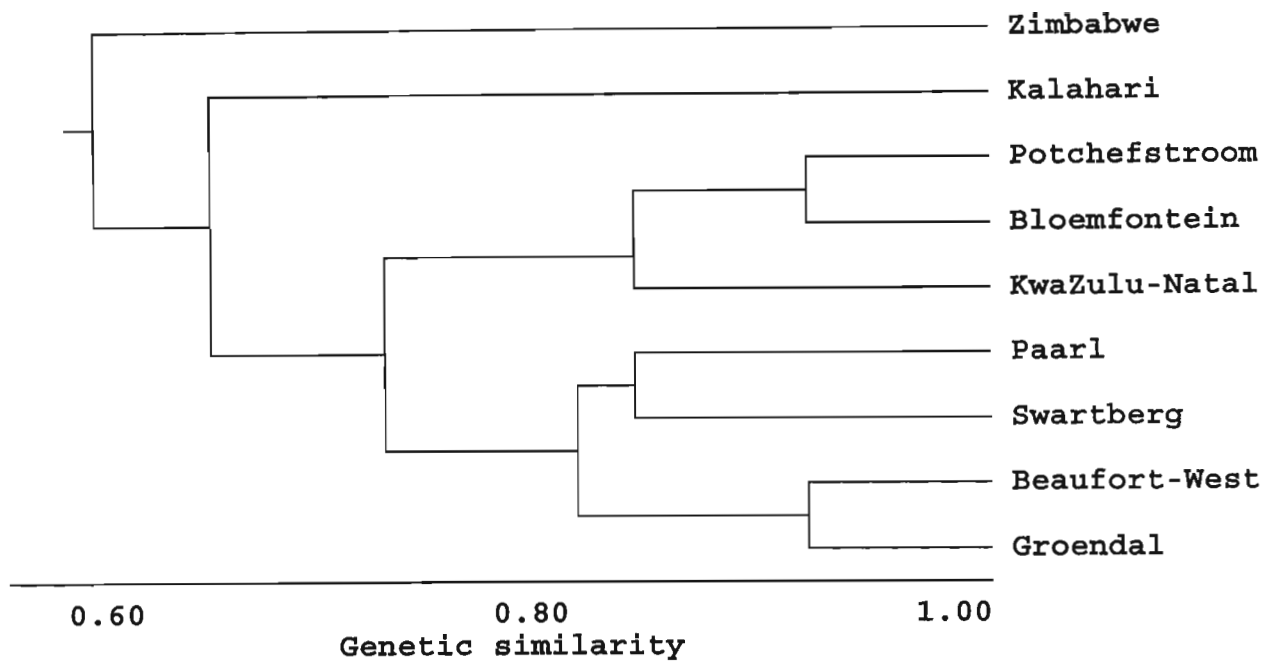
(a) primer 5
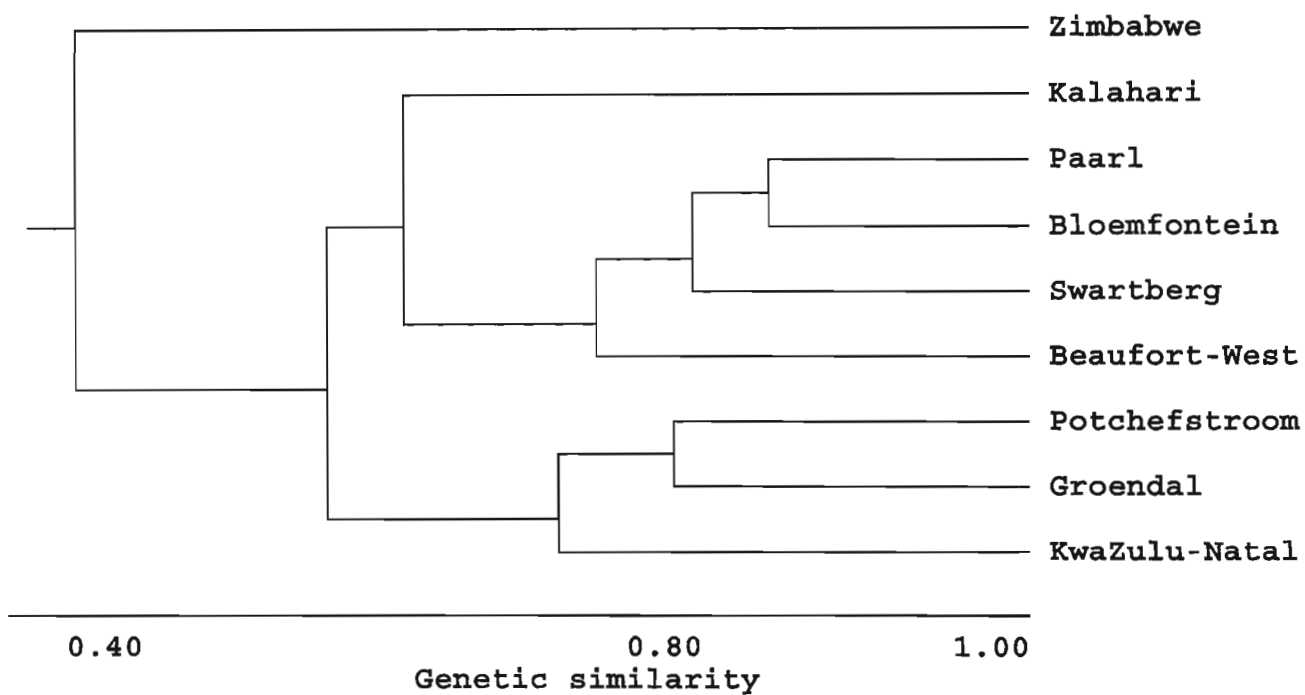
(b) primer 6

Figure 3.4   Phenograms showing the similarity between samples of *Rhabdomys pumilio* from different localities using the Ntsys-pc computer programme (Rohlf, 1989) with (a) primer 5 and (b) primer 6.
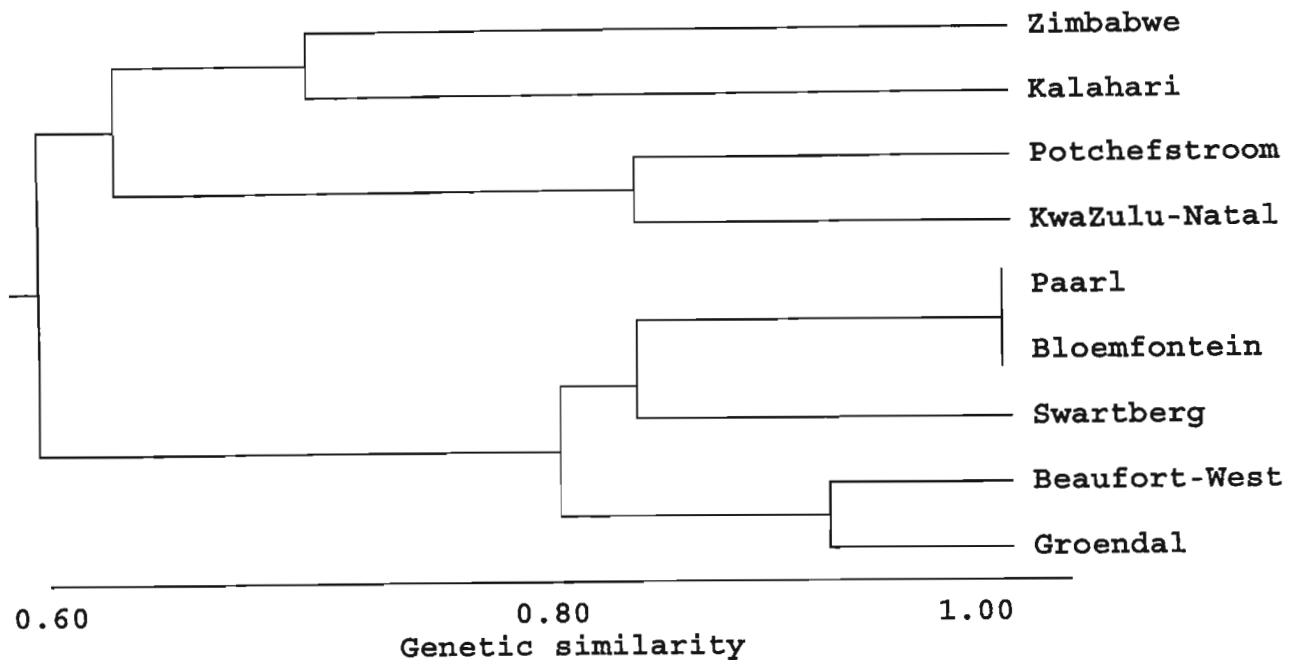
(a) primer 7
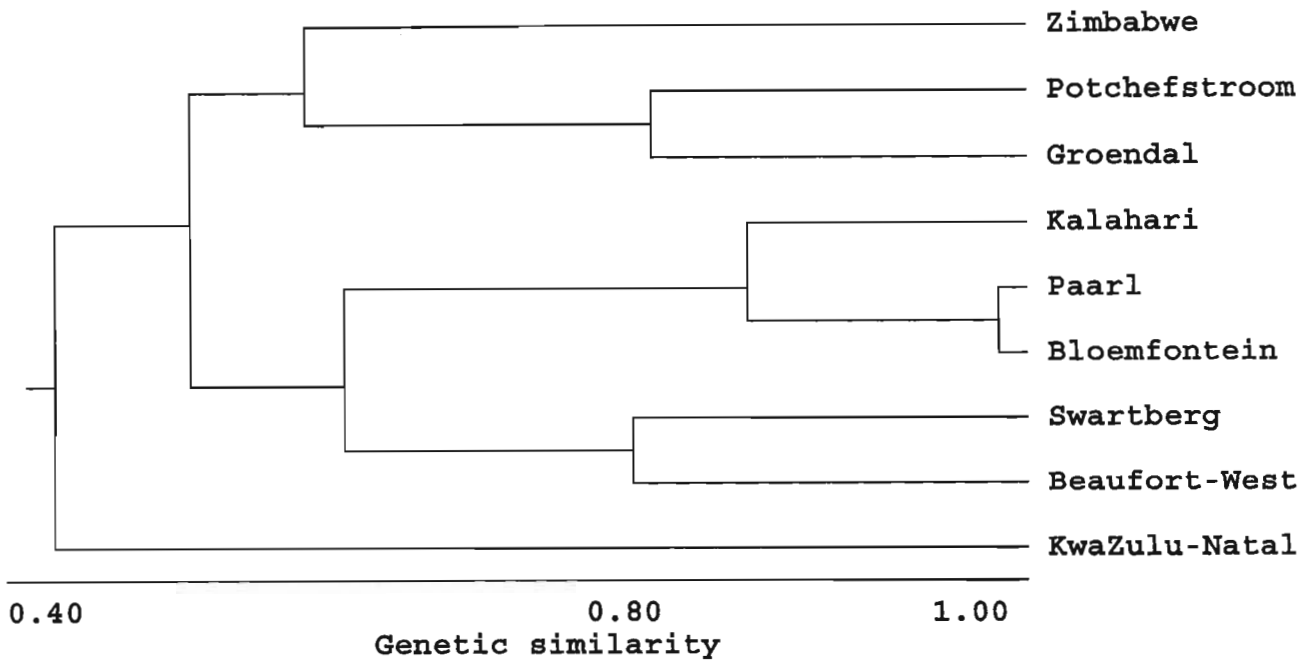
(b) primer 9

Figure 3.5   Phenograms showing the similarity between samples
              of *Rhabdomys pumilio* from different localities using
              the Ntsys-pc computer programme (Rohlf, 1989) with
              (a) primer 7 and (b) primer 9.
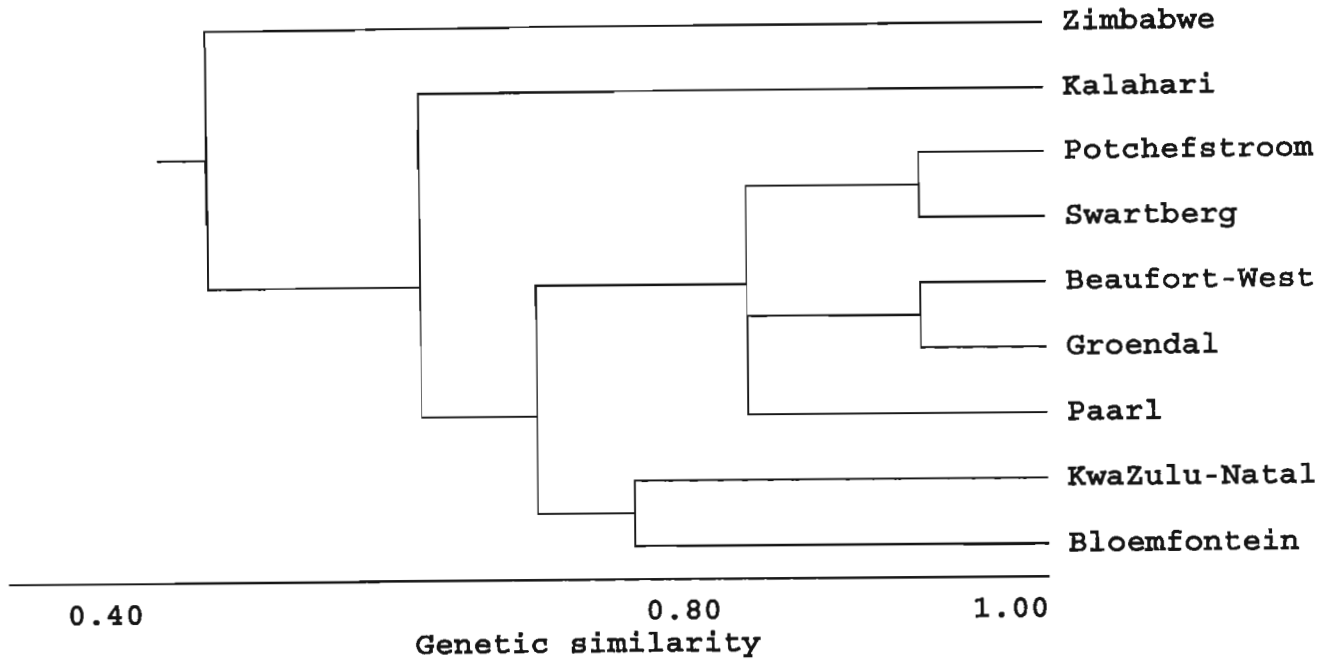
(a) primer 10
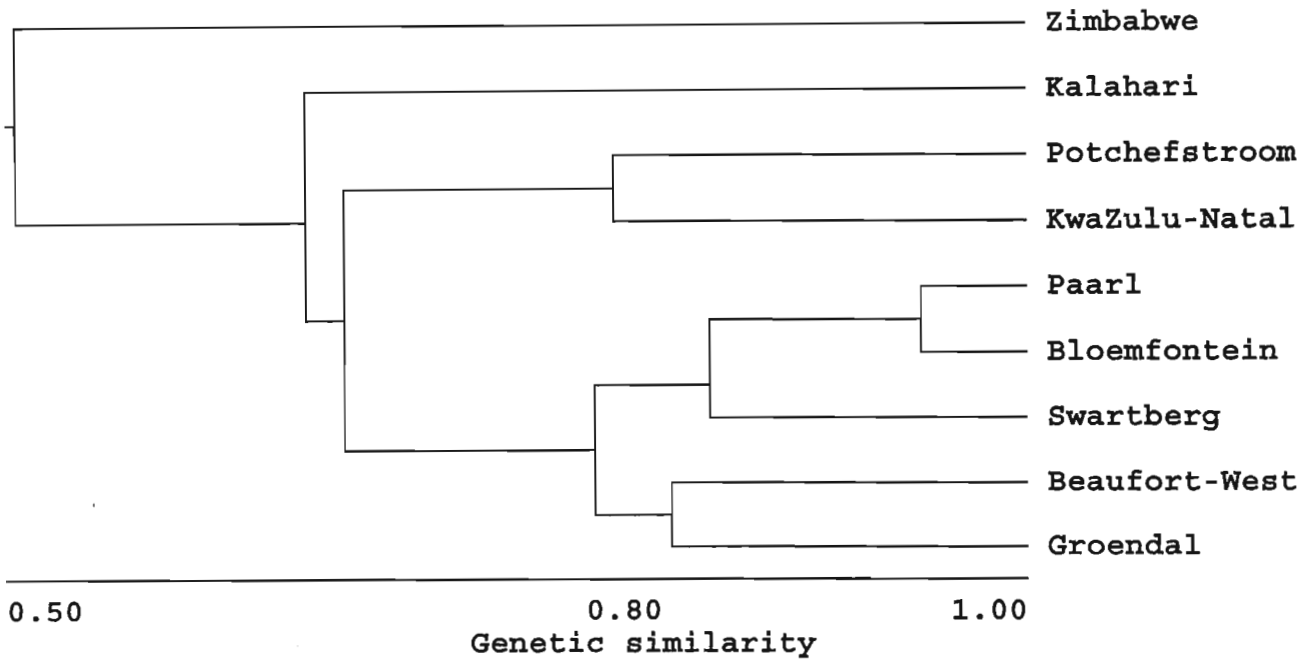
(b) combined primers

Figure 3.6   Phenograms showing the similarity between samples
             of *Rhabdomys pumilio* from different localities using
             the Ntsys-pc computer programme (Rohlf, 1989) with
             (a) primer 10 and (b) combined primers.

a
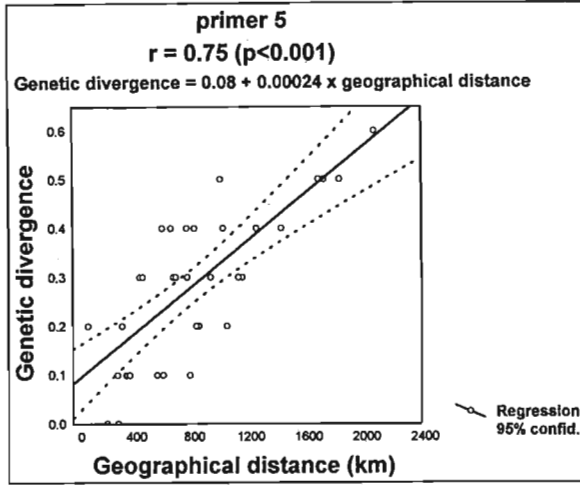
b
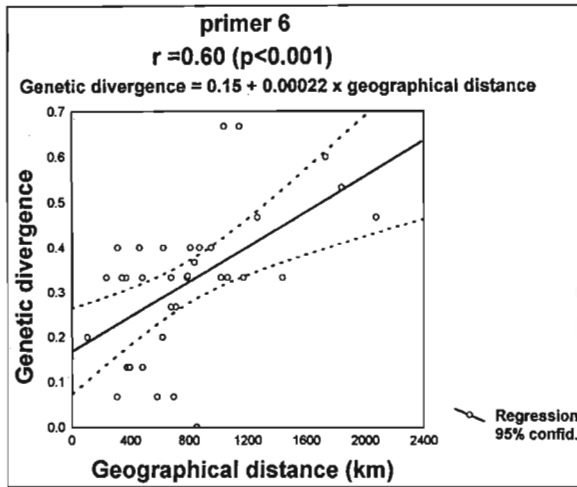
Figure 3.7   Graphs showing the relationship between the
              geographical distance (km) and genetic
              divergence between the different samples of
              Rhabdomys pumilio with (a) primer 5 and
              (b) primer 6.

a
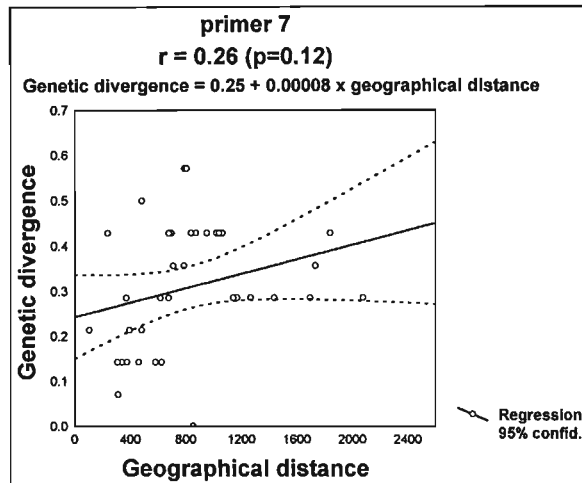
b
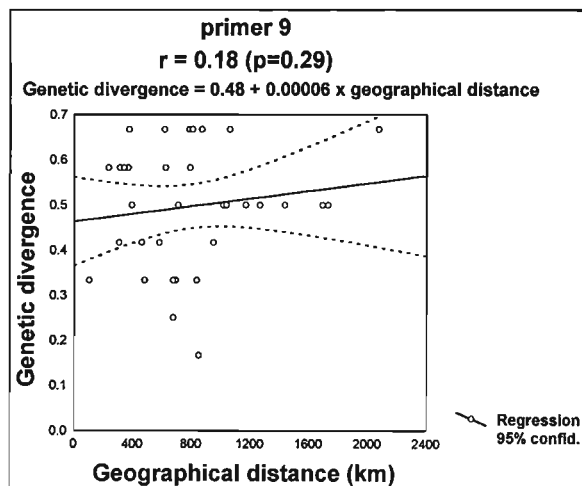
Figure 3.8   Graphs showing the relationship between the
             geographical distance (km) and genetic
             divergence between the different samples of
             Rhabdomys pumilio with (a) primer 7 and
             (b) primer 9.

a
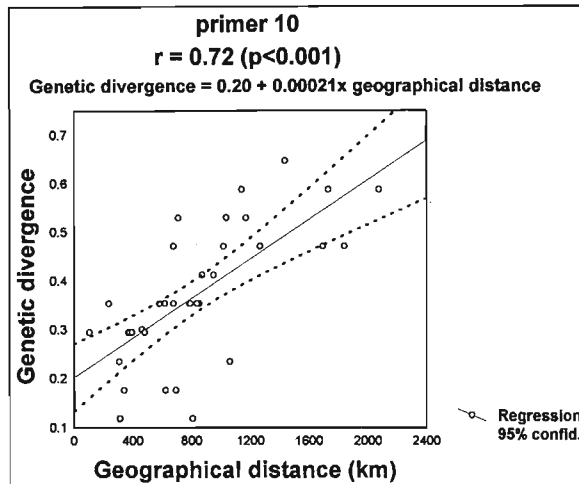
b
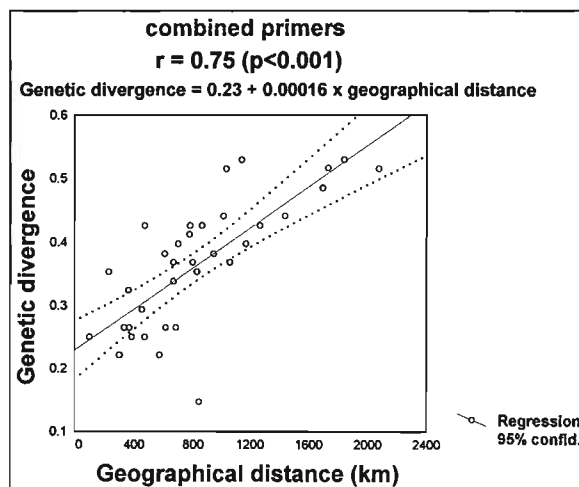
Figure 3.9   Graphs showing the relationship between the geographical distance (km) and genetic divergence between the different samples of *Rhabdomys pumilio* with (a) primer 10 and (b) combined primers.

Discussion

The high genetic similarity expected for the different samples of *R.pumilio* because of its presumed panmictic population structure was not substantiated by the PCR-RAPD study particularly with primer number 6 (genetic similarity ranged between 0.267 and 0.999 between the different samples). The combining of the five primers showed a similarity range of 0.471-0.853 between the samples of *R.pumilio* (Table 3.4). Comparing the above data with that of other workers, for example, Kozol *et al.* (1994) working with the burying beetle *Nicrophorus americanus* from different localities obtained a genetic similarity (simple-match coefficient) of between 0.82 and 1.00; Johnston and Fernando (1995) obtained a mean similarity of 0.70 between different strains of the Protozoan parasite, *Eimeria acervulina*; Bishop (1995) obtained a simple-match similarity coefficient of 0.74 to 0.81 for Zebra populations from different game reserves in KwaZulu-Natal, Van de Zande and Bijlsma (1995) obtained simple-match similarity coefficients between 0.80 and 0.86 between different strains of *Drosophila melanogaster* and values ranging from 0.03 to 0.38 between 9 different species of *Drosophila*, supported genetic divergence in the samples of *R.pumilio*. Genetic divergence between the samples of *R.pumilio* was further supported by the combined primers phenogram (Figure

3.6) which indicated the Kalahari and Zimbabwe samples as outliers, with the Potchefstroom sample associated with KwaZulu-Natal and separated from the Eastern and Western Cape samples. This subgrouping of samples of R.pumilio as a result of genetic differentiation was also recognised by the allozymes (Chapter 2).

Bishop (1995) obtained a percent polymorphism range of 20-39% between the four zebra populations from different game reserves and concluded that the percent polymorphism was not maintained in small isolated zebra populations. Based on this argument the decline in percent polymorphism in some of the samples of R.pumilio (50.6% to 41.6%) could be attributed to isolation which would then result in geographic genetic divergence. However, because of the small sample size of the various populations, error could have been introduced when measuring the genetic diversity and therefore the above argument could not be verified.

In the absence of physical barriers to gene flow, geographical differentiation was expected to exhibit an isolation by distance relationship (Demastes et al., 1996; Patton et al., 1996). The significant correlation coefficient between genetic divergence (coefficient of dissimilarity) and geographic distance obtained with primer numbers 5 (r=0.75), 6 (r=0.60) and 10 (r=0.72) supported an isolation by distance model. Although the

isolation by distance model was not supported by primer number 7 (r=0.26, p=0.12) and primer number 9 (r=0.18, 0.29), this model was supported by the combined primers results with which a correlation coefficient of 0.75 (p<0.001) was obtained. This suggested a certain degree of genetic differentiation between the different samples. Because of the small sample size from the different localities, these results are presented simply as support for the allozyme data (Chapter 2) which proposed an isolation by distance model for genetic variation between samples of *R.pumilio* from different localities. A correlation coefficient of 0.54 (p<0.001) was obtained with the allozyme data, between Nei's (1978) unbiased genetic distances and the geographical distances between the samples of *R.pumilio*.

The combined primers phenogram (Figure 3.6) indicated the Zimbabwe sample of *R.pumilio* as an outlier to the KwaZulu-Natal/Potchefstroom subgroup and the less defined Eastern and Western Cape subgroups. With the allozymes phenogram (Chapter 2: Figure 2.2) the Zimbabwe sample was an outlier to the Eastern Cape and Western Cape subgroups, while bootstrapping (Chapter 2: Figure 2.3) showed that the Zimbabwe sample was an outlier of the KwaZulu-Natal subgroup. However, the Zimbabwe sample was weakly associated with the South African subgroups (Chapter 2: Figure 2.3) and this was supported by the combined primers

phenogram, in which the genetic divergence of the Zimbabwe sample was expressed more strongly.

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CHAPTER 4

CHROMOSOMAL VARIATION

Introduction

The primary techniques used in most evolutionary studies of chromosomal variation are G-banding, C-banding and silver nitrate banding of nucleolar-organizing regions. Correspondence between G-banding and genetic homology has been demonstrated by numerous studies (Yates *et al.*, 1979; Elder, 1980; Viegas-Pequignot *et al.*, 1983; Baker *et al.*, 1987; Searle, 1988; Contrafatto *et al.*, 1992; Volobouev *et al.*, 1996). C-bands are usually equated with constitutive heterochromatin which is regarded as the site of highly repetitive DNA (Arrighi *et al.*, 1970; John and King, 1977; Buckland and Evans, 1978; King, 1993). Although several hypotheses have been advanced (Yunis and Yasmineh, 1971; Hsu, 1975; Miklos *et al.*, 1980), the function of heterochromatin remains obscure. Recently, it has been suggested that heterochromatin may play an important role in the organization and evolution of chromosomes (Holmquist, 1989; Pardue and Hennig, 1990; Ronne, 1990; Whichman *et al.* 1991). Not only are

differences in quantity, position and type of heterochromatin between species exceedingly common (Pathak *et al.*, 1973; King, 1993), but also differences between related species, for example, rats (Yosida, 1975; Contrafatto *et al.*, 1992), hamsters (Gamperl *et al.*, 1976) and shrews (Macholán *et al.*, 1994). Nevertheless, heterochromatin has been used as a cytogenetic marker in spiny rats (Aguilera *et al.*, 1995) and house mice (Ivanitskaya *et al.*, 1996).

Normally it is not possible to determine the direction of chromosomal evolution from banding studies, but there appears to be no doubt that many populations of mice have developed metacentric chromosomes by a process of fusion (Redi and Capanna, 1988). The vast majority of populations of the mouse *Mus musculus* and its close relatives have 20 pairs of telocentric chromosomes. However, certain populations in the Alps and Apennines and some other localities in Europe and North Africa have karyotypes with a smaller number of chromosomes in which pairs of non-homologous telocentrics have fused to form single metacentric chromosomes. A similar situation was found in the common shrew, *Sorex araneus*, in which different European populations have metacentric chromosomes made up of different combinations of acrocentrics (Searle, 1984), all of which remain unfused in the closely related *S. granarius* (Wójcik and Searle, 1988).

Because *R.pumilio* has such a wide distribution in different
climatic regions and altitudes, some degree of chromosomal
variation is possible, however this was expected to be minimal
because small isolated demes are required for fixation (King,
1993). A cytogenetic study was undertaken to compare the
chromosomal numbers and banding patterns in some of the
populations of *R.pumilio*.

Materials and methods

Rhabdomys pumilio specimens were live-trapped in different
regions of southern Africa (Table 4.1) using Sherman-type (Titian
Productions, Cape Town) and PVC traps (Willan, 1979). The
distribution and sampling sites for *R.pumilio* are illustrated in
Figure 4.1. Three specimens each from KwaZulu-Natal (Midmar Dam)
and Zimbabwe (Vumba), one each from Potchefstroom, Umtata and
Wellington were yeast stressed for two days to stimulate bone
marrow mitosis (Lee and Elder, 1980) (Appendix 10). The animals
were sacrificed and standard measurements recorded. The metaphase
cells of the bone marrow of the long bones of the forelimbes and
hindlimbs were harvested (Lee, 1969; Lee and Elder, 1977) and
chromosomal slide preparations were made using the splash
technique (Appendix 11). Chromosomal preparations were also made
from fibroblasts of spleen cultures of a specimen from the

Kalahari (Appendix 12). The spleen was mascerated in Dulbecco's modified eagles medium (Highveld Biological) and cultured with phytohaemaglutinin at 37^0C for 72 hours. The cultures were then incubated for 50 minutes with colchicine (25ng/ml). Prepared slides were G- and C-banded following the method of Wang and Fedoroff (1972) and Sumner (1972) respectively (Appendix 13 and 14). An unbanded karyotype was also prepared from a specimen from the Kalahari Gemsbok National Park (Kalahari).

Silver nitrate staining for nucleolar-organizing regions was done according to the method of Bloom and Goodpasture (1976). However, suitable silver nitrate staining of the nuclear-organising regions were not obtained even with modifications to the method (Appendix 15).

Voucher specimen were deposited in the Durban Natural Science Museum (Appendix 18).

Table 4.1 The sampling sites and number of specimens (N)
          of *Rhabdomys pumilio* used for karyotyping.

Sampling sites	Geographical co-ordinates	Number of specimens (N)
KwaZulu-Natal		
Midmar Dam	$29^0 30'$S $30^0 12'$E	3
Eastern Cape		
Umtata	$31^0 35'$S $28^0 47'$E	1
Western Cape		
Wellington	$33^0 39'$S $19^0 00'$E	1
Northern Cape		
Kalahari Gemsbok National Park	$25^0 30'$S $20^0 30'$E	2
Gauteng		
Potchefstroom	$26^0 42'$S $27^0 06'$E	1
Zimbabwe		
Vumba	$18^0 55'$S $32^0 40'$E	3
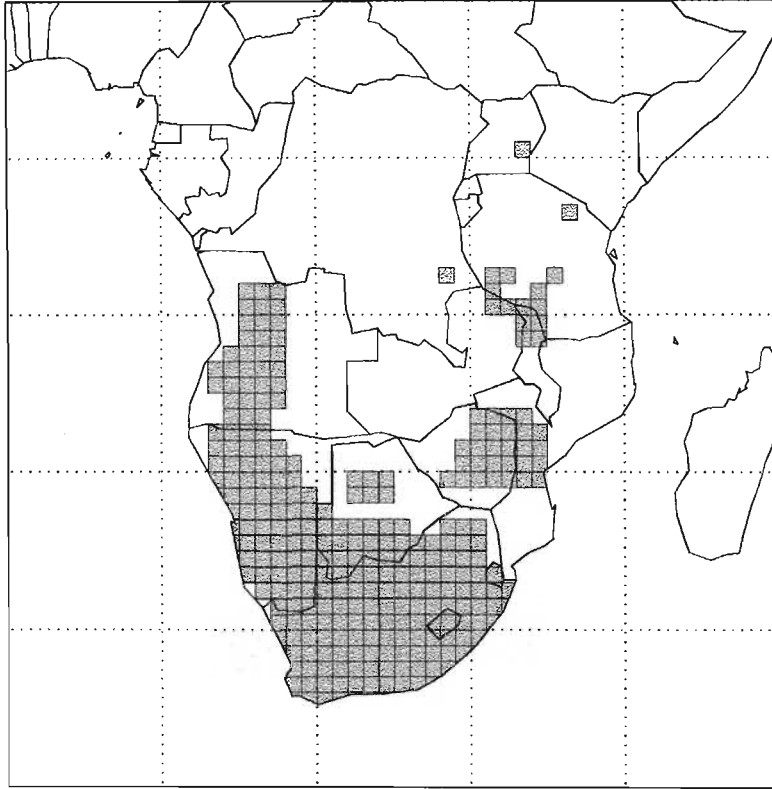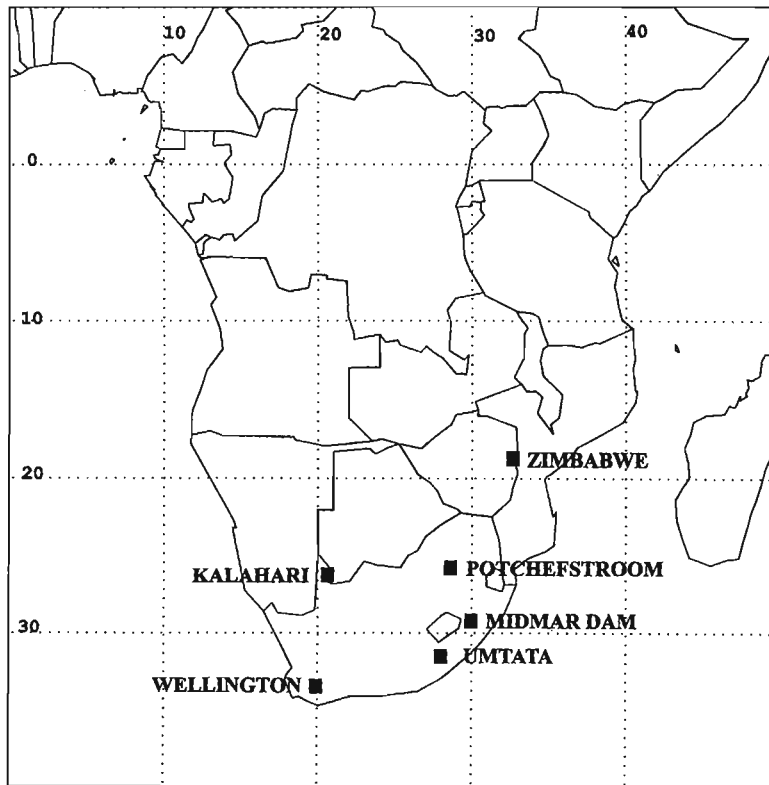
Figure 4.1 Maps showing (A) the distribution of *Rhabdomys
pumilio* (courtesy of Neil Burgess) and (B)
the sampling sites.

Results

Similar results were obtained with the tissue culture technique and the bone marrow preparations. The Kalahari, KwaZulu-Natal, Umtata and Wellington specimens had a chromosomal number of 2n=2x=48(XX/XY) (Figure 4.2). These were composed of two pairs of relatively long metacentric (1 and 2), two pairs of submetacentric chromosomes (4 and 5), 17 pairs of relatively short acrocentric chromosomes (6-22) and two pairs of relatively short metacentric chromosomes (23 and 24). Both the X and Y chromosomes were acrocentric. The Potchefstroom and Zimbabwe samples had a chromosomal number of 2n=2x=46(XX/XY) (Figure 4.3). The difference in chromosomal number was the result of a fusion of two acrocentric chromosomes (present in the 2n=48 chromosomal form) and formed the third pair of relatively long metacentric chromosomes of the Potchefstroom and Zimbabwe samples. No significant difference (p=0.58) was obtained between the comparison of the mean relative total lengths (cm) of the 2n=48 and 2n=46 chromosomal groups (Table 4.2).

The G- and C-banding patterns were similar in the different samples, with only centromeric heterochromatin being displayed (Figures 4.4 and 4.5b). The position of the centromeres is indicated in a G-banded metaphase stage of mitosis in a specimen from Zimbabwe (Figure 4.5a).

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Table 4.2 Mean relative lengths (±se) of 5 spreads of the haploid karyotype of specimens of *Rhabdomys pumilio* from KwaZulu-Natal and Zimbabwe.

Chromosome number	Mean relative lengths (cm)	
	KwaZulu-Natal	Zimbabwe
1	2.09 (0.42)	2.22 (0.46)
2	2.47 (0.31)	2.16 (0.42)
3	----------	1.51 (0.13)
4	1.93 (0.21)	1.69 (0.14)
5	1.57 (0.35)	1.92 (0.22)
6	0.92 (0.42)	----------
7	0.75 (0.14)	----------
8	0.90 (0.07)	0.72 (0.11)
9	0.84 (0.23)	0.69 (0.24)
10	1.13 (0.32)	0.75 (0.23)
11	0.75 (0.03)	0.47 (0.05)
12	0.60 (0.19)	0.65 (0.15)
13	0.73 (0.21)	0.57 (0.12)
14	0.62 (0.14)	0.69 (0.08)
15	0.70 (0.29)	0.47 (0.09)
16	0.64 (0.10)	0.58 (0.18)
17	0.49 (0.11)	0.43 (0.08)
18	0.57 (0.12)	0.57 (0.09)
19	0.62 (0.18)	0.57 (0.19)
20	0.71 (0.14)	0.50 (0.17)
21	0.44 (0.09)	0.48 (0.05)
22	0.69 (0.18)	0.42 (0.15)
23	0.45 (0.07)	0.37 (0.10)
24	0.43 (0.18)	0.49 (0.17)
X	1.24 (0.55)	1.16 (0.47)
Y	0.31 (0.11)	0.30 (0.11)
Mean (±se)	0.90 (0.25)	0.84 (0.27)
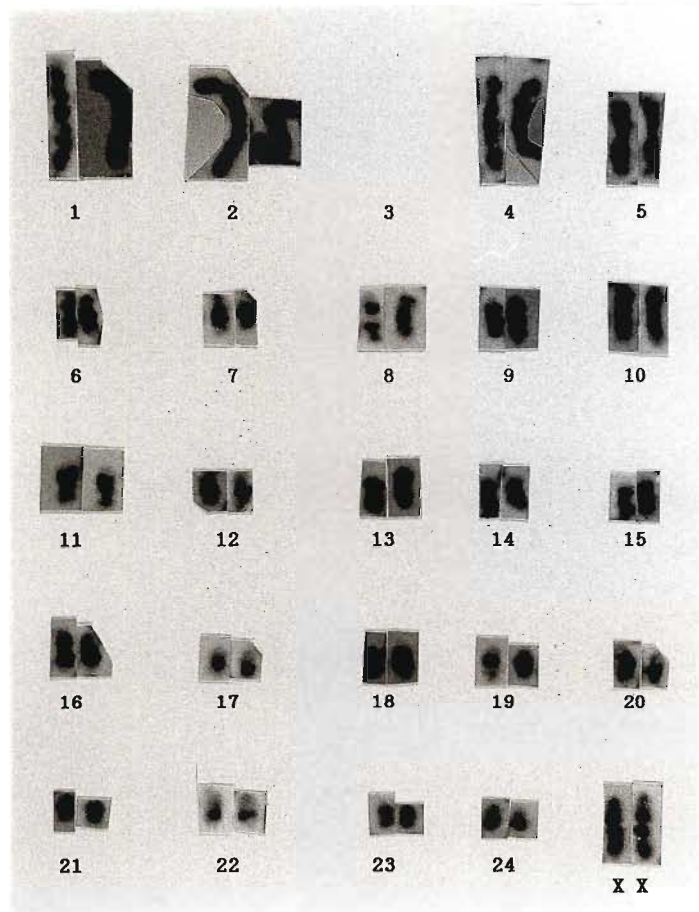
Figure 4.2   G-banded karyogram of a female specimen of
Rhabdomys pumilio from KwaZulu-Natal.

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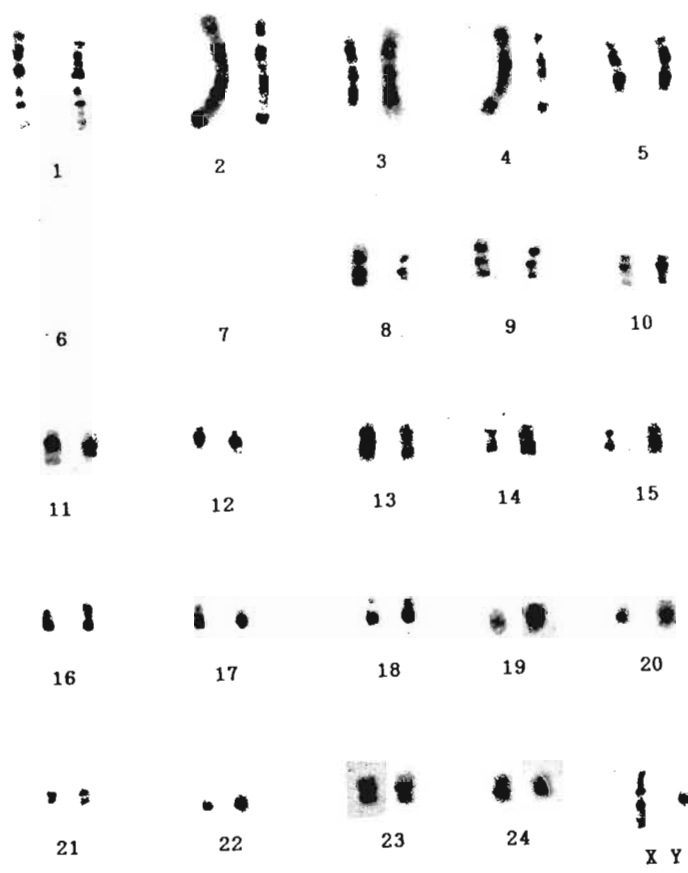
Figure 4.3 G-banded karyogram of a male specimen of
Rhabdomys pumilio from Zimbabwe.

Figure 4.4   C-banded karyogram of different specimens
             of *Rhabdomys pumilio* from KwaZulu-Natal.
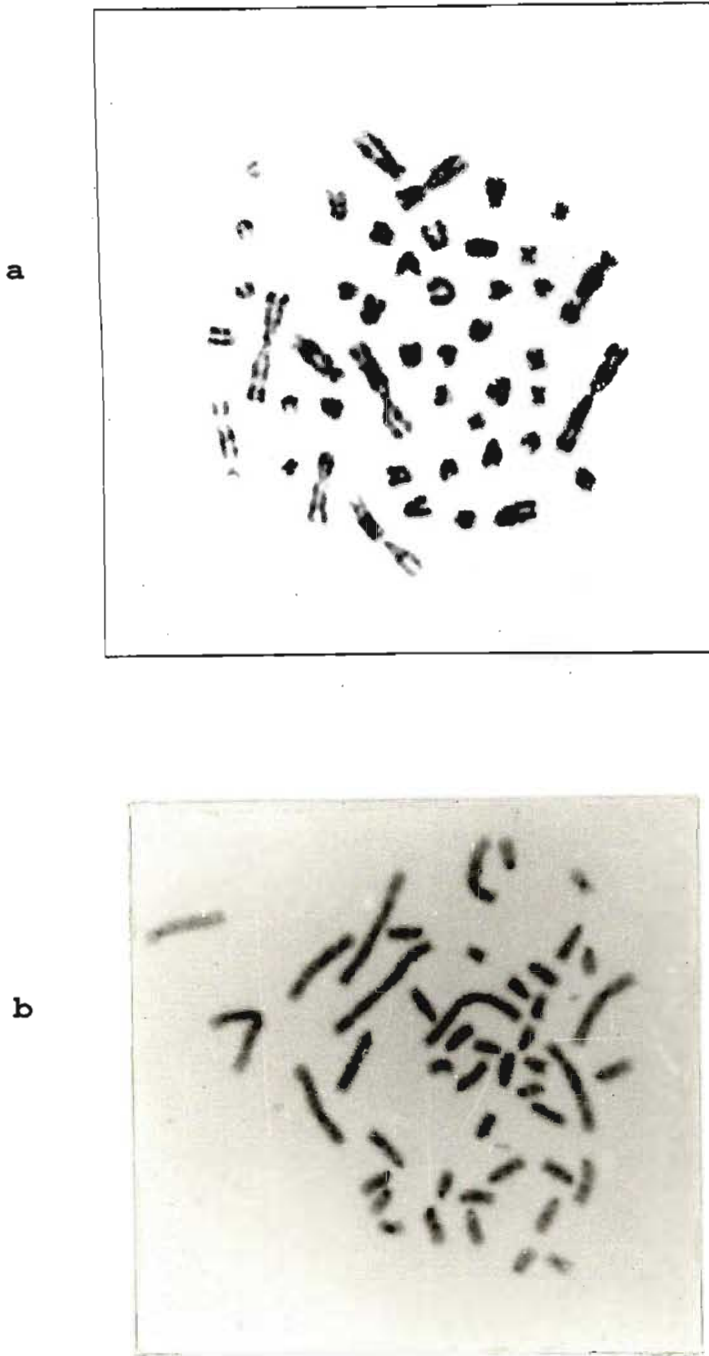
Figure 4.5   (a) G-banded karyogram of *Rhabdomys pumilio*
from Zimbabwe in the metaphase stage
showing the position of the centromeres and
(b) C-banded karyogram of *Rhabdomys pumilio*
from Zimbabwe.

Discussion

According to Gropp *et al.* (1972), Capanna *et al.* (1975), Wilson *et al.* (1975) and Contrafatto *et al.* (1992) chromosomal evolution is rapid in taxa whose ecology or social structure permits the formation of small demes or social groups. In contrast taxa whose members are ecologically wide-ranging tend towards lower rates of chromosomal evolution (Aranson, 1972; Capanna and Corti, 1991). Therefore, notwithstanding a certain degree of geographical isolation in the different samples of *R.pumilio*, the homology in G- and C-banding in the different samples was not suprising.

A diploid number of 2n=48-52 consisting primarily of acrocentric chromosomes was considered the most likely primitive karyotype for both the Muridae and the Cricetidae (Baker and Mascarello, 1969; Koop *et al.*, 1984). Also, karyotypes with the greater number of acrocentric chromosomes in the common shrew were considered the primitive form (Wójcik and Searle, 1988; Zima, 1991; Wòjcik, 1993). Therefore, it is possible that the 2n=48 chromosomal group with the greater number of acrocentric chromosomes, is the primitive condition and the Potchefstroom and Zimbabwe specimens of *R.pumilio* (2n=46) the derived form. This is supported by the peripheral status of the Potchefstroom population of *R.pumilio* which lies close to the boundary

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isolating it from the Zimbabwe population. The South African population of *R.pumilio* is geographically isolated from the Zimbabwe population of *R.pumilio* by savanna vegetation.

The question that arises from the difference in chromosomal numbers is whether it is important as an adaptive role to the environment (Bickham and Baker, 1979; John, 1981) or an adaptive role for re-stabilization of a stressed genome (Elder and Hsu, 1988) or involved in speciation and the primary cause of reproductive isolation (White, 1969; King, 1985, 1993).

Role of chromosomal rearrangements

(a) adaptive role to the environment

The evidence for the argument that changes in the karyotype may facilitate adaptive divergence is twofold: (1) gene function can be altered by changing its position within the chromosome and (2) the frequent occurrence of directed karyotypic change within lineages is difficult to explain by random-drift alone. Position-effect change in *Drosophila* (Spofford, 1975) is commonly cited as an example of the altered gene function resulting from chromosomal rearrangement whereas Wilson *et al.* (1974) makes reference to position-effect changes in mammals. Systematic studies of rodents have shown numerous examples of cryptic chromosomal "species", many of which involve substantial

reorganization of the karyotype, but have no noticeable phenotypic effect (White, 1982; King, 1993). Patton and Sherwood (1983) maintained that the gross karyotypic rearrangements in rodents were unlikely to play a major role in gene regulation. Support for adaptive divergence is provided by similar Robertsonian rearrangements occurring independently in many different populations of *Mus musculus* (Gropp and Winking, 1981). It appears unlikely that the 2n=46 and 2n=48 chromosomal groups of *R.pumilio* provides any support for an adaptive role to the environment. An argument for adaptive convergence in the Potchefstroom and Zimbabwe populations of *R.pumilio* because of the similar fusions in populations which are geographically isolated by savanna vegetation can be counteracted by evidence provided by Whichman *et al.* (1991) showing that interstitially located heterochromatin provided `safe' breakpoints (orthoselection) for chromosomal rearrangements without the risk of damage to the euchromatic portion of the genome.

(b) adaptive role in genomic reorganization

In the genus *Muntiacus*, the diploid number of the Chinese muntjac (*M.reevesi*) is 2n=46, while in the Indian muntjac (*M.muntjak vaginalis*) the diploid number is 2n=6, with a great deal of banding homology. These deer are closely related, and

viable but sterile F_1 hybrids were produced (Shi *et al.*, 1980).

These numerous chromosomal rearrangements have been fixed in a short evolutionary time span and the environmentally adaptive nature of each and every rearrangement is questionable (Elder and Hsu, 1988).

In Drosophila, hybridization of individuals from different populations has been shown to release mutator factors that induced frequent and nonrandom chromosome breakage (Woodruff and Thompson, 1980). Introgression or some other factor may destabilize the complex organization of a genome and this often takes the form of chromosomal rearrangements.

In R.pumilio the role of introgression in chromosomal rearrangement appears to be limited because of the wide-spread distribution of the 2n=48 chromosomal group throughout South Africa except for the peripheral population of Potchefstroom which had 2n=46 chromosomes. It is essential that further sampling be done in the Gauteng, Mpumalanga and the Northern Province area to determine the extent of the distribution of the 2n=46 chromosomal form.

(c) Role in speciation

Chromosomal differences are frequently associated with taxonomic differences at the species level (Patton and Sherwood,

1983) and therefore tends to support the speciation concept, but it still remains a controversial topic. Some researchers believe that chromosomal mutations plays a special role in speciation (Bush *et al.*, 1977; White, 1978a,b; Capanna, 1982; Baker and Bickham, 1986; Meester, 1988; Reig, 1989; Bengtsson and Frykman, 1990; King, 1993; Capanna and Redi, 1994) while others were against a role of chromosomal change in speciation (Carson, 1982; Patton and Sherwood, 1983; Vrba, 1985 and Zima, 1991). King (1993) strongly attacked those who used chromosomal polymorphism as evidence for the absence of a chromosomal role in speciation and concluded "that complex structural rearrangements may segregate in a balanced fashion in some organisms but not in others and that ultimately it depends on the meiotic system of the individual".

Since the Potchefstroom population of *R.pumilio* is situated near the periphery of its range in South Africa and the Zimbabwe population is geographically isolated from the South African populations by savanna vegetation, it can be argued that the chromosomal rearrangement in these populations represents a peripatric (the fixation of chromosomal variants in populations peripheral to the main species distribution) speciation event. In species, such as *R.pumilio* which exhibit a simple fusion, little or no loss in fertility was expected in cross-breeding

experiments (Redi and Capanna, 1988; Searle, 1993) since they frequently form balanced chromosomal polymorphisms (King, 1993). The effect on fertility of a single chromosomal fusion depends on the ability of the organism to segregate the meiotic products in a balanced fashion. Comprehensive cross-breeding trials between the 2n=48 and 2n=46 chromosomal groups are necessary to determine the effect of the single chromosomal fusion on the fertility of the hybrids. F_2 back-crosses have to be carried-out so that "all ramifications of recombinational effects on the genome, which are only realized in the F_2 generation", are included (King, 1993). Since a comprehensive breeding study was beyond the scope of this study, preliminary breeding trials were undertaken between the KwaZulu-Natal (2n=48) and Zimbabwe (2n=46) chromosomal groups. These breeding studies (refer Chapter 5 for further discussion) showed that their was no apparent loss in fertility in the F_1 and F_2 generations. Therefore, it is likely that the chromosomal rearrangement in *R.pumilio* represents an early stage in the speciation process and that subsequent changes could produce a chromosomal imbalance.

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CHAPTER 5

DISCUSSION

Introduction

Because *R.pumilio* has such a wide distribution throughout southern Africa and occupies a variety of habitats with varying temperature and rainfall regimes, with savanna vegetation (Rutherford and Westfall, 1986) separating the more northerly populations such as Zimbabwe from the South African populations, it was necessary to collect samples from various localities to study the population genetic structure of *R.pumilio*. Allozymic analysis (genetic variation based on protein variation) was carried out on specimens from all the sampled localities which included the Eastern Cape, Western Cape, KwaZulu-Natal, Gauteng, the semi-arid regions of Bloemfontein, Karoo and Kalahari, and the eastern highlands of Zimbabwe (Chapter 2). For the PCR-RAPD study (measurement of genetic variation based on nuclear DNA variation), 3 localities were selected from the Western Cape (because of the variation in the haemoglobin (*Hb*) loci detected with the allozyme study), one locality was selected from each of

the remaining sampled provinces in South Africa and Vumba in Zimbabwe. A cytogenetic study was also undertaken of specimens of *R.pumilio* from some of the localities to determine whether chromosomal variation existed in this species.

Comparison of allozymes and PCR-RAPD data

The haemoglobin (*Hb*), nucleoside phosphorylase (*Np*), superoxide dismutase (*Sod*) and creatine kinase (*Ck*) locus to a lesser extent provided a geographic pattern of allelic variation (Chapter 2). The *Hb-2* locus (the "B" allele was assumed to be a "null" allele) was expressed in most of the animals from the peninsular Western Cape but only in a few specimens from Gauteng (0.111). This was supported by primer number 5 with the PCR-RAPD data in which the Zimbabwe sample showed a genetic divergence of 0.60 from the Paarl sample while the genetic divergence between the remaining sample pairs ranged between 0.001 and 0.500 (Appendix 9).

In general, neither the allozymes nor the PCR-RAPD study provided alleles or bands unique to a particular locality or region. This minimal differentiation was expected because *R.pumilio* has high vagility, continuous distribution over much of its range and is an excellent colonizer. However, the allozyme (Chapter 2) and PCR-RAPD phenograms (Chapter 3) showed

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subgrouping of the samples of *R.pumilio*. The allozymes phenogram identified a KwaZulu-Natal, Eastern Cape and peninsular Western Cape subgroup, while the PCR-RAPD's identified a KwaZulu-Natal/Potchefstroom subgroup but a less sharply defined Eastern and Western Cape subgroup. However, with the PCR-RAPD's, the Zimbabwe sample was expressed as an outlier to all the remaining samples of *R.pumilio*, while the allozymes expressed the Zimbabwe sample as an outlier only to the Eastern and Western Cape samples.

Test for the isolation by distance and other models

The allozymes (Chapter 2) and PCR-RAPD (Chapter 3) data supported an isolation by distance model for the population genetic structure of *R.pumilio*. With the allozymes, a correlation coefficient of 0.54 ($p<0.001$) was obtained between the genetic and geographical distances between the different samples of *R.pumilio*, while a similar correlation with the PCR-RAPD data was 0.75 ($p<0.001$). The strength of the relationship with the allozymes ($r^2=0.29$) was not very strong whereas the PCR-RAPD's ($r^2=0.56$) presented a much stronger motivation for the isolation by distance model. However, due regard must be given to the fact that no correction for small sample size was introduced into the PCR-RAPD study when calculating genetic diversity. Furthermore,

although the isolation by distance model was supported by the spatial autocorrelation results (Chapter 2) because of the positive autocorrelation within small distances and the negative autocorrelation between larger distances, only half of the alleles analysed supported this isolation by distance model.

Temperature and rainfall were also important factors in determining the frequency of allelic distribution in the different localities in which *R.pumilio* were found (Chapter 2). However, this requires further investigation.

Little evidence for the niche-width variation hypothesis was provided for by the allozymes (Chapter 2).

Van Staaden (1995) considered breeding tactics and social structure to be important behavioural factors in determining the genetic structure of a population and maintained that the isolation by distance model was insufficient to explain local genetic structure (Van Staaden *et al.*, 1996). A number of studies have shown that despite the potential for individuals to move over large areas, behavioural factors resulting in limited vagility can cause localized variations between the different populations of a species (Chesser, 1983; Pope, 1992; White and Svendsen, 1992; Van Staaden *et al.*, 1996).

Lidicker and Patton's (1987) review of four rodent taxa and Van Staaden *et al.* (1996) working with Richardson's ground

squirrels, similarly concluded that breeding tactics were more important than dispersal characteristics in determining the genetic structure of a population. This was supported by Chesser's (1991a and b) theoretical studies which also indicated that F_{is} is a robust indicator of breeding tactics. Mathematical (Chesser, 1991a) and simulation models (De Jong et al., 1994) showed that for socially structured populations, F_{is} is always negative (a negative F_{is} value was obtained for R.pumilio) and indicated a complicated substructure rather than simply avoiding inbreeding. Chesser (1991a) further emphasized the evolutionary importance of polygynous breeding tactics for maintaining intra-group variation rather than a reduction in migration rates.

Rhabdomys pumilio is a highly social animal, a trait which according to Johnson (1980) presumably evolved in response to the diurnal activity patterns which necessitated a high level of social organization as an anti-predatory defence mechanism. The males of this species form a structured hierarchy, with many of the subadults or nearly mature males emigrating while females formed the breeding nucleus of the group (Johnson, 1980). Only the dominant male is territorial while females are territorial only during the breeding season. The life history traits of R.pumilio suggests that it is r-selected but breeding tactics represent a trend towards K-selection (Perrin, 1980; Willan and

Meester, 1989). These breeding tactics and the social structure within populations of *R.pumilio* could account for the high F_{st} (0.460) value.

What is highlighted from the above scenarios is that a multidisciplinary approach to the population genetic structure of a species is essential.

Taxonomic status of populations

With regard to the taxonomic status, the picture is quite confusing. Roberts (1951) listed twenty subspecies based on differences in tail length within this species. De Graaff (1981) questioned the validity of these described forms and contended that there may be a valid eastern and western subspecies. Misonne (1974) considered all these forms as conspecifics under *R.pumilio*. Meester *et al.* (1986) retained seven of the twenty subspecies listed by Roberts (1951). Recently in Angola, Crawford-Cabral (1998) recognised *R.p.angolae* and *R.p.bechuanae* as good subspecies based on morphological measurements.

The isolation by distance model proposed for *R.pumilio* forms an important basis for speciation. This model represents the classical concept for allopatric speciation (Mayr, 1963; Mayr, 1970; King, 1993). Although several definitions of a species have been advanced with regard to sexually reproducing organisms

(King, 1993; Avise, 1994), the "Biological Species Concept" (Mayr, 1942) is the most popular. It defined species as "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups". The key issue is reproductive isolation. Lewontin (1974), Ayala et al. (1974) and Ayala (1975) divided the processes of speciation into various stages for geographically isolated populations of the same species. However, Zouros et al. (1988) emphasized that behavioural and ecological factors are also important mechanisms for eliminating hybridization in the wild.

(a) Speciation - geographical factor

The classical view of allopatric speciation is that geographically isolated but undifferentiated populations of a species can gradually speciate over time as a result of genetic and morphological differences (Mayr, 1963; Mayr, 1970; King, 1993; Azzaroli-Puccetti et al., 1996). Thereafter, if sufficient differences have accumulated between populations and they come into contact again due to habitat expansion, they will be partially reproductively incompatible, with pre-zygotic isolating mechanisms developing due to "reinforcement" in the hybridization zone (Mayr, 1963; Dobzhansky, 1970). However, Paterson (1978) stresses the importance of the geographical divergence of

signalling systems so that when two populations meet they will be immediately and completely "reproductively incompatible" due to the specific mate recognition system.

Paterson (1985) and McKitrick and Zink (1988) maintained that reproductive isolation was not selected for but rather a secondary effect of the accumulation of genetic differences and suggested that reproductive isolation should not be a part of the species concept. Avise and Ball (1990) (The Concordance principles) accepted that reproductive barriers were intrinsic (Biological Species Concept) but suggested subspecies status "when phylogenetic concordance was exhibited across genetic characters solely because of extrinsic (geographic) barriers to reproduction". Although the South African samples of *R.pumilio* displayed a certain degree of geographical divergence as determined by the allozymes and PCR-RAPD's data, they were not completely isolated from each other. However, the savanna vegetation does provide a definite geographic barrier between the South African and Zimbabwean populations of *R.pumilio* which according to the Concordance principles could qualify for subspecies status.

(b) Speciation - behaviour factor

In Australia, the bush rats *Rattus lutreolus*, *R.tunneyi* and

R.fuscipes are morphologically and genetically distinct but chromosomally similar (Baverstock *et al.*, 1983a and b). These species are allopatric, with *R.lutreolus* and *R.tunneyi* sympatric in certain areas. They do not form interspecific hybrids in the wild as a result of ecological or behavioural factors. However, laboratory experiments produced F_1 hybrids and backcrosses which were fertile and viable. This is one of the problems of the Biological Species Concept - the difficulty in determining the specific status of related forms in allopatry. Because reproductive isolation can develop between geographically isolated populations as a secondary effect of genetic divergence, the test for the biological species status (the retention of separate identity in sympatry) has quite often not been carried out in nature. Although the test for pre-zygotic isolation in nature can be confirmed in the laboratory, it is also possible that pre-zygotic isolation barriers can be broken-down under laboratory conditions and fertile and viable F_1 hybrids and backcrosses produced. Bock (1984) showed that many *Drosophila* species can hybridize in laboratory experiments due to a disturbance of pre-mating isolating mechanisms which prevented hybridization in the wild. Breeding studies between different populations of *O.irroratus* (Pillay *et al.*, 1995), showed high levels of aggression between the morphologically similar KwaZulu-

Natal populations of Kamberg and Karkloof. The Kamberg population differed from the Karkloof in that it had a tandem fusion of chromosomal pairs 7 and 12.

Preliminary breeding trials between the morphologically indistinguishable KwaZulu-Natal (2n=48) and Zimbabwe (2n=46) samples of *R.pumilio* did not indicate any unusual levels of aggression and viable and fertile F_1 and backcross F_2 hybrids were produced. However, the biological species status of these two populations of *R.pumilio* is still questionable because the prediction for pre-zygotic isolating mechanisms has not been confirmed in laboratory studies. Pre-mating isolating mechanisms may exist between the 2n=48 and 2n=46 chromosomal groups of *R.pumilio* but these barriers may have been broken down in the laboratory experiments. Genetically, the Potchefstroom sample of *R.pumilio* was more closely related to the South African samples than the Zimbabwe samples (Chapters 2 and 3) and it is unlikely that pre-zygotic isolation exists between the two chromosomal groups in the Potchefstroom vicinity. Further studies are required in the Potchefstroom and surrounding areas to determine the extent of the distribution of the 2n=46 and 2n=48 chromosomal groups and whether pre-zygotic isolation exists between these forms.

The question that arises is, can the genetic information

obtained for *R.pumilio* contribute to resolving the taxonomic status of populations within this species. Ehrlich and Raven (1969) maintained that some degree of genetic differentiation exists between populations of nearly all species, the question then arises is how much genetic diversity is required to warrant species or subspecies status.

(c) Species status

Richardson *et al.* (1986) suggested that allopatric populations with differences at more than 20% of their loci based on electrophoretic data alone can be regarded as separate species, while Avise and Aquandro (1982) recorded a genetic distance of 0.30 between congeneric mammal species. Similar values were obtained with the common shrew (Wójcik *et al.*, 1996) but their karyotypes were different (Dannelid, 1994; Zima *et al.*, 1996) and exhibited clear differences in social behaviour and feeding habits (Churchfield, 1990). Similar results were also obtained between different genera of the elephant-shrew by Raman and Perrin (1997) but the two genera were morphologically, chromosomally and behaviourally distinct. Nei's (1978) unbiased genetic distance between the different samples of *R.pumilio* (0.000 and 0.189) was insufficient to warrant species status.

Patton *et al.* (1972) obtained a genetic similarity (Rogers,

1972) of 0.845 between two species of the pocket gopher, *Thomomys bottae* and *T.umbrinus*. However, clear evidence of reproductive failure associated with abnormal meiosis was provided (Patton, 1973). Rogers (1972) mean coefficient of genetic similarity was 0.763 between the allopatric species, *Sigmodon hispidus* and *S.arizonae* (Johnson *et al.*, 1972). Hybrids have not been found in nature and behavioural incompatibilities prevented breeding in the laboratory. Rogers (1972) genetic similarity for *R.pumilio* ranged between 0.796-0.988 (mean=0.833, sd=0.04) and again species status can be ruled out unless it is characterized by some feature which acts as a reproductive barrier.

Bishop (1995) using PCR-RAPD's obtained genetic similarity (simple-match coefficient) values ranging between 0.74 and 0.81 for Zebra populations from different game reserves in KwaZulu-Natal. Using the same technique, Van de Zande and Bijlsma (1995) obtained genetic similarity values between 0.80 and 0.86 between different strains of *Drosophila melanogaster* and values ranging from 0.03 to 0.38 between 9 different species of *Drosophila*. Comparing the above with the simple-match similarity coefficient (0.47-0.85) for *R.pumilio* suggests insufficient dissimilarity to warrant species status.

Although differences in autosomal number existed between the Zimbabwe (2n=46) and KwaZulu-Natal (2n=48) specimens, it did not

form a reproductive barrier in preliminary breeding experiments in the laboratory and therefore cannot be considered at this stage as contributing to a species status.

(d) Subspecies status

Ayala (1975) considered allopatric populations in the first stage of the speciation process as subspecies (Chapter 1). This initial period of speciation involves only a slight amount of genetic variation and is a reversible process.

The average Rogers (1972) genetic similarity between populations of the subspecies of the house mouse, *Mus musculus musculus* and *M.m.domesticus* was 0,769 ± 0.001 (Hunt and Selander, 1973). Avise *et al.* (1974) obtained similarity values of 0.793 ± 0.026 between subspecies of the *Peromyscus boylii* group. But, in these cases the subspecies status of these animals was also supported by other evidence, such as morphology and chromosomal rearrangements. The Rogers (1972) genetic similarity for the allozyme data ranged between 0.796 and 0.988 (mean=0.883, sd=0.04) between the different samples of *R.pumilio* and can be considered sufficiently genetically divergent for subspecies status, if it is supported by other evidence. This evidence was provided by the difference in autosomal numbers, with the South African samples (excluding Potchefstroom) having a chromosomal

number of 2n=48 whereas the Potchefstroom and Zimbabwe samples have a chromosomal number of 2n=46. The PCR-RAPD data provided similar support but due regard must be taken of the small sample size used in the study. Comparing the genetic similarity (simple-match coefficient) for *R.pumilio* for the combined primers (0.471-0.853) with the PCR-RAPD of Bishop (1995) and Van de Zande and Bijlsma (1995) who obtained genetic similarities (simple-match coefficient) values ranging between 0.74 and 0.81 for Zebra populations from different game reserves in KwaZulu-Natal and 0.03 to 0.38 between 9 different species of *Drosophila* respectively, does provide support for subspecies status. If the Zimbabwe sample was excluded, the genetic similarity between the different samples of *R.pumilio* using the combined primers results ranged between 0.559-0.853. Although the Potchefstroom specimen displayed the same chromosomal number as the Zimbabwe animals, the allozyme phenogram (Figure 2.2) and the PCR-RAPD combined primers phenogram (Figure 3.6b) suggested that genetically the Potchefstroom samples were more closely related to the remaining South African samples than the Zimbabwe specimens.

Therefore, although the Zimbabwe population of *R.pumilio* must be considered for subspecies status, further evidence is required before the South African populations of *R.pumilio* can be considered for subspecies status.

Historical perspective

Slatkin (1987) emphasized historical and contemporary gene flow as being responsible for the geographical patterns of genetic variation within species. *R.pumilio* is distributed throughout most of southern Africa (Skinner and Smithers, 1990) and except for the Potchefstroom specimen which had a chromosomal number of 2n=46, the remaining South African samples of *R.pumilio* had a chromosomal number of 2n=48. It is essential to determine the extent of the distribution of the 46 cytotype in the Gauteng, Northern Province and Mpumalanga provinces of South Africa. A diploid number of 2n=48-52 consisting primarily of acrocentric chromosomes is considered the most likely primitive karyotype for rodents (Baker and Mascarello, 1969; Koop *et al.*, 1984). Since both the Potchefstroom and Zimbabwe populations of *R.pumilio* represent geographically peripheral groups to the remaining South African populations, further support is provided for the likelihood that the 2n=46 specimens represent the derived form and the 2n=48 specimens represent the ancestral primitive form. The Zimbabwe population could have spread to Potchefstroom during favourable environmental conditions such as would exist during a pluvial. At a later stage the Zimbabwe population could have become isolated from the Potchefstroom

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population. This could have occurred during an interpluvial period which caused a barrier of unfavourable conditions which could have arisen in the Limpopo river basin and south-eastern Zimbabwe during dry periods (Meester, 1958). It is currently still a barrier. Alternatively, orthoselection for the same chromosomal rearrangement could have occurred separately in the Potchefstroom and Zimbabwe populations after being isolated from each other.

Work done by Meester (1958) on *Myosorex* regarded this species as originating in Zimbabwe and radiating towards South Africa during two successive invasions coinciding with pluvials. Both *Rhabdomys* and *Myosorex* have an isolated Zimbabwe population and a South African population and co-occur in similar mesic grasslands. Argument for a similar scenario could be presented for *R.pumilio*, with the Zimbabwe population radiating towards and becoming widespread in South Africa during a pluvial. It is possible that this ancestral form represented the 2n=48 chromosomal group. Extending this argument, it is possible that during an interpluvial the 2n=46 chromosomal group could have became fixed in a peripheral population of *R.pumilio* in Zimbabwe which subsequently radiated southwards reaching the Potchefstroom area. A subsequent interpluvial isolated the Zimbabwe and Potchefstroom populations of *R.pumilio* by the dry Limpopo river

basin. Thereafter, genetic divergence of the Zimbabwe population occurred.

Matthee and Robinson (1996) working with the South African populations of Smith's red rock rabbit, *Pronolagus rupestris*, revealed two major groups. A south-eastern group extending along the Great Escarpment and a north-western group including the Free State, Northern Cape and North West Province. This species also has an isolated east African population and could represent the ancestral population which radiated into South Africa with subsequent isolation being caused by the Limpopo basin. Prinsloo and Robinson (1992) identified two major South African groups of the rock hyrax, *Procavia capensis*, a large south-eastern clade following the Great Escarpment and a northern clade following the northern mountains of the Gauteng and the North West Province. Although this species extends throughout the dry Limpopo basin, the east African population of the rock hyrax could represent the ancestral form.

Lawes (1990) working with the forest dwelling Samango monkey, *Cercopithecus mitis*, regarded the Zimbabwe population as the ancestral population with a southward radiation of this species towards the Eastern Cape in South Africa. He further maintained that climatic conditions caused the extinction of the Samango monkey in some areas, resulting in an isolated Eastern

Cape population. Subsequently when favourable climatic conditions returned, the ancestral Eastern Cape population radiated towards KwaZulu-Natal. However, the re-establishment of forests particularly in the eastern highlands of Zimbabwe was aggrevated by the drier conditions after the last glacial maximum (Lawes, 1990). This is consistent with Tomlinson's (1974) account of the dominance of grasslands for the past 12000 years at Inyanga in Zimbabwe.

Paralleling this argument, but based on chromosomal work, Contrafatto (1996) regarded the Zimbabwe population of *Otomys irroratus* as the ancestral population, with an isolated but chromosomally "relic population" in the Eastern Cape. This relic Eastern Cape population subsequently radiated northwards towards KwaZulu-Natal and westwards to the Western Cape giving rise to 5 distinct cytotypes. The occupation of similar habitats between *Otomys* and *Rhabdomys* does provide for a similar argument.

The above scenarios do provide a strong argument for the ancestral population of *R.pumilio* occurring in east Africa and Zimbabwe with a southern radiation of this species into South Africa. Radiation probably occurred along the Drakensberg mountains via the highveld of Gauteng from where it spread into KwaZulu-Natal and the Free State. The KwaZulu-Natal population possibly extended into the Eastern Cape, while the Free State

population radiated towards the North West Province and towards the central and Western Cape. Some measure of genetic differentiation as measured by allozymes and PCR-RAPD's has occurred between these geographical regions.

Conclusions

(1) Environmental factors such as temperature and rainfall are important factors in the geographic allelic frequency distribution of some genetic loci of *R.pumilio*.

(2) The evidence supports an isolation by distance model for the population genetic structure of *R.pumilio* on a macro-geographic scale, with social structure and breeding tactics contributing to this model on a micro-geographic scale.

(3) Sufficient evidence exists to support a subspecies status for the Zimbabwe population of *R.pumilio* whereas further evidence is required to warrant a subspecies status for the remaining South African populations.

(4) The 2n=48 chromosomal group probably represents the ancestral type, with the 2n=46 chromosomal group probably fixed in the peripheral Zimbabwe population during a previous (Pleistocene) interpluvial with subsequent southward radiation into South Africa.

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Appendix 1

Protein electrophoresis

Tissue homogenization

1. 0.2 g of frozen liver tissue was placed in a clean centriguge tube and mechanically homogenized with 3 ml of distilled water.
2. The homogenate was centrifuged at 10000 g for 20 minutes to separate the extracted proteins from cellular debris.
3. The tissue was kept ice-cold at all times.

Preparation of starch gel

1. 70g of hydrolyzed starch was weighed in a 500 ml pyrex beaker, to which was added 400 ml of the selected buffer. The mixture was stirred until the starch was well emulsified.

   The two primary types of buffer systems are continuous and discontinuous buffers. In continuous systems the starch-gel buffer is usually a 10% or less dilution of the tray (electrode) buffer. In discontinuous systems, the tray and gel buffer are made of different electrolytes.

2. The starch suspension was heated to boiling with frequent stirring.
3. A rectangular gel mould was placed on a glass plate and the hot starch was rapidly poured into the mould in an even distribution until the gel almost overflowed.
4. Air bubbles, if any, were immediately removed using a pasteur pipette.
5. The gel was covered with a plate glass, avoiding the formation of any air bubbles.
6. The gel was allowed to cool to ambient temperature (about 45-60 minutes) and then placed in a refrigerator for 1 hour.
7. The buffer wells (trays) were filled with buffer.

Appendix 1 continued

Continuous buffers

1. Whitt (1970)

Stock solution
Tris   0.15 M
Citric acid.H_2O 0.05 M

Gel buffer pH 6.1
A 1:29 dilution of electrode solution was made.

Electrode solution pH 6.1
A 1:1 dilution of the stock solution was made.

2. Markert and Faulhaber (1965)

Stock solution
Tris   0.18 M
Boric acid   0.10 M
Sodium EDTA   0.004 M

Gel buffer pH 8.6
A 1:4 dilution of the electrode solution was made.

Electrode solution
The stock solution was used.

Disontinuous buffer

3. Ridgway et al.(1970)

Stock solution
LiOH.H_2O 0.06 M
Boric acid   0.03 M
10% stock solution   100 ml/L

Gel buffer pH 8.7
Tris   0.03 M
Citric acid. H_2O 0.005 M

Electrode buffer
The stock solution was used.

Appendix 1 continued

Gel loading

The inoculation of protein extracts into horizontal gels is generally accomplished by the use of sample wicks which are rectangular pieces of filter paper (Whatman No.3), measuring 2-4 mm in width and 1 mm longer than the gel mould. Wicks can be hand-cut or purchased.

1. The top glass plate was removed and the edges of the gel freed from the mould.
2. The gel was cut vertically about 3 cm from the edge.
3. The narrow strip of gel was gently separated from the larger piec by about 5 mm.
4. Using narrow-tip forceps, the wick was immersed in the supernatan of the tissue extract. The drip-dried saturated wick was then placed vertically against the cut surface, making contact with th bottom of the gel and extending slightly above it.
5. The remaining samples were loaded in the same way, with the wicks spaced about 1.5-2.0 mm apart.
6. A tracking dye was placed after every 10 samples. The last wick was soaked with tracking dye and placed about 5 mm from the edge of the gel mould.
7. Once all the samples were loaded, the gel was covered with a plastic-food wrap.

Electrophoresis

1. The loaded gel was placed between and resting on two electrolyte wells and electrical continuity was established by soaking one en of a sponge-cloth in the electrolyte solution in the well and the other end on the starch gel (about 1 cm) beneath the plastic-food wrap. This entire operation is carried out in a cold-room. An electical current was introduced, running from the cathode to the anode. The current was maintained between 50-75 mA to prevent overheating of the gel.
2. After 15 minutes of electophoresis, the electric current was stopped and the wicks were removed with a forceps. The current was reconnected and was continued until the tracking dye had reached the end of the gel.
3. The plastic-food wrap and the buffer wells were removed and the gel was ready for slicing.

Gel slicing

1. The left edges (3-5 mm) of the gel was cut away to serve as a marker for the arrangement of the samples. The gel was towel dried.
2. The gel was sliced into thin layers by means of a platinum wire drawn through the length of the gel.
3. The sliced sections were placed in staining trays and labelled with the gel number, enzyme system or locus to be stained, gel buffer and the date.

Histochemical staining

The distance of migration of specific proteins through a starch gel is visualized by histochemical staining. These stains consist of a "substrate" on which a specific enzyme reacts and a "detection mechanism" such as a dye or substance that fluoresces under ultra-violet light.

Unless otherwise indicated, the stained gel slices were incubated in the dark at 37^0C.

Agar overlays were prepared by boiling a 2.0 % (w/v) agar solution. The agar solution was cooled to just below 50^0C before the staining mixture was added and poured onto the gel slice.

The following stain recipes were used in this study:

Adenosine deaminase (Ada)
(EC 3.5.4.4)
Monomer
The stain was prepared as an agar overlay

0.2 M Tris-HCl, pH 8.0	15.0 ml
adenosine	30.0 mg
arsenic acid	80.0 mg
xanthine oxidase	0.4 units
nucleoside phosphorylase	1.8 units
1 % MTT	0.5 ml
1 % PMS	3 drops
2 % Agar	10.0 ml

Appendix 1 continued

Albumin and general proteins

0.5% naphthol blue black
stain fixing solution   500.0 ml
(1:5:5 mixture of glacial acetic acid, methanol and water)

The gel slice was soaked in 50 ml of the naphthol blue black for 20 minutes at 20^{0}C. The slice was washed several times with the fixin solution until background became pale.

Aspartate aminotransferase (Aat)
(EC 2.6.1.1)
Dimer.

0.5 M Tris-HCl, pH 8.0   50.0 ml
aspartic acid   100.0 mg
alpha-ketogutaric acid   100.0 mg

The pH was adjusted to 8.0 with 4.0 N NaOH
then the following substances were added.

pyridoxal 5-phosphate   200.0 mg
fast blue BB salt   50.0 mg

Creatine kinase (Ck)
(EC 2.7.3.2)
Dimer
The stain was prepared as an agar overlay

0.1 M Tris-HCl, pH 8.0   10.0 ml
adenosine 5'-diphosphate   10.0 mg
glucose   30.0 mg
hexokinase   10.0 mg
phosphocreatine   20.0 mg
G6pdh   5.0 units
NAD   5.0 mg
MTT   0.5 ml
PMS   2 drops
2% agar   10.0 ml

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Appendix 1 continued

Esterase (Est)
Monomer or Dimer

0.2 M Tris-HCl, pH 7.0	50.0 ml
! % alpha-napthyl proprionate	1.0 ml
fast blue BB salt	5.0 mg

Incubation was done at ambient temperature.
The stock solution (1% solution in 50% acetone) was prepared by dissolving the alpha-napthyl acetate in the acetone and thereafter the water was added.

Fructose-biphosphatase (Fbp)
(EC 3.1.3.11)
Dimer or Tetramer

0.2 M Tris-HCl, pH 8.0	50.0 ml
$MgSO_4.7H_2O$	2.5 mg
D-fructose-1,6-diphosphate	2.0 mg
Glucose-6-phosphate isomerase	50.0 units
G6pdh	40.0 units
2-mercaptoethanol	1 drop
(1 drop in 10.0 ml H_2O)	
NADP	20.0 mg
1 % MTT	0.5 ml
1 & PMS	3 drops

The gel slice was incubated at 37^0C in the dark in a fume cupboard.

Glucose-6-phosphate dehydrogenase (G6pdh)
(EC 1.1.1.49)
Dimer?
The stain was prepared as an agar overlay

0.2 M Tris-HCl, pH 8.0	10.0 ml
0.1 M $MgCl_2.6H_2O$	3.0 ml
glucose-6-phosphate	100.0 mg
NADP	10.0 mg
1 % MTT	0.5 ml
1 % PMS	3 drops
2 % Agar	10.0 ml

Appendix 1 continued

Glucose phosphate isomerase (Gpi)
(EC 5.3.1.9)
Dimer
The stain was prepared as an agar overlay

0.2 M Tris-HCl, pH 7.0	50.0 ml
0.1 M MgCl$_2$.6H$_2$O	5.0 ml
D-fructose-6-phosphate	40.0 mg
G6pdh	40.0 units
NAD	10.0 mg
1 % MTT	0.5 ml
5 mg/ml PMS	3 drops

Glycerol-3-phosphate dehydrogenase
(EC 1.1.1.8)
The stain was prepared as an agar overlay

0.2 M Tris HCl pH 8.0	10.0 ml
DL alpha glycerophosphate	200.0 mg
0.1 M MgCl$_2$	1.0 ml
NAD	20.0 mg
1 % MTT	0.5 ml
1 % PMS	3 drops
2% Agar	10.0 ml

Haemoglobin

0.2 M sodium acetate pH 4.7	50.0 ml
o-dianisidine	30.0 mg
Hydrogen peroxide (100 vol)	4 drops
0.1 M Calcium chloride	1.0 ml

To stop the reaction a 1 M sodium thiosulphate solution was added dropwise to reduce excess hydrogen peroxide.

Appendix 1 continued

Isocitrate dehydrogenase (Idh)
(EC 1.1.1.42)
Dimer
The stain was prepared as an agar overlay

0.2 M Tris-HCl, pH 8.0	10.0 ml
0.1 M MgCl$_2$	3.0 ml
isocitrate	30.0 mg
NADP	5.0 mg
1% MTT	0.5 ml
1 % PMS	3 drops
2 % Agar	10.0 ml

Lactate dehydrogenase (Ldh)
(EC 1.1.1.27)
Tetramer

0.2 M Tris-HCl, pH 8.0	50.0 ml
1.0 M lithium lactate pH 8	8.0 ml
NAD	10.0 mg
1 % MTT	0.5 ml
! % PMS	3 drops

The stock subtrate solution was prepared by using lactic acid solution. The pH was adjusted to 8.0 by the addition of lithium hydroxide.

Malate dehydrogenase (Mdh)
(EC 1.1.1.37)
Dimer

0.2 M Tris-HCl, pH 8.0	50.0 ml
2.0 M DL-malic acid	5.0 ml
NAD	10.0 mg
1 % MTT	0.5 ml
1 % PMS	3 drops

Appendix 1 continued

Malic enzyme (Mal)
(EC 1.1.1.40)
Tetramer

0.2 M Tris-HCl, pH 8.0	50.0 ml
0.1 M $MgCl_2$	1.0 ml
2.0 M DL-malic acid pH 8	5.0 ml
NADP	20.0 mg
1 % MTT	0.5 ml
1 % PMS	3 drops

It is important that NADP be use in solid form in this stain.

Nucleoside phosphorylase

The stain was prepared as an agar overlay

0.1 M Dipotassium hydrogen phosphate	10.0 ml
Inosine	10.0 mg
Xanthine oxidase	2.5 mg
1 % MTT	0.5 ml
1 % PMS	3 drops
2% Agar	10.0 ml

Peptidase (Pep)
(EC 3.4.11)
Subunit structure variable
The stain was prepared as an agar overlay

0.2 M Tris-HCl, pH 8.0	50.0 ml
di/tripeptide	40.0 mg
snake venom	10.0 mg
peroxidase	20.mg
o-dianisidine dihydrochloride	10.0 mg

   Snake venom was used as a source of L-amino acid oxidase.

Appendix 1 continued

Phosphoglucomutase (Pgm)
(EC 2.7.5.1)
Monomer
The stain was prepared as an agar overlay

0.2 M Tris-HCl, pH 8.0	10.0 ml
0.1 M MgCl$_2$	5.0 ml
glucose-1-phosphate	20.0 mg
G6pdh	40.0 units
NAD	10.0 mg
1 % MTT	0.5 ml
1 % PMS	3 drops
2% Agar	10.0 ml

6-Phosphogluconate dehydrogenase (Pgdh)
(EC 1.1.1.44)
Dimer
The stain was prepared as an agar overlay

0.2 M Tris-HCl, pH 8.0	5.0 ml
0.1 M MgCl$_2$	5.0 ml
6-phosphogluconic acid	10.0 mg
NADP	10.0 mg
1 % MTT	0.5 ml
1 % PMS	3 drops

Sorbitol dehydrogenase (Sdh)

0.2 M Tris HCl pH 8.0	10.0 ml
Sorbitol	400.0 mg
NAD	20.0 mg
1 % MTT	0.5 ml
1 % PMS	3 drops
2% Agar	10.0 ml

Superoxide dismutase (Sod)
(EC 1.15.1.1)
Dimer and Tetramer

0.2 M Tris-HCl, pH 9.0	50.0 ml
1 % MTT	0.5 ml
1 % PMS	0.5 ml

Appendix 1 continued

Xanthine dehydrogenase (Xdh)
(EC 1.1.1.37)
Monomer or Dimer

0.2 M Tris-HCl, pH 8.0	5.0 ml
0.1 M KOH	5.0 ml

This solution was stirred for 10 minutes, then the following substances were added.

0.2 M Tris-HCl, pH 8.0	40.0 ml
NAD	10.0 mg
1 % MTT	0.5 ml
1 % PMS	3 drops

The pH was adjusted to 8.0.

Stain fixing solution
1:5:5 glacial acetic acid:methyl alcohol:water

Appendix 2

RHABDOMYS PUMILIO, ALL POPULATIONS,GENEPOP NOTATION, JULY 1998
ADA,CK,EST-1,EST-2,HB-1,G3PD,G6PD,GPI,HB-2,ICD,PNP,PEP-2,PGD,PGM,SOD
POP
Kamberg
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Fort Nottingham
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Karkloof
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Appendix 2 continued

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Appendix 2 continued

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Appendix 2 continued

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POP
Zimbabwe
ZM02, 0102 0202 0101 0102 0102 0101 0202 0101 0202 0101 0202 0101 0202 0101 0202
ZM03, 0202 0102 0101 0103 0202 0101 0202 0101 0202 0101 0102 0101 0102 0101 0202
ZM04, 0101 0102 0101 0103 0202 0101 0102 0101 0202 0101 0102 0101 0202 0101 0202
ZM05, 0101 0202 0101 0101 0102 0101 0102 0101 0202 0101 0102 0101 0102 0101 0202
ZM06, 0101 0202 0101 0102 0202 0101 0102 0101 0202 0101 0102 0101 0102 0101 0202
ZM07, 0101 0101 0101 0102 0202 0101 0102 0101 0202 0101 0102 0101 0101 0101 0202
ZM08, 0101 0102 0101 0103 0202 0101 0202 0101 0202 0101 0102 0101 0101 0101 0202
ZM09, 0101 0202 0101 0102 0202 0101 0202 0101 0202 0101 0101 0101 0101 0101 0202
ZM10, 0101 0202 0101 0102 0202 0101 0101 0101 0202 0101 0202 0101 0202 0101 0202
ZM11, 0101 0202 0101 0102 0202 0101 0102 0101 0202 0101 0101 0101 0102 0101 0202
ZM12, 0102 0202 0101 0102 0202 0101 0102 0101 0202 0101 0102 0101 0202 0101 0202
ZM13, 0102 0102 0101 0102 0202 0101 0101 0101 0202 0101 0202 0101 0102 0101 0202
ZM14, 0102 0102 0101 0102 0102 0101 0202 0101 0202 0101 0202 0101 0202 0101 0202
ZM15, 0101 0101 0101 0102 0202 0101 0102 0101 0202 0101 0101 0101 0102 0101 0202
ZM16, 0102 0101 0101 0102 0202 0101 0101 0101 0202 0101 0101 0101 0102 0101 0202
ZM17, 0102 0101 0101 0102 0202 0101 0101 0101 0202 0101 0101 0101 0101 0101 0202
ZM18, 0101 0101 0101 0102 0102 0101 0102 0101 0202 0101 0202 0101 0202 0101 0202
POP
Malawi
MA01, 0202 0202 0101 0102 0202 0101 0101 0101 0101 0101 0301 0101 0102 0101 0202
MA02, 0202 0202 0101 0102 0202 0101 0101 0101 0101 0101 0301 0101 0102 0101 0202
MA03, 0202 0202 0101 0102 0202 0101 0101 0101 0101 0101 0101 0101 0101 0101 0202
MA04, 0202 0202 0101 0102 0202 0101 0101 0101 0101 0101 0301 0101 0101 0101 0202

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Appendix 3

Nei's (1978) unbiased genetic distance

Kamberg	*****							
Linwood	0.001	*****						
Boschoek	0.000	0.000	*****					
Fort Nottingham	0.000	0.004	0.005	*****				
Karkloof	0.015	0.006	0.013	0.015	*****			
Midmar Dam	0.005	0.005	0.014	0.006	0.008	*****		
Good Hope	0.000	0.000	0.000	0.000	0.017	0.011	*****	
Cathedral Peak	0.016	0.014	0.023	0.015	0.011	0.012	0.019	*****
Van Reenen	0.018	0.016	0.024	0.019	0.014	0.015	0.022	0.000
Groendal	0.099	0.090	0.092	0.099	0.082	0.106	0.102	0.072
KWS	0.094	0.090	0.089	0.098	0.083	0.106	0.097	0.066
Umtata	0.080	0.076	0.075	0.085	0.078	0.085	0.084	0.060
Beaufort West	0.128	0.120	0.127	0.122	0.108	0.137	0.130	0.103
Cape Point	0.136	0.139	0.136	0.136	0.146	0.155	0.134	0.129
Cedarberg	0.084	0.085	0.075	0.090	0.096	0.100	0.092	0.081
Paarl	0.119	0.122	0.112	0.121	0.132	0.146	0.122	0.116
Swartberg	0.089	0.094	0.087	0.089	0.101	0.110	0.097	0.083
Wellington	0.116	0.123	0.108	0.117	0.139	0.152	0.118	0.122
Bloemfontein	0.099	0.096	0.098	0.094	0.109	0.119	0.091	0.085
Kalahari	0.089	0.075	0.087	0.087	0.060	0.088	0.089	0.038
Potchefstroom	0.115	0.105	0.112	0.114	0.097	0.119	0.115	0.077
Zimbabwe	0.118	0.109	0.124	0.115	0.109	0.113	0.111	0.091
Malawi	0.174	0.160	0.162	0.173	0.152	0.189	0.173	0.140

Nei's (1978) unbiased genetic distance continued

Van Reenen	*****													
Groendal	0.065	*****												
KWS	0.062	0.021	*****											
Umtata	0.054	0.018	0.007	*****										
Beaufort West	0.100	0.011	0.060	0.061	*****									
Cape Point	0.129	0.077	0.058	0.070	0.092	*****								
Cedarberg	0.073	0.015	0.022	0.011	0.051	0.076	*****							
Paarl	0.112	0.050	0.042	0.047	0.076	0.009	0.038	*****						
Swartberg	0.073	0.009	0.029	0.023	0.030	0.008	0.005	0.041	*****					
Wellington	0.119	0.051	0.046	0.052	0.071	0.015	0.035	0.001	0.035	*****				
Bloemfontein	0.079	0.095	0.147	0.123	0.077	0.167	0.131	0.155	0.103	0.143	*****			
Kalahari	0.028	0.060	0.096	0.091	0.078	0.184	0.104	0.149	0.084	0.157	0.064	*****		
Potchefstroom	0.069	0.009	0.037	0.026	0.025	0.101	0.042	0.078	0.029	0.081	0.076	0.054	*****	
Zimbabwe	0.087	0.062	0.092	0.068	0.074	0.147	0.103	0.140	0.085	0.145	0.092	0.090	0.036	*****
Malawi	0.131	0.046	0.093	0.090	0.054	0.068	0.084	0.047	0.065	0.058	0.129	0.091	0.052	0.101
*****														

Geographic distance (km)

```
  40
  50    7
  25   20   27
  57   30   30   30
  57   20   15   30   15
  39   10   15   24   40   27
  80  121  127  102  125  134  120
 107  135  141  115  126  137  187   65
 595  610  615  615  640  630  602  592  660
 410  425  425  431  452  440  415  432  495  207
 232  236  237  245  265  277  229  270  327  390  190
 757  786  792  784  812  806  780  720  780  312  480  620
1220 1250 1255 1245 1275 1267 1240 1185 1245  702  905 1069  462
1085 1119 1125 1112 1141 1137 1110 1041 1095  640  824  965  345  207
1142 1170 1177 1167 1205 1200 1162 1115 1162  622  845 1005  392   75  141
 845  877  876  870  897  890  864  815  875  340  530  690  105  377  302  307
1140 1172 1177 1167 1197 1190 1162 1105 1162  642  840  996  385   86  130   10  307
 340  382  387  368  395  396  375  277  322  460  395  355  480  935  775  850  580  850
 991 1035 1040 1016 1040 1045 1030  915  932  947 1010 1027  707  900  695  835  787  825  675
 357  395  401  375  395  402  394  275  272  692  600  500  480 1135  960 1060  805 1050  235  675
1150 1155 1155 1140 1127 1140 1162 1122 1060 1692 1550 1385 1730 2152 1960 2075 1837 2065 1265 1435 1035
1930 1940 1940 1925 1915 1930 1945 1895 1835 2455 2325 2165 2440 2820 2620 2745 2559 2735 2002  200 1765
 790
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Appendix 4

The temperature and rainfall at weather stations nearest the localities at which *R.pumilio* specimens we sampled. Altitude and geographical co-ordinates of the sampled sites are included.

Locality (weather station)	A	B	C	D	E	F	G	H	I	J	K	L
1. Kamberg (Shaleburn)	13.6	21.9	5.3	35.5	-13.0	1038.0	727.0	1221.0	127.0	348.0	3.0	34.
2. Linwood (Cedara)	16.2	22.4	9.9	37.3	-4.2	861.0	567.0	1462.0	313.0	558.0	3.0	26.
3. Boschoek (Cedara)	16.2	22.4	9.9	37.3	-4.2	861.0	567.0	1462.0	313.0	558.0	3.0	26.
4. Fort Nottingham (Shaleburn)	13.6	21.9	5.3	35.5	-13.0	1038.0	727.0	1221.0	127.0	348.0	3.0	34.(
5. Karkloof (Cedara)	16.2	22.4	9.9	37.3	-4.2	861.0	567.0	1462.0	313.0	558.0	3.0	26.(
6. Midmar Dam (Cedara)	16.2	22.4	9.9	37.3	-4.2	861.0	567.0	1462.0	313.0	558.0	3.0	26.(
7. Good Hope (Cedara)	16.2	22.4	9.9	37.3	-4.2	861.0	567.0	1462.0	313.0	558.0	3.0	26.(
8. Cathedral Peak (Golden gate)	13.7	20.7	6.7	33.6	-9.8	788.0	611.0	943.0	107.0	233.0	4.0	28.0
9. Van Reenen (Golden gate)	13.7	20.7	6.7	33.6	-9.8	788.0	611.0	943.0	107.0	233.0	4.0	28.0
10. Groendal (Uitenhage)	18.5	24.7	12.3	45.0	-2.0	417.0	207.0	766.0	140.0	191.0	4.0	11.0
11. King William's Town (King William's Town)	18.0	23.8	12.3	43.0	-1.6	606.0	372.0	929.0	120.0	360.0	1.0	18.0
12. Umtata (Umtata)	17.5	24.1	10.9	44.0	-3.3	650.0	430.0	901.0	86.0	244.0	2.0	19.0
13. Beaufort West (Beaufort West)	17.7	25.2	10.1	41.4	-5.6	236.0	129.0	472.0	83.0	164.0	9.0	7.0
14. Cape Point (Cape Point)	15.8	18.7	12.8	35.8	3.5	353.0	202.0	510.0	55.0	155.0	5.0	9.0
15. Cedarberg (Clan William Dam)	19.7	27.1	12.4	45.9	1.3	257.0	199.0	352.0	47.0	88.0	7.0	6.0
16. Paarl (Paarl)	17.7	23.8	11.5	42.5	-0.3	886.0	593.0	1387.0	94.0	381.0	3.0	30.0
17. Swartberg (Oudtshoorn)	18.1	25.5	10.7	44.0	-2.6	239.0	142.0	442.0	55.0	98.0	5.0	7.0
18. Wellington (Wellington)	18.3	24.8	11.9	43.2	-0.1	640.0	434.0	979.0	81.0	285.0	5.0	23.0
19. Bloemfontein (Bloemfontein)	15.9	24.4	7.5	39.3	-9.7	559.0	326.0	1013.0	142.0	530.0	5.0	18.0
20. Kalahari Gemsbok National Park (KGNP)	20.2	29.4	11.0	43.4	-10.3	213.0	90.0	560.0	61.0	219.0	12.0	7.0
21. Potchefstroom (Potchefstroom)	17.2	25.1	9.4	38.6	-9.3	631.0	443.0	979.0	95.0	258.0	5.0	21.0

A : Mean annual temperature (^{0}C)
B : Mean monthly maximum temperature (^{0}C)
C : Mean monthly minimum temperature (^{0}C)
D : Absolute maximum temperature (^{0}C)
E : Absolute minimum temperature (^{0}C)
F : Mean annual precipitation (mm)
G : Minimum annual precipitation (mm)
H : Maximal annual precipitation (mm)
I : Maximum precipitation in 24 hours (mm)
J : Highest maximum monthly precipitation (mm)
K : Months with potentially zero rainfall (mm)
L : Average days with greater than 10 mm rainfall

Appendix 4 continued

Locality (weather station)	M	N	O
1. Kamberg (Shaleburn)	1614.0	29°24'S	29°40'E
2. Linwood (Cedara)	1350.0	29°33'S	30°05'E
3. Boschoek (Cedara)	1450.0	29°21'S	30°06'E
4. Fort Nottingham (Shaleburn)	1756.0	29°25'S	29°55'E
5. Karkloof (Cedara)	1438.0	29°21'S	30°13'E
6. Midmar Dam (Cedara)	1118.0	29°30'S	30°12'E
7. Good Hope (Cedara)	1400.0	29°39'S	29°58'E
8. Cathedral Peak (Golden gate)	2000.0	28°55'S	29°01'E
9. Van Reenen (Golden gate)	1943.0	28°22'S	29°24'E
10. Groendal (Uitenhage)	32.0	33°40'S	25°28'E
11. King William's Town (King William's Town)	400.0	32°53'S	27°24'E
12. Umtata (Umtata)	742.0	31°35'S	28°47'E
13. Beaufort West (Beaufort West)	842.0	32°18'S	22°36'E
14. Cape Point (Cape Point)	226.0	34°18'S	18°26'E
15. Cedarberg (Clan William Dam)	152.0	32°21'S	19°10'E
16. Paarl (Paarl)	166.0	33°45'S	18°58'E
17. Swartberg (Oudtshoorn)	314.0	33°13'S	22°03'E
18. Wellington (Wellington)	170.0	33°39'S	19°00'E
19. Bloemfontein (Bloemfontein)	1351.0	29°07'S	26°14'E
20. Kalahari Gemsbok National Park (KGNP)	879.0	25°30'S	20°30'E
21. Potchefstroom (Potchefstroom)	1350.0	26°42'S	27°06'E

M : Altitude (m)
N : Latitude
O : Longitude

Appendix 5

Mean heterozygosity (Het.) and frequency of alleles at the various polymorphic loci in the samples of *R.pumilio* from different localities in South Africa.

	Het.	Ada	Ck	Est-1	Est-2 (A)	Est-2 (B)	Est-2 (C)	Gpi	G3pdh	Hb-1	Hb-2	Icdh	Np (A)	Np (A
Kamberg	0.070	0.500	0.000	1.000	0.500	0.167	0.333	0.967	1.000	0.967	0.000	0.967	0.033	0.96
Linwood	0.073	0.429	0.000	1.000	0.429	0.429	0.143	1.000	1.000	0.857	0.000	1.000	0.000	1.00
Boschoek	0.056	0.333	0.000	1.000	0.500	0.500	0.500	1.000	1.000	1.000	0.000	1.000	0.000	1.00
Fort Nottingham	0.064	0.500	0.000	1.000	0.583	0.000	0.417	1.000	1.000	0.875	0.000	1.000	0.000	1.00
Karkloof	0.077	0.187	0.000	1.000	0.375	0.375	0.250	1.000	1.000	0.688	0.000	0.937	0.000	1.00
Midmar Dam	0.072	0.625	0.000	1.000	0.125	0.250	0.625	1.000	1.000	0.900	0.000	1.000	0.000	1.00
Good Hope	0.075	0.583	0.000	1.000	0.667	0.250	0.083	1.000	1.000	0.917	0.000	1.000	0.000	1.00
Cathedral Peak	0.099	0.441	0.000	1.000	0.525	0.125	0.350	0.950	1.000	0.725	0.000	1.000	0.475	0.52
Van Reenen	0.101	0.444	0.000	1.000	0.444	0.222	0.333	1.000	1.000	0.722	0.000	1.000	0.611	0.38
Groendal	0.085	0.000	0.000	1.000	0.400	0.400	0.200	1.000	1.000	0.400	0.000	1.000	0.450	0.00
King William's Town	0.061	0.000	0.000	1.000	0.800	0.200	0.000	1.000	1.000	1.000	0.000	1.000	0.571	0.00
Umtata	0.094	0.192	0.269	1.000	0.417	0.417	0.167	1.000	1.000	1.000	0.000	1.000	0.615	0.0?
Beaufort West	0.055	0.036	0.036	1.000	0.536	0.179	0.286	1.000	1.000	0.000	0.000	1.000	0.071	0.00
Cape Point	0.044	0.333	0.000	1.000	1.000	0.000	0.000	1.000	1.000	1.000	0.000	1.000	0.000	0.00
Cedarberg	0.055	0.000	0.000	1.000	0.250	0.417	0.333	1.000	1.000	1.000	0.000	1.000	0.500	0.00
Paarl	0.054	0.000	0.000	1.000	0.800	0.100	0.100	1.000	1.000	1.000	0.846	1.000	0.380	0.00
Swartberg	0.073	0.000	0.000	1.000	0.500	0.071	0.429	1.000	0.857	0.643	0.000	1.000	0.571	0.00
Wellington	0.036	0.000	0.000	1.000	0.929	0.000	0.071	1.000	1.000	1.000	0.714	1.000	0.214	0.00
Bloemfon-tein	0.068	0.571	0.714	1.000	0.786	0.214	0.000	1.000	1.000	0.000	0.000	1.000	0.286	0.00
Kalahari	0.056	0.143	0.000	1.000	0.429	0.429	0.143	1.000	1.000	0.000	0.000	1.000	1.000	0.00
Potchef-stroom	0.135	0.222	0.333	0.667	0.500	0.389	0.111	1.000	1.000	0.222	0.111	1.000	0.667	0.00

Appendix 5   continued

	Np (C)	Pep-2	Pgdh	Pgm	Sod
Kamberg	0.000	1.000	0.733	1.000	1.000
Linwood	0.000	1.000	0.615	1.000	1.000
Boschoek	0.000	1.000	0.833	1.000	1.000
Fort Nottingham	0.000	1.000	0.727	1.000	1.000
Karkloof	0.000	1.000	0.250	1.000	1.000
Midmar Dam	0.000	1.000	0.400	1.000	1.000
Good Hope	0.000	1.000	0.750	0.833	1.000
Cathedral Peak	0.000	1.000	0.316	1.000	1.000
Van Reenen	0.000	1.000	0.357	1.000	1.000
Groendal	0.555	1.000	0.600	1.000	0.000
King William's Town	0.429	0.857	0.286	1.000	0.000
Umtata	0.308	1.000	0.462	1.000	0.000
Beaufort West	0.929	1.000	0.679	1.000	0.036
Cape Point	1.000	1.000	0.500	1.000	0.000
Cedarberg	0.500	1.000	0.917	1.000	0.000
Paarl	0.692	1.000	0.808	1.000	0.000
Swartberg	0.429	1.000	1.000	1.000	0.000
Wellington	0.786	1.000	1.000	1.000	0.000
Bloemfontein	0.714	1.000	1.000	1.000	1.000
Kalahari Gemsbok National Park	0.000	1.000	0.429	1.000	1.000
Potchefstroom	0.333	1.000	0.500	1.000	0.000

Appendix 6

Isolation of DNA

1. About 0.2 g of liver tissue was ground in liquid nitrogen with a pestle in a mortar.
2. The ground tissue was placed in a 1.5 ml microcentrifuge tube and 500 ul of STE and 25ul of 20% SDS was added.
3. The sample was then placed in a shaking waterbath at 55^0C for 2 hours.
4. An equal volume of PCI was added and mixed gently but thoroughly and incubated at room temperature for 5 minutes.
5. The sample was centrifuged for 5 minutes at 7000 g.
6. The aqueous layer was carefully removed with a micropipette and a wide-bore tip and transferred to a clean tube.
7. The aqueous phase was re-extracted with PCI and steps 4-6 was repeated.
8. An equal volume of CI was added, mixed gently, and incubated at room temperature for 5 minutes. The sample was remixed once a minute during this time to prevent the phases from separating.
9. The sample was centrifuge for 3 minutes at 7000 g.
10. The upper aqueous layer was carefully removed with a micropipette and a wide-bore tip and transferred to a clean tube. Care was taken not to disturb the interface.
11. The aqueous phase was re-extracted with CI.
12. One-tenth volume (about 45 ul) of 2 M NaCl and 1 ml of cold (-20^0C) absolute ethanol was added to precipitate the DNA.
13. The sample was incubated on ice for 10-20 minutes.
14. The DNA precipitate was spinned down (Note: if large wisps of DNA are visible, centrifuge for 20 seconds at 7000 g and if the DNA is not clearly visible, centrifuge for 1-2 minutes at 7000 g).
15. The ethanol was decanted and the pellet dried under a vacuum until the ethanol had just evaporated.
16. The pellet was resuspended in 250 ul of 1 X TE (this may require up to 24 hours).
17. The concentration and purity of the DNA measured in a spectrophotometer by taking readings at 260 and 280 nm. An optical density of 1 at 260 nm corresponds to a double stranded DNA concentration of approximately 50ug/ml. The ratio of the readings at 260 nm/280 nm should be approximately 1.8. Lower readings indicate contamination with protein and/or phenol.

Appendix 6 continued

Stock solutions

CI
A solution of chloroform and isomyl alcohol, in the ratio (v/v) of 24:1.

PCI
A solution of phenol, chloroform and isomyl alcohol, in a ratio of 25:24:1. A layer of water will form on the surface; the PCI is the lower layer.

The phenol is first equilibrated to pH 7.5 as follows:
To 500 g phenol (solid) add 100 ml 2 M Tris, pH 7.4 and 100 ml water.
Heat slowly to 37^0C, mix layers, and let stand.
Remove aqueous layer.
Add an equal volume of 1 M Tris, pH 7.5, mix and let stand. Remove aqueous layer.
Repeat until Tris remains at pH 7.5.
Add 500 mg 8-hydroxyquinoline.
Store at 4^0C under 1 M Tris pH 7.5.

SDS
A 20% sodium lauryl sulphate solution. Do not refrigerate or autoclave.

STE
0.1 M NaCl
0.05 M Tris HCl, pH 7.5
0.001 M EDTA

10 x TE
0.01 M Tris HCl, pH 7.5
0.001 M EDTA
  autoclave.

Appendix 7

Random amplified polymorphic DNA using the polymerase chain reaction (PCR-RAPD)

This process uses short oligonucleotide primers (5-20 bases). These are designed without any prior knowledge of the DNA sequence. This is the difference between PCR-RAPD and other PCR techniques where primers are homologous to sequences flanking the area or gene of interest. The genomic DNA was denatured at a relatively high temperature in the presence of the 4 different nucleotides, enzyme (Taq polymerase), buffer and magnesium chloride. The reaction mixture was allowed to cool so that the primers could anneal to homologous sequences. Thereafter, the temperature was increased to allow for the extension of the primer. Successive cycles of denaturation, annealing and extension resulted in an exponential increase in the number of DN copies.

RAPD reactions were setup on ice with 8 ul (1.5 ng/ul) of template in each reaction tube. The master mix was formulated by adding the following constituents in the order indicated:

Sterile distilled water	555.75 ul
Stoffel buffer	182.40 ul
dNTP's	18.05 ul
Magnesium chloride solution	380.00 ul
Primer	60.80 ul
Enzyme	19.00 ul
Total	1216.00 ul

The total volume was sufficient for 76 reactions
The master mix was thoroughly mixed.

The primers were supplied in a lyophilised state and were diluted with 50 ul of sterile distilled water to produce stock solutions. Working solutions were made by diluting stock solutions to 6 ul. 16ul of master mix was aliquoted into each reaction tube to give a final volume of 24ul. Contamination was checked for by including a negative control in which DNA was excluded from the reaction mixture. All reaction tubes were covered with mineral oil.

The reaction tubes were inserted immediately into the thermocycler. Amplifications were performed according to that originally recommended by Williams *et al.* (1990) but were modified slightly. For the first cycle in the thermal cycler (ESU Programmable Temperature Cycler), denaturation, annealing and extention was 94°C for three minutes, 39°C for two minutes

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and 72°C for three minutes respectively. Denaturation time was decreased by one minute for the following forty cycles, with the extension time being increased by four minutes in the final cycle.

Appendix 8

Non-ammoniacal silver staining method

1. The polyacrimide gel was placed in a polyethylene container and 100 ml of fixing solution was added. The gel was agitated gently for 30 minutes.
2. The mixing solution was poured out and the destaining solution was added to the gel. The gel was agitated slowly for 30 minutes.
3. The destaining solution was poured out and the gel was covered with 50 ml of 10% glutaraldehyde and the gel was agitated slowly for 10 minutes in a fume cupboard.
4. The glutaraldehyde was poured out and the gel was thoroughly washed with several changes of water for two hours to ensure low background levels.
5. The water was poured out and the gel was soaked in 100 ml of 5ug/ml DTT for 30 minutes.
6. The DTT was poured out and without rinsing the gel, 100 ml of a 0.1% silver nitrate solution was added. The gel was agitated slowly for 30 minutes.
7. The siver nitrate solution was poured out and the gel was quickly washed with a small amount of water, then rapidly two times with a small amount of carbonate developing solution.
8. The gel was soaked in 100 ml of the carbonate developing solution and agitated slowly until te desired level of staining was achieved.
9. The staining was stopped by adding 5 ml of a 2.3 M citric acid per 100 ml of carbonate developing solution for 10 minutes.
10. The solution was poured off and the gel was washed several times with water.
11. The gel was stored by soaking in 0.03% sodium carbonate for 10 minutes.

Fixing solution
50 % (v/v) methanol
10% (v/v) acetic acid
40% water

Destaining solution
7% acetic acid
5% methanol
88% water

DTT
5 ug/ml dithiothreitol

Carbonate developing solution
0.5 ml 37% formaldehyde per liter solution
3 % (w/v) sodium carbonate
distilled water

APPENDIX 9

Geographic distance (km)

Fort Nottingham	---								
Groendal	615	---							
Beaufort West	784	313	---						
Paarl	1167	623	393	---					
Swartberg	870	340	105	308	---				
Bloemfontein	369	460	480	850	580	---			
Kalahari	1016	948	708	835	788	675	---		
Potchefstroom	375	693	480	1060	805	235	675	---	
Zimbabwe	1140	1693	1730	2075	1838	1265	1435	1035	---

Matrix of dissimilarity

Primer number 5

Fort Nottingham	-----								
Groendal	0.400	-----							
Beaufort West	0.400	0.001	-----						
Paarl	0.300	0.100	0.100	-----					
Swartberg	0.200	0.200	0.200	0.100	-----				
Bloemfontein	0.100	0.300	0.300	0.200	0.100	-----			
Kalahari	0.500	0.300	0.300	0.400	0.300	0.400	-----		
Potchefstroom	0.100	0.300	0.300	0.200	0.100	0.001	0.400	-----	
Zimbabwe	0.300	0.500	0.500	0.600	0.500	0.400	0.400	0.400	-----

Primer number 6

Fort Nottingham	-----								
Groendal	0.200	-----							
Beaufort West	0.333	0.400	-----						
Paarl	0.333	0.400	0.133	-----					
Swartberg	0.400	0.333	0.200	0.067	-----				
Bloemfontein	0.333	0.400	0.133	0.001	0.067	-----			
Kalahari	0.333	0.400	0.267	0.267	0.333	0.267	-----		
Potchefstroom	0.133	0.067	0.333	0.333	0.400	0.333	0.337	-----	
Zimbabwe	0.667	0.733	0.600	0.467	0.533	0.467	0.337	0.667	-----

Primer number 7

Fort Nottingham	-----								
Groendal	0.286	-----							
Beaufort West	0.357	0.071	-----						
Paarl	0.286	0.143	0.214	-----					
Swartberg	0.429	0.143	0.214	0.143	-----				
Bloemfontein	0.286	0.143	0.214	0.001	0.143	-----			
Kalahari	0.429	0.429	0.357	0.429	0.571	0.429	-----		
Potchefstroom	0.143	0.429	0.500	0.429	0.571	0.429	0.286	-----	
Zimbabwe	0.286	0.286	0.357	0.286	0.429	0.286	0.286	0.429	-----

Primer number 9

Fort Nottingham	----								
Groendal	0.667	-----							
Beaufort West	0.667	0.583	-----						
Paarl	0.500	0.583	0.500	-----					
Swartberg	0.667	0.583	0.333	0.583	-----				
Bloemfontein	0.583	0.417	0.333	0.167	0.417	-----			
Kalahari	0.500	0.417	0.500	0.333	0.583	0.250	-----		
Potchefstroom	0.667	0.333	0.750	0.667	0.667	0.583	0.333	-----	
Zimbabwe	0.750	0.500	0.500	0.667	0.750	0.500	0.500	0.500	----

Appendix 9 continued

Matrix of dissimilarity

Primer number 10

	Fort Nottingham	Groendal	Beaufort West	Paarl	Swartberg	Bloemfontein	Kalahari	Potchefstroom	Zimbabwe
Fort Nottingham	----								
Groendal	0.353	-----							
Beaufort West	0.353	0.118	-----						
Paarl	0.529	0.176	0.294	-----					
Swartberg	0.412	0.176	0.294	0.235	-----				
Bloemfontein	0.294	0.300	0.294	0.353	0.353	-----			
Kalahari	0.471	0.412	0.529	0.353	0.353	0.471	-----		
Potchefstroom	0.294	0.176	0.294	0.235	0.118	0.353	0.353	-----	
Zimbabwe	0.588	0.471	0.582	0.588	0.471	0.471	0.647	0.530	----

Combined primers

	Fort Nottingham	Groendal	Beaufort West	Paarl	Swartberg	Bloemfontein	Kalahari	Potchefstroom	Zimbabwe
Fort Nottingham	-----								
Groendal	0.382	-----							
Beaufort West	0.412	0.221	-----						
Paarl	0.397	0.265	0.250	-----					
Swartberg	0.426	0.265	0.250	0.221	-----				
Bloemfontein	0.324	0.294	0.250	0.147	0.221	-----			
Kalahari	0.441	0.382	0.397	0.353	0.426	0.368	-----		
Potchefstroom	0.265	0.265	0.426	0.368	0.368	0.353	0.338	-----	
Zimbabwe	0.529	0.485	0.516	0.515	0.529	0.426	0.441	0.515	-----

Appendix 10

Yeast method for mitotic chromosomes
from small vertebrates

1. The rodent was injected subcutaneously with an active Yeast suspension (0.5 ml/25 g body weight). After a 24 hour incubation period, a second dose of freshly prepared active yeast suspension was injected. A 24 hour incubation period was allowed.
2. Thereafter the animal was injected with 1 mg/ml colchicine, 0.1 ml/10 g body weight, and a 1 hour incubation period was allowed.
3. The animal was sacrificed and dissected to remove the upper leg (femur) and upper arm (humerus) bones. As much as possible of the soft tissue was removed.
4. Both ends of each long bone was cut and a syringe full of hyptonic KCl (0.075 M) was inserted at one end to flush out the marrow into a small volume (3 ml) of warm hypotonic KCl in a 15 ml centrifuge tube. The tube was flicked to disperse the cells and more hypotonic KCl was added to bring the volume up to 10 ml.
5. The cell suspension was incubated at 35^0C for 15 minutes in the hypotonic solution.
6. The cell suspension was centrifuged (200 g, 5 minutes) and the supernatant discarded.
7. The tube was flicked vigorously to loosen the pellet and 5 ml of freshly prepared fixative was added and left to fix for 20 minutes.
8. A pipette was used to remove any tissue particles that settled to the bottom of the tube.
9. The cell suspension was centrifuged and thereafter the pellet was resuspended in 5 ml of fixative and left for 10 minutes.
10. Step 9 was repeated twice.
11. Slide preparations were made using the splash technique (appendix 8).

Yeast suspension
   2-3 g dry yeast
   5-6 g dextrose
   25 ml warm water
The suspension was incubated at 40^0C until it began to foam vigorously (30 minutes).

Fixative
   3 parts of methanol and 1 part of glacial acetic acid.

Appendix 11

Splash technique for slide preparations
of mitotic chromosomes

1. Several drops of a fixed cell suspension was splashed from a heigh of 0.5 m onto a clean ice-cold slide.
2. The slide was dried on a slide warmer at 40^{0}C.
3. The cell density was checked and the cell concentration was adjusted, if necessary, by diluting or spinning down and resuspending the cells in a smaller volume of fixative.

Appendix 12

Mitotic chromosomes from fibroblasts
of the spleen

1. The rodent was sacrificed and the spleen was dissected out aseptically.
2. The spleen was transferred to a petri dish, cut into small fragments and cultured in 2 ml of Dulbecco's medium with 20% fetal calf serum (FCS) in a culture flask at 35^0C in an incubator supplied with 5% carbon dioxide.
3. When confluent sheets of cells were seen (>24 hr), 0.02 ml of 0.16% colchicine was added and incubated for 1 hour at 35^0C.
3. The cells were harvested by detaching them with 0.125% trypsin in 0.02% EDTA.
4. The suspension was centrifuged and the supernatant was discarded. The tube was flicked to disperse the cells and 10 ml of hypotonic KCl (0.075 M) was added.
5. The cell suspension was allowed to incubate for 30 minutes at 35^0C in the hypotonic solution.
6. The cell suspension was centrifuged (200g, 5 minutes) and the supernatant was discarded.
7. The tube was flicked vigorously to loosen the pellet and immediately thereafter 5 ml of fresh fixative was added and allowed to fix for 20 minutes.
8. After centrifuging, the pellet was resuspend in 5 ml of fixative and left to stand for 10 minutes.
9. Step 8 was repeated twice.
10. Slide preparations were made using the splash technique (appendix 8).

Appendix 13

G-banding

1. After fixing and making chromosome preparations in the standard way, the slide was placed horizontally and flooded with 0.25% trypsin in PBS solution for 10-20 seconds.
2. The slide was rinsed twice with the PBS solution.
3. The slide was stained for 5 minutes in 5% Giemsa solution in phosphate buffer.
4. The slide was rinsed with distilled water and air dried.

PBS solution
   0.15 M NaCl
   0.05 M NaHPO$_4$

Adjusted to pH 7.4

Phosphate buffer, pH 6.8
   0.025 M KH$_2$PO$_4$

Titrated to pH 6.8 with 50% NaOH

Appendix 14

C-banding

1. Prepared chromosome preparations were aged for 2 weeks at 35^0C.
2. The slide was treated with 0.2N HCl for 1 hour at room temperature, followed by rinsing with distilled water.
3. The slide was placed in a freshly prepared 5% aqueous solution of barium hydroxide octahydrate $(Ba(OH)_28H_2O)$ at 50^0C for 2-5 minutes
4. This was followed by thorough rinsing with distilled water.
5. The slide are incubated for 1 hour at 60^0C in 2 X SSC (0.3 M sodium chloride containing 0.03 M tri-sodium citrate).
6. The slide was rinsed with distilled water.
7. The slide was stained with Giemsa (1 ml in 50 ml of phosphate buffer (pH 6.8) for 45 minutes.
8. The slide was rinsed in distilled water air dried.

<u>Phosphate buffer, pH 6.8</u>
   0.025 M KH_2PO_4

Titrated to pH 6.8 with 50% NaOH

Appendix 15

AgNO₃-banding for nucleolar organizing regions (NOR's)

1. The chromosome slide preparation was aged for 2 weeks.
2. 2 parts of 50% (w/v) silver nitrate solution and 1 part developer were mixed in a glass vial.
3. 3 drops of the solution was added to the chromosomal slide preparation and quickly overlaid with a coverslip.
4. The slide was incubated at 90^0C until the staining solution turned muddy yellowish-brown.
5. The coverslip was rinsed off with distilled water and air dried.

<u>Developer</u>
  2% gelatin
  1% formic acid

The gelatin powder was mixed with 50 ml of distilled water and heated to dissolve. The formic acid was added to the cooled solution.

Appendix 16

Specimen number for the tissues of
Rhabdomys pumilio used in the allozyme study

1. **Kamberg**

DM 6025	DM 6093
DM 6026	DM 6094
DM 6027	DM 6095
DM 6028	DM 6096
DM 6089	UN 1
DM 6090	UN 2
DM 6091	UN 3
DM 6092	

2. **Linwood**

DM 3375	DM 3400
DM 3376	DM 3401
DM 3377	DM 3402
DM 3378	DM 3403
DM 3380	DM 3409
DM 3397	DM 3410
DM 3398	DM 2734

3. **Boschoek**

DM 6034	DM 6036
DM 6035	

4. **Fort Nottingham**

DM 3385	DM 3405
DM 3386	DM 6029
DM 3387	DM 6030
DM 3388	DM 6031
DM 3389	DM 6032
DM 3390	DM 6033

5. **Karkloof**

UN 4-11

6. **Midmar Dam**

DM 6037	DM 6040
DM 6038	UN 12
DM 6039	

7. **Good Hope**

DM 3372	DM 3414
DM 3373	DM 3415
DM 3374	DM 3423

Appendix 16   continued

8. Cathedral Peak

DM 1604	DM 2734
DM 1605	DM 2735
DM 2726	DM 2736
DM 2727	DM 2737
DM 2728	DM 2738
DM 2729	DM 2739
DM 2730	DM 2740
DM 2731	DM 2741
DM 2732	DM 2742
DM 2733	DM 2743

9. Van Reenen

DM 6083	DM 6061
DM 6084	DM 6062
DM 6085	DM 6063
DM 6059	DM 6064
DM 6060	

10. Groendal

DM 4225	DM 4230
DM 4226	DM 4231
DM 4227	DM 4237
DM 4228	DM 4242
DM 4229	DM 4243

11. King William's Town

DM 4249	DM 4253
DM 4250	DM 4254
DM 4251	DM 4255
DM 4252	

12. Umtata

DM 6041	DM 6048
DM 6042	DM 6049
DM 6043	DM 6050
DM 6044	DM 6051
DM 6045	DM 6052
DM 6046	DM 6053
DM 6047	

Appendix 16 continued

13. Beaufort West

DM 4083	DM 4101
DM 4084	DM 4102
DM 4085	DM 4103
DM 4086	DM 4110
DM 4087	DM 4111
DM 4099	DM 4112
DM 4100	DM 4113

14. Cape Point

DM 4175	DM 4178
DM 4177	

15. Cedarberg

DM 4184	DM 4188
DM 4185	DM 4189
DM 4186	DM 4192

16. Paarl

DM 4120	DM 4129
DM 4121	DM 4130
DM 4122	DM 4140
DM 4124	DM 4143
DM 4125	DM 4144
DM 4126	DM 4145
DM 4128	

17. Swartberg

DM 4199	DM 4206
DM 4200	DM 4207
DM 4203	DM 4208
DM 4205	

18. Wellington

DM 4166	DM 6069
DM 4168	DM 6070
DM 6067	DM 6071
DM 6068	

19. Bloemfontein

DM 4088	DM 4115
DM 4089	DM 4116
DM 4090	DM 4117
DM 4114	

Appendix 16   continued

20. Kalahari

        UN 13-18
        DM 6100

21. Potchefstroom

TM 44953	TM 44966
TM 44954	TM 49967
TM 44955	TM 49968
TM 44956	TM 49969
TM 44960	

22. Zimbabwe

    Inyanga

DM 4649	DM 5007
DM 4690	DM 5008
DM 5006	

    Vumba

DM 4625	DM 4635
DM 4626	DM 4636
DM 4627	DM 4637
DM 4628	DM 4638
DM 4629	DM 4639
DM 4632	DM 4640
DM 4633	

23. Malawi

    Chelinda

CC 751	CC 753
CC 752	CC 757

UN = Biology Department, University of Natal, Durban.
DM = Durban Natural Science Museum.
TM/CC = Transvaal Museum, Pretoria.

Appendix 17

Specimen number for the tissues of
Rhabdomys pumilio used in the PCR-PAPD study

1. **Fort Nottingham**

DM 3385	DM 3405
DM 3386	DM 6029
DM 3387	

2. **Groendal**

DM 4225	DM 4228
DM 4226	DM 4229
DM 4227	

3. **Beaufort West**

DM 4083	DM 4086
DM 4084	DM 4087
DM 4085	

4. **Paarl**

DM 4120	DM 4124
DM 4121	DM 4125
DM 4122	

5. **Swartberg**

DM 4199	DM 4205
DM 4200	DM 4206
DM 4203	

6. **Bloemfontein**

DM 4088	DM 4114
DM 4089	DM 4115
DM 4090	

7. **Kalahari**

UN 13-16	DM 6100

8. **Potchefstroom**

TM 44953	TM 44956
TM 44954	TM 49960
TM 44955	

8. **Vumba**

DM 4625	DM 4628
DM 4626	DM 4629
DM 4627	

UN = Biology Department, University of Natal, Durban.
DM = Durban Natural Science Museum.
TM/CC = Transvaal Museum, Pretoria.

Appendix 18

Specimen number of *Rhabdomys pumilio* used for karyotyping.

1. Midmar Dam

   DM 6037   DM 6040

   DM 6038

2. Umtata

   DM 6053

3. Wellington

   DM 6071

4. Kalahari

   UN 13-14

5. Potchefstroom

   UN 19

6. Vumba

   UN 20-22

UN = Biology Department, University of Natal, Durban.

DM = Durban Natural Science Museum.