MASTOMYS NATALENSIS AND M. COUCHA:

SPECIES IDENTIFICATION, HABITAT PREFERENCES AND POPULATION GENETICS

MASTOMYS NATALENSIS AND *MASTOMYS COUCHA*: IDENTIFICATION, HABITAT PREFERENCES AND POPULATION GENETICS

By

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ABSTRACT

Two species of multimammate mice occur in South Africa, namely *Mastom*ys *natalensis* and *M. coucha*. These species, both reservoirs for different and equally important disease pathogens, are morphologically identical with variations described in chromosome number, sperm morphology, pheromones, ultrasound and allozyme and isozyme markers. Their identical macro morphologies also raise problems where habitat and distribution is concerned. The need to identify various disease threats based on the carrier organism makes their identification important because they occur sympatrically in many areas of South Africa. Both species were expected to occur within the Roan Camp, Kruger National Park, South Africa and therefore toe samples were obtained from D. MacFadyen who was able to capture the *Mastomys* genus during his biodiversity survey of the area. He was unable to assign species designations to the captured individuals based on external morphology, but identification would allow exploration into the habitat preferences of each species when compared to the vegetation data gathered by MacFadyen using cross tabulation methods. DNA was extracted from 90 toe samples obtained during August 2003 (30 samples) and 2004 (60 samples) and the cytochrome-*b* region was amplified using the polymerase chain reaction (PCR). Amplified PCR products were cut using the restriction enzyme *Bsm*AI to produce restriction fragment length polymorphism (RFLP) profiles from which the two species were identified. Once identified, the species were associated to vegetation data received from MacFadyen and samples were selected for further analysis using 5 microsatellite loci. *Mastomys natalensis* was found in very low numbers, as expected, based on the average rainfall and altitude of the sample area, which is preferred by *M. coucha.* Further insight was gained into the preferences of each species concerning habitat and their respective status as pioneer species in habitat recolonisation. Genetic differences were obtained within the two species with 100% and 80% polymorphic loci for *M. coucha* and *M. natalensis* respectively. Allele classes at most of the loci deviated from Hardy-Weinberg equilibrium, probably due to small sample sizes, null alleles and/or heterozygote deficiencies with average heterozygosities of 0.754 for *M. coucha* and 0.526 for *M. natalensis.* The genetic distance between the two species was 0.465 and an *Rst* value of 0.503 indicated a significant differentiation between the two species. Fixed allele differences between the species were not obtained, but private alleles were found at four loci in *M. coucha* and one locus in *M. natalensis*. This study therefore contributes to the global information on species identification, genetic variation and ecology of the two cryptic *Mastomys* species; the three pillars on which the Convention of Biological Diversity (of which South Africa is a signatory) rests upon.

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LIST OF ABREVIATIONS

AAT-1 - Aspartate aminotransferase-1

DNA – Deoxyribose nucleic acid

dNTP's – dinucleotide triphosphates

EMCV – Encephalomyocarditis virus

GPI-2 – Glycosylphosphatidylinositol-2

KNP – Kruger National Park

Mya – Million years ago

PCR – Polymerase chain reaction

RFLP – Restriction fragment length polymorphism

RNA – Ribose nucleic acid

S.A. – South Africa

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CHAPTER 1

INTRODUCTION

INTRODUCTION

1. 1 THE PRAOMYS COMPLEX

The *Praomys* complex is comprised of five taxa namely *Praomys*, *Mastomys*, *Myomys*, *Myomyscus* and *Hylomyscus*. All existing members are exclusively found in Africa with *Praomys* and *Mastomys* being primarily terrestrial and the other three taxa inhabiting or spending large amounts of time in trees or bushes (Chevret *et al*., 1994). Typically these taxa have been regarded as related genera or sub-genera within the *Praomys* complex (Davis, 1962), but their taxonomic status within Murinae, and the relationships between them are not well understood mainly due to the lack of morphological differentiation between the many species described for the complex. In the past authors such as Thomas (1915) considered *Praomys*, *Mastomys* and *Myomys* to be members of the genus *Epimys* while *Hylomyscus* was a separate genus. In 1941, Ellerman considered all of them to belong in the genus *Rattus* until 1962 when Davis revised their taxonomic status to that previously mentioned. Following this Rosevear (1969) and other authors such as Robbins and Van der Straeten (1989) and Nowak (1991) gave full generic rank to all the taxa based on skull, tooth and body morphology. The use of morphology has however encountered problems in species identification within a genus such as that of *Mastomys*.

1.2. GENUS MASTOMYS - THE MULTIMAMMATE MOUSE

1.2.1. GENERAL DESCRIPTION

Multimammate mouse is the term used to describe two morphologically identical rodent species in the KNP, S.A., namely *Mastomys natalensis* (Smith, 1834) and *M. coucha* (Smith, 1836). Based on qualitative external and/or cranial morphology

however, these two species cannot be reliably distinguished in the field (Gordon and Watson, 1986).

Fig. 1.1 – *Mastomys coucha* (After Mills and Hes, 1997).

Fig. 1.2 – *Mastomys natalensis* (After Mills and Hes, 1997).

Mastomys coucha (Fig. 1.1) and *M. natalensis* (Fig. 1.2) have a typical mouse-like appearance and are smaller than ordinary rats (*Rattus* spp.). The fur is moderately long and soft and ranges in colour from pale gray to grey brown. Typically the dorsal colour is buffy grey with a brownish tinge and suffused black hairs. The under-parts are paler than the upper parts, varying from white to darkish grey, with the individual hairs being grey at the base with a white tip. Juveniles and older specimens have a distinct difference in colour with the former being a dull smokey grey. When the rodent matures, the dull, smokey grey colour becomes paler and rustier with a grizzled appearance (De Graaff, 1981; Stuart and Stuart, 2001).

The relatively short tail is finely scaled and bears a sparse covering of short, rigid hairs. The length of the tail can vary in specimens from the same area but is rarely as long as, or slightly longer than, the length measured from the tip of the head to the end of the body. Rings present on the tail are fairly close to each other. Multimammate mice have fairly narrow hands and feet with the hallux of the hind foot falling just short of the base of the second digit. The moderately sized ears are ovate and thinly covered with sparse, short hair (De Graaff, 1981).

They are nocturnal, terrestrial and frequently encountered in close association with humans (Stuart and Stuart, 2001). It is therefore well known as the “common” house mouse in many parts of southern Africa. As the common and generic name suggests, there is a large number of mammae present, between 8-12 pairs from breast to groin, especially noticeable in the female. This allows for positive identification of the genus by both layman and specialist alike, because the rows of mammae are clearly visible. It should be noted that the teats do not always occur in pairs but each one is usually ringed by lighter coloured hairs (De Graaff, 1981).

Mastomys have great reproductive potential with reports that the average number of pups in a litter may vary between 10-16 individuals, depending on

environmental conditions. Breeding occurs throughout the year but a decline may occur during the colder months. The young are born helpless and hairless with pink translucent skins, staying in the nest until they can fend for themselves. They are able to begin breeding at about three and a half months. Due to this fact the numbers of *Mastomys* can increase noticeably to unprecedented numbers in a short period of time leading to a population explosion, associated with health concerns (De Graaff, 1981).

For many years both species were regarded as Smiths' (1836) *M. coucha* until it was determined that *M. natalensis* was the prior name. Both species were therefore considered as *M. natalensis* until subsequent karyological, cytogenetic and protein electrophoretic studies revealed the presence of two electrophoretically distinct cytotypes, namely *M. natalensis* with a diploid number of 2n = 32 or a "slow" HAEMOGLOBIN electromorph and *M. coucha* having 2n = 36 or a "fast" HAEMOGLOBIN electromorph (Green *et al*., 1980). The two cytotypes also differ in reproductive behaviour, pheromones, cranial morphology, gross sperm morphology and ultrasonic vocalisations (Skinner and Smithers, 1990). The absence of hybrids in areas where the two species are sympatric furthermore suggests there is reproductive isolation between the two chromosomal races (Gordon and Watson, 1986; Skinner and Smithers, 1990). More insight into the reproductive isolation between *M. natalensis* and *M. coucha* might be revealed by studying the habitat and distribution preferences of these two species.

1.2.2. HABITAT AND DISTRIBUTION

The multimammate mouse has a wide habitat tolerance (Stuart and Stuart, 2001) being found from sea level to high lying ground, tending to be absent from excessively dry or arid regions (De Graaff, 1981). Skinner and Smithers (1990) provided a provisional distribution, which shows that in some areas the two species are sympatric whilst in others allopatiric, however their respective

distributional ranges remain uncertain as *M. coucha* and *M. natalensis* cannot be easily distinguished morphologically.

The distribution of *M. natalensis* and *M. coucha* was studied by Venturi *et al.* (2004) using locality data obtained from various sources and predictions of occurrence. The geographic distributions of Venturi *et al*. (2004) were based on positively identified specimens of *M. natalensis* and *M. coucha* obtained from the Transvaal Museum's mammal collection, the Durban Natural Science Museum's mammal collection and the findings of Hallett (1977) and Smit *et al.* (2001). The 77 localities yielded from the collation of the locality data obtained in S.A. provided 31 localities for *M. natalensis* and 46 for *M. coucha*. From this data it was evident that there was a distinct pattern of segregation along the eastern escarpment of S.A. largely influenced by altitude and rainfall (Fig. 1.3). *Mastomys natalensis* seems to occur along the low altitude and high rainfall eastern coastal region, extending up to the northeastern corner of S.A. whereas *M. coucha* occurs in the high altitude and moderate rainfall zones of the central and northeastern parts of S.A. This is in accordance with Gordon (1984) who stated that *M. natalensis* occurs in areas with an annual rainfall of greater than 600 mm and *M. coucha* in areas with less than 700 mm of rainfall. Gordon (1984) also suggested that the overlapping distribution of the two species seen in the 600-700 mm rainfall zone may represent an area in which the species specific distribution alters continually. The verified locality data of Venturi *et al.* (2004) furthermore showed that the two species occurred in close proximity to one another at four localities namely Pretoria, Satara in the KNP, Grahamstown and the Addo Elephant Park.

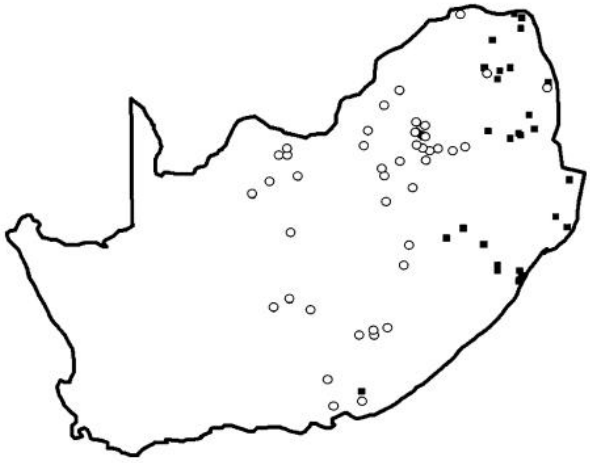


Fig. 1.3 – The geographic distribution of *M. natalensis* (squares, ■) and *M. coucha* (circles, ○) as determined by Venturi *et al.* (2004) showing the segregation along the eastern escarpment of S.A.

In addition to the locality data, Venturi *et al.* (2004) made predictions of the most likely areas of occurrence for the two species based on spatial analysis using a multi-criteria evaluation procedure and fuzzy set theory. A fuzzy set attempts to evaluate the vagueness in a class of elements that do not have well defined boundaries between entities that either partly belong or do not belong to a class.
The procedure attempts to define descriptive variables such as small, medium or large in terms of a base variable, the values of which are real numbers in a specific range such as temperature and precipitation. Consequently the values of basic eco-geographic variable statistics were used to construct sigmoidal curves, the functions of which were in turn used to define eco-geographic variable ranges for each of the two species. Predictions of the most likely areas of distribution (darker shading) for *M. natalensis* (Fig. 1.4) and *M. coucha* (Fig. 1.5) in S.A. broadly coincide with the geographic distributions derived from verified locality data (circles). These predictions reflect the generally wide distributions of the two species but also suggest that a possible zone of overlap occurs along the eastern escarpment and not only the four localities mentioned previously (Venturi *et al.,* 2004).

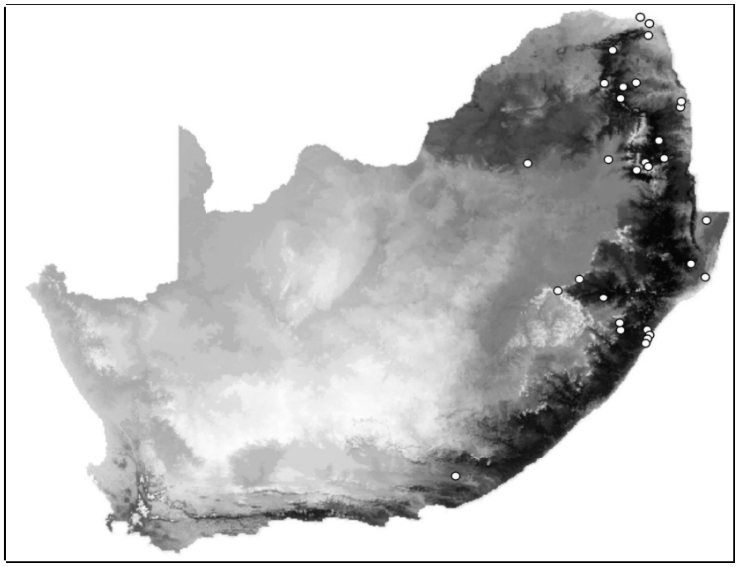


Fig. 1.4 – Prediction of the most likely distribution (dark shading) for *M. natalensis* in S.A. from verified locality data (circles, ○) (After Venturi *et al.*, 2004).

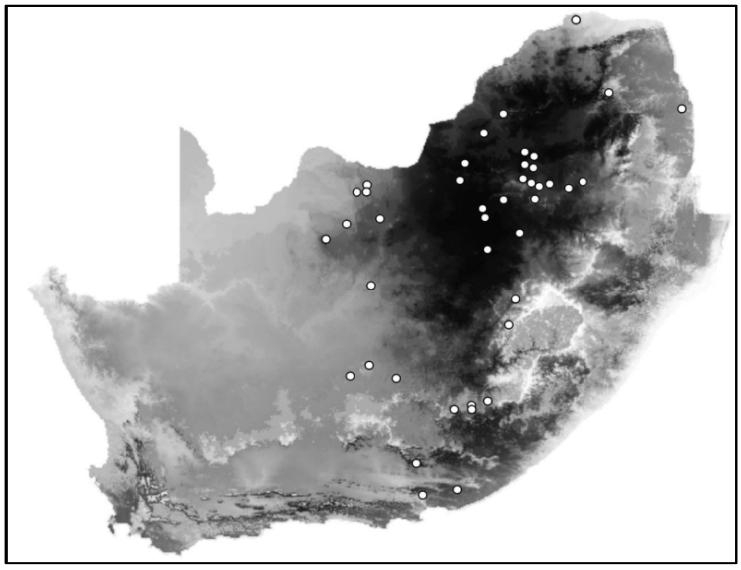


Fig. 1.5 – Prediction of the most likely distribution (dark shading) for *M. coucha* in S.A. from verified locality data (circles, ○) (After Venturi *et al.*, 2004).

Venturi *et al.* (2004) additionally found that potential evaporation was one of the eco-geographic variables to be influential in the distribution of the two species in S.A. The high average potential evaporation value associated with the localities from where *M. coucha* was collected is almost double that of the collecting localities for *M. natalensis.* This, together with the lower mean annual temperature associated with the collecting localities for *M. coucha*, suggest that the species is more likely to be affected by drought than *M. natalensis*.

Water is a limiting factor for many terrestrial rodents and physiological adaptation is often achieved through the concentrating ability of the kidneys. Ntshotsho *et al.* (2004) hypothesised that the differences in distribution between the two species might be attributed to the differences in kidney function based on aridity tolerance differences. The aspects of renal physiology were analysed by subjecting individuals of each species to different levels of salinity. The two species were found to have similar rates of water consumption and urine production with the salinity treatments causing sodium diuresis in both species. The results did not support the hypothesis.

The delineated distributional ranges of the two species within S.A. may be associated with a specific vegetation type. The predicted distributional limits, derived by Venturi *et al.* (2004) of *M. natalensis* seem to coincide with the savanna/grassland transitional zone as suggested by Rutherford and Westfall (1986). Smit *et al.* (2001) however noted that *M. natalensis* seems to occupy the warm, moist savannah regions whereas the grassland regions appear to predominantly support *M. coucha*. Vegetation preferences are therefore still uncertain. Where altitude is concerned, the central part of S.A. seems to be dominated by *M. coucha* (Venturi *et al.*, 2004). A similar preference for high altitude by the same species exists in the central and northern highlands of Zimbabwe (Gordon, 1978).

De Graaff (1981) noted that both *Mastomys* species were partial to sandy ground with an abundant overgrowth of scrub and grass. This was a similar finding to that of Veenstra (1958) who found that *M. natalensis* was mainly seen in dense bush. Veenstra (1958) found that, if forced to do so, *M. natalensis* would excavate their own burrows in this habitat type but more commonly they will use alternative sources of cover such as crevices between rocks, holes in termite mounds, spaces under fallen logs or deserted burrows of other animals. In Botswana, *Mastomys* is found in dry water courses, on the edges of swamps in Ngamiland and in *Acacia*, mopane scrub and woodland. In Zimbabwe the same wide habitat range is evident with the multimammate mouse occupying dry grassland with *Terminalia* scrub on Kalahari sand, as well as, areas close to water on basalt soils containing mopane woodland (De Graaff, 1981).

As Mastomys spp. are well known commensals with humans they are abundant where population concentrations are high. In these circumstances they are common on the fringes of, and within, agricultural lands where crops such as maize are cultivated and therefore the scrub fences erected around such cultivated lands provide cover. In 1999, Monadjem did a study on small mammal distributions in Swaziland. The author found that in Swazi Nation Land localities all the small mammals were caught in abandoned maize fields, of which most of the captures were of *Mastomys*. In comparison to the two ecological reserves studied, *Mastomys* was only captured in the main camp grounds at low frequency. Monadjem (1999) therefore suggests that *Mastomys* is rare in natural mountainous habitats in southern Africa but managed to colonise high lying areas in Swaziland with the aid of human disturbance (settlements and cultivation).

Mastomys are familiar to households where they find abundant food and water supplies as well as shelter. In African kraals they can occur in large numbers especially in the thatch roofs of traditionally constructed huts. The multimammate mouse is less common in modern towns, perhaps due to the different construction types used for building and the presence of better adapted species such as

Rattus (Veenstra, 1958; De Graaff, 1981). The habitat preferences and distribution of both species therefore has an effect on the role that these rodents play in the ecology of an area.

1.2.3. ECOLOGICAL ROLE

Through field work, Meester *et al.* (1979) showed that *M. natalensis* has generalised ecological requirements and, in localities where the species occurred, it was often one of the first species to become established in an area recovering from habitat destruction. Such destruction includes that by fire as well as habitats modified by people (e.g. crop cultivation/deforestation). Habitat destruction or fragmentation can influence ecological processes including population dynamics. Regardless of the type of disturbance, if the vegetation is changed or the habitat altered the population of one rodent species may be adversely affected whilst that of another may benefit. Few studies have quantified the factors within a locality that influences a species abundance and distribution but a trend towards relating these two factors to habitat structure has emerged (Geier and Best, 1980). *Mastomys natalensis* and *M. coucha* may look alike but their preferences to habitat and their tolerance to habitat destruction may be quite different. The influences of human management and other means of natural or anthropological destruction should be explored not only in terms of the impact of these factors on *M. natalensis,* but also that of the *Mastomys* species complex.

Meester *et al.* (1979) recorded that the greater diversity of plant species, often with a high proportion of weeds and exotics, associated with the succession seen after the destruction of habitats, provides varied food choices and shelter for *M. natalensis*. These conditions are often suboptimal for other smaller mammals. It is considered that as succession precedes the multimammate mouse is replaced by other specialist small mammal species, which are more closely adapted to the more specialised and homogeneous plant communities characteristic of later stages of plant succession. *Mastomys natalensis* is therefore considered as a

pioneer species and its presence is an indication of an area in an early stage of succession after habitat damage (Meester *et al.,* 1979; De Graaff, 1981). Little is said about the ecological role of *M. coucha* , but *M. natalensis* and *M. coucha* are both considered to live under diverse climatic and geographic circumstances being abundant in cultivated areas and around human settlements as well as coexisting with a variety of other small mammal species (Meester *et al*., 1979; De Graaff, 1981). This abundance and close association with humans draws attention to the need to address the medical importance of these species.

1.2.4. MEDICAL IMPORTANCE

The *Mastomys* species complex is the reservoir host for a number of organisms which cause various diseases in humans. This increases the likelihood that a disease carried by these rodents will be transmitted to people due to close contact (Skinner and Smithers, 1990). It is therefore of great medical importance to identify not only the species but also the pathogens they may transmit.

The various health risks that are posed by *M. natalensis* and *M. coucha,* respectively, are particularly difficult to identify due to the high degree of morphological similarity and the lack of a clear habitat and distribution pattern between the two species within S.A. To add to this it has, up to now, not been determined which of the two species has a greater tendency towards commensalism with humans. Whether a difference in this respect does indeed exist is of interest.

The pathogens for which the multimammate mice are reservoirs include the plague causing bacterium *Yersina pestis* as well as pathogenic bacteria such as *Salmonella typhimurium*, *Pasturella pneumotropica* and *Escherischia coli*. Viruses such as the Lassa fever virus, Witwatersrand and Banzi viruses are also associated with the multimammate mice (Murray *et al*., 1995). The rodent bourne hemorrhagic fevers such as Lassa fever are infectious diseases caused by single

stranded RNA viruses of either the family Arenaviridae or Bunyaviridae. Each disease pathogen is primarily associated with a single species of rodent host generally of the family Muridae. In the specific host the virus establishes a prolonged infection which rarely causes disease in the host. The infected rodent host however sheds the virus into the environment either via its faeces, urine or saliva (Mills and Childs, 1998).

Only *M. natalensis* is found to carry the Lassa fever virus, an arenavirus (Fig. 1.6.) and this is of medical importance. Although *M. natalensis* is found throughout most of Africa the disease, Lassa fever, is found only in West Africa specifically Nigeria, Sierra Leone, Liberia, Guinea, Senegal and Mali. The disease is passed on to humans via direct contact with their excretions or via flea bites, eating food contaminated with saliva from the rodents, eating mice directly and/or breathing in aerosolised urine. The disease has a high mortality rate even in hospitalised cases (Beltz, 2004).

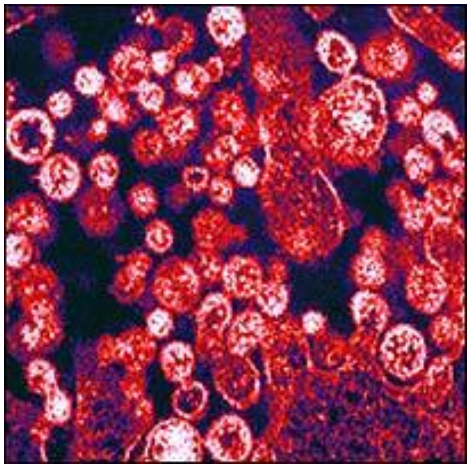


Fig. 1.6 – The causative agent of Lassa fever, an arenavirus (spherical structures), as seen under an electron microscope (http://newsimg.bbc.co.uk/media/images/41237000/jpg/_41237485_lassa203.jpg).

It has been discovered that *M. natalensis* is more resistant to experimental infection with *Y. pestis* (Fig. 1.7) than *M. coucha* and that the geographic distribution of plague in southern Africa corresponds significantly with the

distribution of *M. coucha* (Skinner and Smithers, 1990; Venturi *et al.*, 2004). Plague, also known in history as the Black Death, is typically a disease of rodents. Roberts and Janovy (1996) describes plague to occur in three forms, namely bubonic, primary pneumonic and primary septicaemic plague. The pneumonic and septicaemic forms of plague are rare forms that are usually fatal. The former is airborne and highly contagious whilst the latter is a generalised blood infection. Bubonic plague is the most common of the three forms, especially in epidemics, and is fatal in about 25-50% of untreated cases. The formation of growths known as buboes is characteristic of bubonic plague (Fig. 1.8). Plague, which has become widespread in the southern African subregion, has reached an endemic status. It has occurred in southern Africa since 1899 and by 1974 the disease had spread east into Zimbabwe. By 1987 it was found in northern Namibia (Skinner and Smithers, 1990).

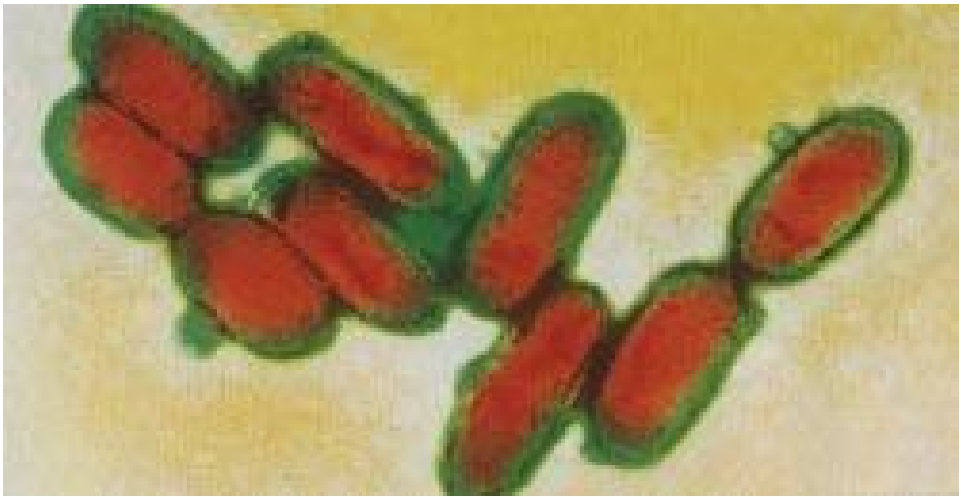


Fig. 1.7 – The bacterium, Yersinia pestis, that is the causative agent in all three forms of plague (http://newsimg.bbc.co.uk/media/images/41998000/jpg/_41998414_plague300spl.jpg).

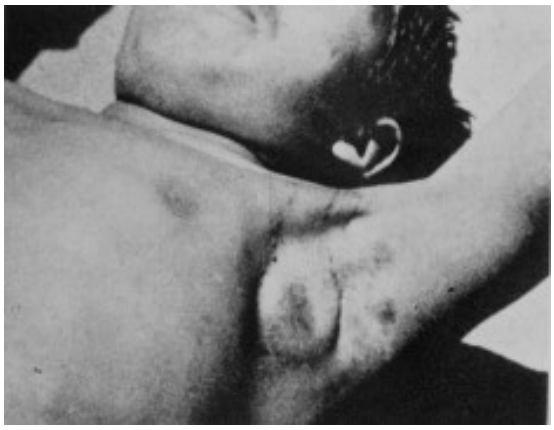


Fig. 1.8 – The buboes that develop in the bubonic form of plague (After Roberts and Janovy, 1996).

Wild rodent populations are the reservoirs for the plague causing bacterium which is transmitted to humans when they are bitten by infected fleas. The disease vector, *Y. pestis*, was originally introduced to Africa at coastal ports by ship rats (*Rattus* spp.), which transmitted the bacteria to local rodent populations via the fleas they carried. The disease was transferred to the multimammate mouse which came into close contact with the ship rats in warehouses and storage facilities at the ports. The free movement of multimammate mice from these ports to their natural homes in the veld and back into areas of human habitation passed the disease on to other rodents such as gerbils (*Tatera* spp.), as well as people, causing the vector to increase its distribution (Skinner and Smithers, 1990).

The *Mastomys* genus is also associated with diseases of animals, specifically that of the EMCV belonging to the genus *Cardiovirus* (Knowles *et al.,* 1998). EMCV typically affecting and maintained in nature by rodent populations (Zimmerman, 1994), was identified as the cause of fatal heart disease in African elephant populations of the KNP in 1993. The genus *Mastomys*, specifically *M. coucha* and *M. natalensis*, was linked with the outbreak of this virus (Grobler *et al*., 1995) as they occur in the same area (Skinner and Smithers, 1990; Smit, 2001). More study is however required to determine whether one of the species, or both, is the natural reservoir of the virus.

1.2.5. DISTINGUISHING BETWEEN *MASTOMYS NATALENSIS* AND *MASTOMYS COUCHA*

The *Mastomys* genus and the implications that some species are resistant to certain disease vectors, and merely carriers of them, is of medical significance and therefore the ability to distinguish between *M. natalensis* and *M. coucha* is important. Gordon in 1984 provided insight into the chromosomal variation of these two cryptic but genetically distinct species using G and C chromosomal banding patterns. These patterns are used to compare the structure of the karyotype. Specimens from Zimbabwe, S.A. and Namibia provided samples of two diploid chromosome numbers as mentioned previously. These distinct chromosomal complements were diagnostic of different gene pools and the extensive G-banding differences in the chromosomes suggested that many chromosomal rearrangements had occurred since the two species last shared a common ancestor.

Electrophoretic patterns of haemoglobin were also studied by Gordon in 1984. The gel electrophoresis of proteins is used to interpret genetic structure of, and variation within, populations and for tracking gene flow between populations. Gordon (1984) found two distinctive monomorphic haemoglobin patterns for the various *Mastomys* specimens when referenced to a human blood marker standard (Fig. 1.9). These double banded electromorphs were designated as either fast or slow migrating and were diagnostic for the morphologically similar species.

During both the haemoglobin electromorph and chromosomal studies there was no indication of specimens with a hybrid haemoglobin pattern or intermediate hybrid diploid chromosome number of 34 from natural populations. Through assortative mating tests, Gordon (1984) showed that hybrids could be produced in the laboratory, although mating preferences still lay within the species limits. All

hybrids were infertile. The absence of hybrids from the studied natural populations indicated that mating was positively assortative and provided evidence for the genetical status of both species.

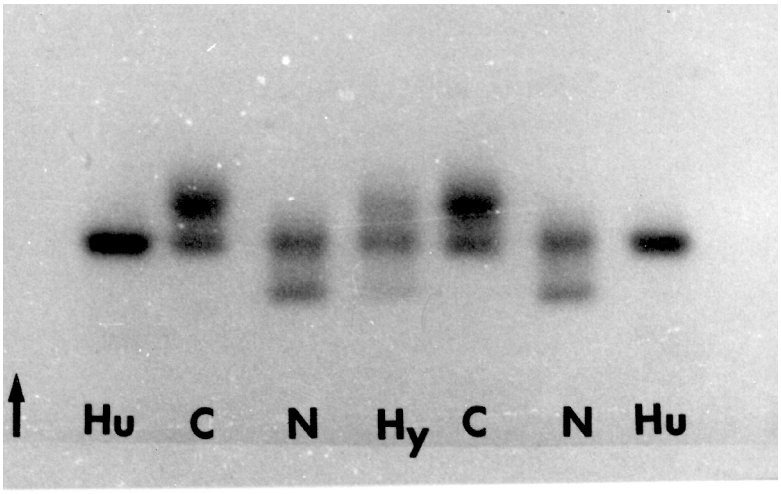


Fig. 1.9 – The haemoglobin markers used by Gordon (1984) to distinguish between *M. coucha* and *M. natalensis*. One allele for both species had the same mobility as a human standard (Hu) but *M. coucha* (C) had a fast migrating allele whilst *M. natalensis* (N) had a slow migrating allele. “Hy” indicates the hybrid form which shares all three alleles (Gordon, 1984).

In 1993, Dippenaar *et al*. did a study based on the diagnostic morphometrics of *M. natalensis* and *M. coucha* to determine if they differed diagnostically in their cranial configuration. It was initially found that although the two species were cranially very similar they could be distinguished using advanced multivariate statistical procedures such as principal component and discriminant function analyses. They used only the sculls of adult specimens, previously identified cytogenetically, and obtained from the Transvaal Museum, S.A. The results from discriminant function analysis showed that it was possible to positively identify specimens of both species, and with careful application of advanced statistical procedures, it could be possible to develop a scheme or key for the reliable identification of the two species. Univariate results however showed that it was unlikely for such a scheme or key to be simplistic as discrepancies in identification

of 50% of the specimens collected at a sympatric locality occurred when using cranial measurements. It was furthermore stated that the results obtained in the study were not recommended for use to positively identify specimens in localities not covered by the original study, because the geographic variation in each species is not known.

The morphological diversity and evolutionary trends of the spermatozoa of Murid rodents was determined by Breed in 1994. The structural organisation of the spermatozoa of both species was described at the light and transmission electron microscopical levels of resolution. Light microscopy indicated that the sperm heads for both species were between 10-12 µm long and about 2 µm wide, with an apical hook that tapers towards its tip. The connecting piece of tail attaches to the lower concave surface of the sperm head and has a midpiece length of 50 µm and a principle end piece of about 110 µm. With this description it was determined that the sperm morphology of *M. natalensis* and *M. coucha* were extremely similar if not identical, but that the dimensions of the sperm head and tail were slightly different as seen in Table 1.1. *Mastomys coucha* was identified by Breed (1994) based on the average sperm head and tail length dimensions of 10 µm and 147 µm, respectively (Table 1.1). *Mastomys natalensis* was identified based on longer sperm head and tail length dimensions of an average of 12 µm and 156 µm, respectively (Table 1.1). This method for identification is however only useful for male specimens.

Table 1.1 – Average sperm head and tail dimensions of *M. coucha* and *M. natalensis* as determined by Breed (1994).

	HEAD DIMENSIONS (µm)			TAIL DIMENSIONS (µm)		
SPECIES	LENGTH	BREADTH	HOOK	MIDPIECE	PRINCIPLE & END PIECE	TOTAL LENGTH
Mastomys natalensis	12	2	6	49	107	156
Mastomys coucha	10	2	7	42	105	147

Smit and Van der Bank and Smit *et al*. (2001) sampled *Mastomys* individuals from two populations in S.A. where it is known that they are not sympatric. These authors applied tissue samples such as muscle, liver, heart and kidney onto standard horizontal starch gels for electrophoreses analysis of various allozyme and isozyme markers. These authors found genetic differentiation between *M. natalensis* and *M. coucha* by identifying fixed allele mobility differences between the two species. Some of these fixed allele differences include those found at the GPI-2, PT-2, PT-3 and AAT-1 gene coding loci. The study also suggested that there is a large amount of genetic differentiation between the two species and confirmed the present taxonomic status of *M. natalensis* and *M. coucha*. It also suggested a high degree of positive assortative mating and showed very little if any gene flow between both species in the wild.

On the basis of external characters, the identification of the *Mastomys* species is impossible and the methods that involve karyotypes, allozyme and isozyme markers are not suitable for population studies involving large numbers of individuals. Fresh material is required for karyotypes, allozyme and isozyme markers and quick transport from the field to the laboratory is not always possible. Lecompte *et al.* (2005a) therefore searched for molecular markers that allow a clear discrimination of *Mastomys* individuals using ethanol preserved samples. They devised two tests using the sequenced cytochrome-*b* region from Lecompte *et al.* (2002). The first test devised was based on species specific primers and the second on generating species specific profiles using restriction enzymes for *M. coucha*, *M. natalensis*, *M. erythroleucus* and *M. huberti*.

The strategy used for test 1 was to find mutations specific to each *Mastomys* species by examining the cytochrome-*b* sequences for short sequences of approximately 20 nucleotides with no or little variation among specimens of a given species, but differing by several mutations from other species. Four pairs of species specific primers were selected by Lecompte *et al.* (2005a), one for each of the species tested, each of which are situated at a different position along the

sequence. This provides PCR products of different sizes that identified the specific species based on the PCR product size by using the different primer pairs in one reaction.

Test 2 involved the generation of PCR restriction maps to show potential restriction sites for various restriction enzymes. The only enzyme that had specific restriction sites yielding different profiles for each of the four species was *Bsm*AI. Lecompte *et al.* (2005a) showed that when the cytochrome-*b* sequences amplified are digested using *Bsm*AI, *M. natalensis* has one restriction site whilst *M. coucha* has two.

Based on the findings by Gordon (1984), Smit and Van der Bank (2001) and Smit *et al.* (2001), Murid rodents are considered biologically informative subjects for genetic analyses. This is due to their rapid evolutionary radiation and the cytogenetic differences that are common between genera and species. These phenomena that are common between the genera and species of the Murid rodents, especially that of *Mastomys*, made research into molecular and chromosomal methods more popular. These methods provide insight into the systematics of the *Praomys* complex and, in turn, *Mastomys*.

1.2.6. SYSTEMATICS

The genus *Mastomys* has a history of systematic debate. The lack of morphological characters which distinguish the cryptic species make them systematically overlooked. The absence of diagnostic features in cryptic species is normally the result of a lack of information with regards to simple aspects such as distribution, life history or the role they play in the species community (Volobouev *et al*., 2002). Comments on the systematics of *Mastomys* were provided by Robbins and Van der Straeten (1989). They studied the holotypes, original descriptions and associated museum specimens of 56 taxa associated

with the genus. The taxa were not allocated into species but it was determined that 36 taxa did belong to the genus.

The problem still exists however that many species in the *Mastomys* genus are morphologically identical and taxonomic problems still remain. Previous systematic studies were all based on body, skull and tooth morphology and as such errors might have occurred. Chevret *et al.* (1994) did a series of DNA/DNA hybridisation experiments, followed by a reconstruction of the phylogeny, involving different murinae species of which the *Praomys* complex (including *Mastomys*) formed part thereof. The different taxa of the *Praomys* group were closely related to each other in this study. In addition, biometrical studies showed that within the complex, *Praomys*, *Mastomys* and *Myomyscus* were well differentiated and the monophyly of *Mastomys* clearly separated it from the other genera.

Chromosomal evolution has shown that the amount and type of chromosomal modifications vary among taxa and that karyotypic changes can occur rapidly so that little morphological or genetic differentiation will accompany it (Baverstock and Adams, 1987; Meester, 1988). Since chromosomal analysis is a reliable method to identify a species correctly and clarify taxonomy, the chromosomal phylogeny of *Mastomys* was studied by Britton-Davidian *et al.* (1995). Through use of chromosomal techniques, various reproductively isolated species were identified by their diploid number. The resulting chromosomal phylogenies generated using a cladistic approach in conjunction with independent datasets, favored *Mastomys* as a monophyletic group. Britton-Davidian *et al.* (1995) further made the conclusion that *M. coucha* was the sister group of *M. huberti* and *M. natalensis.* The latter two species were the most closely related to one another.

The evolutionary consequences of chromosomal change on diversification was also studied by Britton-Davidian *et al.* (1995). The most frequent chromosomal rearrangement was that of pericentric inversions which, although never occurring

alone, are the most common in causing species separation. Pericentric inversions are almost entirely limited to the *Mastomys* branch of the phylogeny (Britton-Davidian *et al.,* 1995) and appear as a recurrent event in chromosomal evolution of *Mastomys*. They do not change the diploid number, but rather the fundamental chromosome number, indicating an event which modified diploid number occurred at the same time as a major speciation event. This was followed by chromosomal diversification within each diploid form to cause changes in fundamental number by pericentric inversions or wide-spread polymorphisms. Species with the same diploid number would therefore be more closely related to one another, which is indicated by the related nature of *M. huberti* and *M. natalensis* shown in the phylogenies of Chevret *et al.* (1994) and Britton-Davidian *et al.* (1995).

Granjon *et al.* (1997) reviewed the systematics of the *Mastomys* genus by taking into account the work of Gordon (1984), Chevret *et al.* (1994) and Britton-Davidian *et al.* (1995). The data sets showed that the species diversification of *Mastomys* had occurred recently and was accompanied by extensive chromosomal rearrangements. The difficulty in identifying the various species within the genus is due to the small morphological divergence.

In line with studying chromosomal rearrangements, Volobouev *et al*. (2002) studied the patterns of karyotype evolution in sibling species complexes belonging to three African murid genera, namely *Arvicanthis*, *Acomys* and *Mastomys*. Using cytogenetic and molecular data the study showed that each complex was characterised by a distinct pattern of karyotype evolution and a specific mutation rate. The chromosomal and molecular data for *Mastomys* was partially congruent but resulted in a more detailed understanding of the groups' evolution than either data set alone. The research showed that chromosomal changes are valuble to phylogenetic characters with every cladogenetic event in the genus *Mastomys* being supported by two pericentric inversions. These became fixed in a population, a similar finding to that of Britton-Davidian *et al.* (1995). The overlapping pericentric inversions were probably selected against therefore

Volobouev *et al*. (2002) hypothesised that the genetic differentiation of *Mastomys* was initiated by “the suppression of recombination within inverted segments and that the accumulation of multiple pericentric inversions reinforced genetic isolation leading to subsequent speciation and genetic diversity”.

A nearly complete molecular phylogeny amongst the *Praomys* complex was established using the cytochrome-*b* gene by Lecompte *et al.* (2002). A wide range of representative species from the recognised genera (Robbins and Van der Straeten, 1989) were included in the study with subsequent phylogenetic analysis. In contrast with the studies by Chevret *et al.* (1994) and Britton-Davidian *et al.* (1995), *Mastomys* appeared to be paraphyletic and not monophyletic but *M. erythroleucus*, *M. natalensis*, *M. huberti* and *M. coucha* formed a strongly supported clade. Lecompte *et al.* (2002) confirmed that *M. coucha* is the sister group to *M. natalensis* and *M. huberti*, in congruence with Chevret *et al.* (1994).

The sequencing of the cytochrome-*b* gene allowed Galan *et al.* (2004) to present data on the isolation of polymorphic microsatellites in *M. huberti* based on the genetic sequences obtained. After DNA extraction and sequencing, 12 microsatellite loci were selected for their high polymorphism and wide range of allele sizes. Tests were done on 31 *M. huberti* specimens. The sets of markers isolated for *M. huberti* and the cross amplifications done using *M. coucha*, *M. natalensis* and *M. erythroleucus* provided 12 loci that can be amplified in multiplex reactions and used to study the population genetics and evolutionary trends of any of the four cryptic species.

1.3. THE PRESENT STUDY – AIMS AND HYPOTHESES

The Roan Camp (22º 46' 21.0" S, 31º 15' 45.0" E) is situated in the central part of the vlakteplaas region in the northern plains of the KNP at an altitude of 384m above sea level (Fig. 1.10). It lies on olivine-rich basalt with the landscape classified as basaltic plains with mopane scrub savanna. The vleis’ (wetlands)

associated with the enclosure are seasonally flooded with an average annual rainfall between 350-450mm (MacFadyen, 2007). Originally the enclosure had a total area of 254 ha and was erected in the center of the roan antelope study area of 1967 for the conservation of rare antelope species in the KNP. It excludes large herbivores and large predators (Joubert, 1974). In 1994, 48 ha of land was added to include an adjacent watercourse and the associated vegetation in the eastern area of the enclosure. The enclosure now encompasses 302 ha (Solomon *et al*., 1999) and is surrounded by a network of fire breaks used for controlled burning to protect the inner vegetation from fire (Trollope *et al*., 1998). In this way intensive scientific research of rare ungulate species could be undertaken and a breeding nucleus of free ranging herds could be established (Joubert, 1974).

MacFadyen (2007) studied the species richness, abundance and habitat preferences of small mammals between June 2003 and April 2005 in, around and outside an enclosure site as well as identification and monitoring of the effect of different management actions on the small mammal populations. For the purposes of the study MacFadyen (2007) divided the catena of the Roan Camp into three broad categories, namely the top, middle and bottom lands (Fig. 1.11). The top land (Fig. 1.11, grid B) refers to the highest point of the catenal gradient. It is comprised of relatively flat, dry ground which is covered by low, stunted mopane, zebrawood and sparse herbaceous material. The middle land (Fig. 1.11, grid A) is the western area represented by substantial grass cover as well as apple leaf and marula trees. The bottom land (Fig. 1.11, grid C) is the eastern area and is a relatively flat, seasonally inundated vlei which was covered by tall grass and sedge. The woody component was represented by lala palms and the herbaceous cover was determined to be dense. In each of the above mentioned land categories, three transect lines were chosen and numbered 1-3 for each region. Fig. 1.11: 1A, 1B and 1C refers to the area outside the enclosure, Fig. 1.11: 2A and 2B refers to the area around the enclosure in the fire break and Fig. 1.11: 3A, 3B and 2C refers to areas inside the enclosure. 3C is representative of a

sodic area found inside the enclosure. All the above mentioned terms will be adopted in the present study.

Fig. 1.10 – A Schematic representation of the KNP indicating the location of the Roan Camp (KNP Official web site, www.sanparks.org/parks/kruger)

MacFadyen (2007) found that between the three regions the frequency of rodent captures differed significantly with regards to the different transects. Transects 1

from each region had the highest captures followed by transects 2 and 3 respectively. The aim of the transect lines was to analyse the effects of different management practices on small mammal assemblages. These management practices results in significant differences in the diversity, density and height of the vegetation. The transect lines outside the enclosure (Fig. 1.11: 1A, 1B and 1C) showed normal veld conditions influenced by ad hoc fire and elephant impact. Outside the enclosure the vegetation was less dense with a lower percentage of woody components than inside. The transect lines from outside the enclosure represented 40.63% of the total captures followed by the area around and inside the enclosure with total captures of 36.37% and 23.0% respectively. In general the transect lines show that the species richness is similar under each management practice but that differences in abundance do occur. The lower capture percentage inside the enclosure was attributed to the greater amount of herbaceous material and woody plants which made the habitat less favourable for species that prefer a less dense habitat.

The number of captures in the artificial fire break differed insignificantly between transect 2A and 2B (Fig. 1.11) but the higher percentage of captures compared to inside the enclosure was attributed to a cold burn (some areas had partial burning) which allowed for sufficient grass seeds and cover to still be present as well as the ability of the rodents to forage on the outskirts of the burn area. In fact no species occurred at a higher density in the fire break when compared to the inside and outside of the enclosure and it was determined that direct fire inflicted mortality did not occur due to the cold burn which allowed animals to shelter in burrows. Joubert (1974) considered fire to be a limiting factor which influences the population growth of rodents. From Joubert (1974) it was expected that burning would have a devastating and immediate affect on the dynamics and structure of small mammal populations but MacFadyen (2007) found that the results indicated an adaption of the rodent populations to post-fire conditions, in some instances favouring such conditions. With regard to the *Mastomys* population there was a large number of captures before, during and after burning. The capture success

decreased four months after burning but this was attributed to the local movements of the species into surrounding areas as the food became less readily available and other, more specialist species moved in. This is in conjunction with the findings of Meester *et al*. (1979). The fire break was considered as a volatile habitat in a pioneer stage of development with 83.04% of the total captures from the transect 2A and 2B (Fig. 1.11) being *Mastomys* (MacFadyen, 2007).

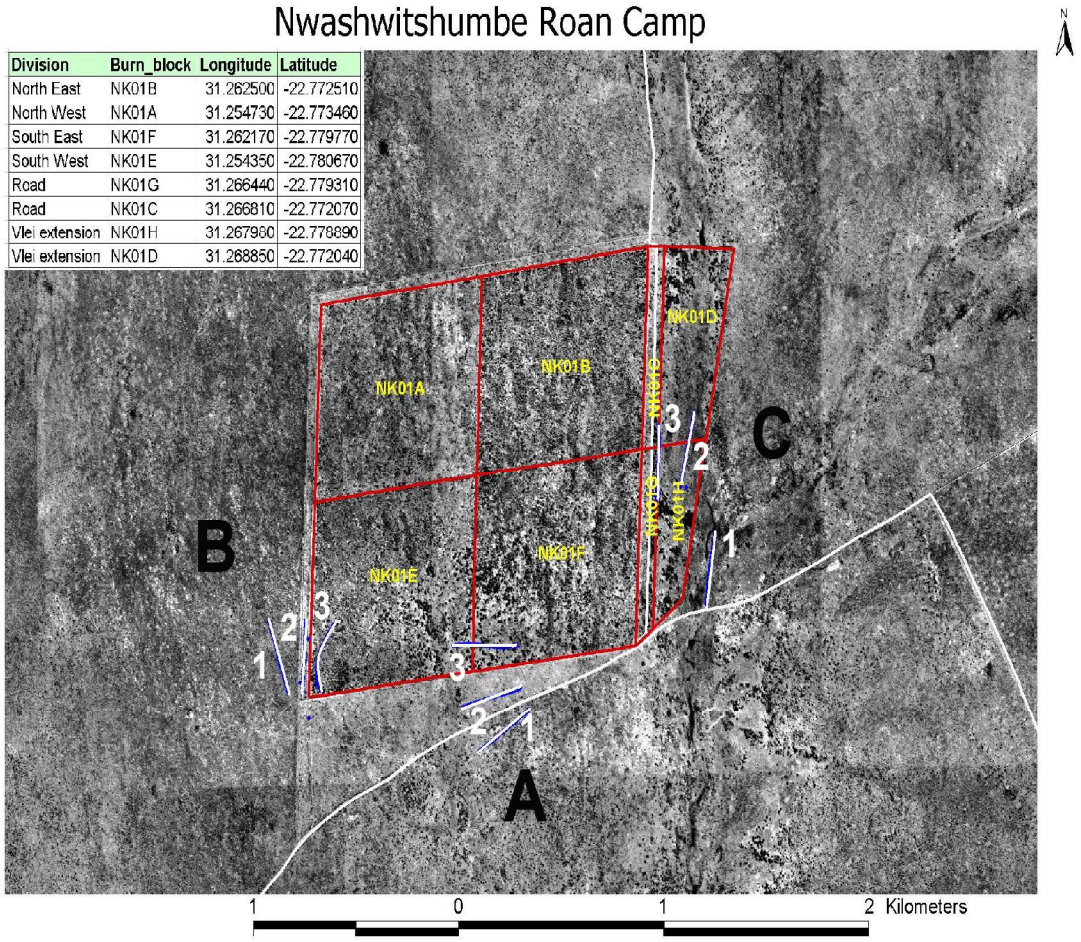


Fig. 1.11 – Aerial photograph showing the three broad regions (A, B and C) selected at the Roan camp and the placement of the transect lines (1, 2 and 3) (MacFadyen, 2007)

MacFadyen (2007) further investigated the vegetation communities of the area and the result that it may play in species richness and abundance. Thirteen

vegetation communities were identified using the Braun blanquet approach (MacFadyen, 2007). This approach has three main ideas:

1. the plant communities are conceived as types of vegetation recognised by their holistic composition,
2. amongst the species that make up the floristic composition of a community some are a more sensitive expression of a given relationship than others, i.e. the approach uses those species whose ecological relationships make them the most effective indicators and
3. diagnostic species are used to organise the communities into a hierarchical classification of which the association is the basic unit.

The main elements of the approach were proposed in 1921 and include the careful description of structure, the combined estimation of cover abundance and the sociability of all the participating species, and the systematical description of the superficial features of the site. It is therefore considered a floristic instead of ecological classification (Van der Maarel, 1975). After the identification of the vegetation communities within the Roan Camp, Analysis of Variance (ANOVA) was used to determine the influence of the vegetation on the small mammal assemblages. For *Mastomys*, 12 of these communities were statistically significant ($P<0.005$) (Table 1.2; MacFadyen, 2007).

The *Mastomys* genus was recorded as the most abundant genus throughout the study, in each region, especially during the months of May and August of each year due to population explosions during these periods. Of the total captures, 80.74% were *Mastomys* and originally MacFadyen (2007) thought only *M. natalensis* was captured. However after allozyme identification of nine *Mastomys* individuals caught from the enclosure, Kneidinger (2005) showed both *M. natalensis* and *M. coucha* to be present. As previously stated the absence of distinguishing features is normally the result of a lack of information regarding distribution, habitat and life history (Volobouev *et al.,* 2002). The presence of both species within the same study area allows for research into the distribution and vegetation aspects of the two cryptic species as well as comparing their genetics.

Table 1.2 – Summary of the 12 different vegetation communities found within and outside the Roan Camp that were statistically significant (P<0.005) (MacFadyen, 2007).

	VEGETATION COMMUNITY	LOCATION	*GRID AND TRANSECT NO.
1	*Schmidtia pappophoroides Heteropogon contortus*	Outside	A1
2	*Schmidtia pappophoroides Setaria incrassate*	Outside	A1
3	*Panicum coloratum Setaria incrassate*	Around, inside	A2, A3
4	*Schmidtia pappophoroideS Urochloa mosambicensis*	Around	A2
5	*Panicum maximum Panicum coloratum*	Inside	A3
6	*Aristida bipartite Colophospermum mopane*	Around	B2
7	*Panicum coloratum Colophospermum mopane*	Outside	B1
8	*Cenchrus ciliaris Colophospermum mopane*	Inside	B3
9	*Setaria incrassate Dalbergia melanoxylon*	Outside, around, inside	A1, A2, A3, B2, B3
10	*Panicum maximum Urochloa mosambicensis*	Around, inside	A2, A3, B2, B3
11	*Cyperus textiles Andropogon spp.*	Outside, inside	C1, C2
12	*Sporobolus pyramidalis Andropogon spp.*	Inside	C2

* Refer to grid and transect numbers as indicated in Fig. 1.11.

Based on the above literature survey the following aims were identified to:

1. successfully use the PCR-RFLP based *Mastomys* species identification technique in accordance with the method described by Lecompte *et al.*;
2. Relate the abundance and distribution of the cryptic *Mastomys* species to vegetation type as well as the influences of fire and natural habitat destruction within the Roan Camp, KNP. Although a lot of research has been done concerning the preferences of each species, all are based on samples obtained from areas where the species are allopatric and often samples from different years are pooled together. The same situation arises when vegetation preferences are considered. Based on broad geographic features such as rainfall and altitude it is expected that *M. coucha* will be more prevalent in the study area but that some *M. natalensis* specimens will be identified allowing for comparison of sympatric specimens. It is hypothesised that the two species have different habitat preferences based on vegetation type, structure and density which therefore has an influence in species distribution.
3. Investigate the population genetics and evolutionary trends of *M. coucha* and *M. natalensis* using microsatellites. From Kneidinger (2005) it was shown that *M. coucha* had more genetic variation than *M. natalensis*, within the KNP region, at the AAT-1 locus. Rapid evolutionary radiation of rodent species was considered as a possible reason for differences in genetic variation and based on the conclusion by Smit (2001) that a chromosomal mutation in *M. natalensis* gave rise to a younger *M. coucha* which became reproductively isolated. It is hypothesised that the use of microsatellite markers will provide information on the population genetics and evolution of *M. natalensis* and *M. coucha*.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1. SPECIMEN COLLECTION

Toe samples were obtained from *Mastomys* specimens captured by MacFadyen between June 2003 and April 2005 in and around the Roan Camp, KNP. Specimens were caught alive using Sherman traps (Fig. 2.1) baited with a mixture of honey and oats. The traps were placed at 10 m intervals along the three transect lines of each of the three grids to include the top, middle and bottom slopes (Fig 1.11), within and surrounding the Roan Camp. Each transect line had 30 traps along its length with a total of 270 traps set out. Around each trap a vegetation sample plot with all its floristic and environmental data was observed and the plant species present were noted and classified in a Braun-Blanquet table (MacFadyen, 2007). The same trapping sites were used for two to three days each month by using markers along each transect line and above each trap (Fig. 2.1).

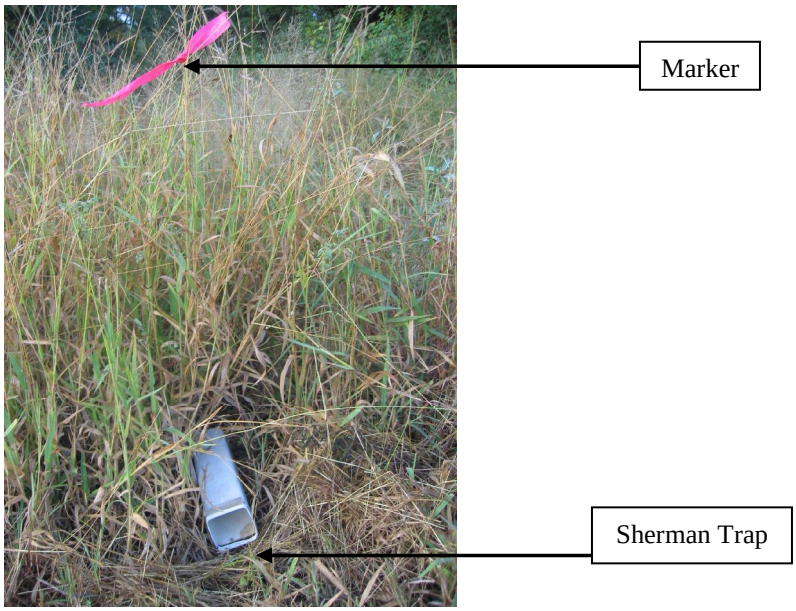


Fig. 2.1 – Photograph of a Sherman trap and marker used to capture rodents.

Once captured each mouse was removed from the trap and anesthetised using ethyl acetate (MacFadyen, 2007). Specimens were identified and sexed visually, weighed using a Pesola pull scale, measured and marked using the 1-2-4-7 toe clipping system modified from DeBlase and Martin (1981) as required for the capture-mark-recapture method followed by D. MacFadyen for his research on rodent and shrew diversity. Each digit was allocated a number as listed in Block 1 to obtain a possible 9 999 different toe clipping combinations for identification. Only the last phalange was removed during the toe clipping procedure following the guidelines set out by the American Society of Mammologists (1998) and stored in 70% ethanol in eppendorf tubes. All specimens were released in the same location as trapped (MacFadyen, 2007).

BLOCK 1 – THE MARKING SYSTEM USED TO IDENTIFY RODENT SPECIMENS FOR THE MARK-RECAPTURE METHOD

FRONT LEFT FOOT – SINGLES

Toe 1(1), 2(2), 3(4) and 4 (7) (gives numbers 1-9 with combinations of toes cut)

For example: first toe cut = 1, second toe cut = 2, first and second toes cut = 3, third toe cut = 4, first and third toe cut = 5, second and third toes cut = 6, fourth toe cut = 7, first and fourth toes cut = 8 and second and fourth toes cut = 9.

FRONT RIGHT FOOT – TENS

Toe 1(10), 2(20), 3(40) and 4 (70) (gives numbers 10-90 with combinations of toes cut)

BACK LEFT FOOT – HUNDREDS

Toe 1(100), 2(200), 3(400) and 4 (700) (gives numbers 100-900 with combinations of toes cut)

BACK RIGHT FOOT – THOUSANDS

Toe 1(1 000), 2(2 000), 3(4 000) and 4 (7 000) (gives numbers 1 000-9 000 with combinations of toes cut)

In the resent study, 30 specimens captured during the first week of August 2003 and 60 captured during the same period of August 2004 were randomly chosen from samples collected by MacFadyen. The larger sample size in August 2004 was because more specimens were captured in 2004. The samples from August 2003 were used as a comparison to the 2004 sample set, and for use as control samples, two specimens of M*. coucha* were caught at Montgomery Park, Johannesburg (26º 09' 26.5" S, 27º 59' 02.7" E). *Mastomys coucha* is known to be allopatric in this area (Skinner and Smithers, 1990). August 2004 was selected because the fire break surrounding the enclosure was burnt in July 2004 as part of the management strategy in the area. Sampling the month after the burn may provide insight into which species, if not both, is more likely to be the pioneer.

2.2 DNA EXTRACTION

The individual toe samples from each specimen as well as muscle samples from the two control animals were frozen in liquid nitrogen and crushed to sufficiently break open the cells. DNA was further extracted using the Qiagen DNeasy blood and tissue kit (Fig. 2.2., http://www1.qiagen.com/SelectLocation.aspx). The kit contents are listed in Table 2.1.

Fig. 2.2 – The contents of the Qiagen DNeasy blood and tissue kit.

Table 2.1 – List of contents for the Quiagen DNeasy blood and tissue kit (50 preparations)

CONTENT DESCRIPTION	NUMBER/VOLUME
DNeasy Mini Spin column in 2ml Collection Tube	50
2ml Collection Tubes	100
Buffer ATL	10ml
Buffer AL	12ml
Buffer AW1	19ml
Buffer AW2	13ml
Buffer AE	22ml
Proteinase K	1.25ml

One hundred and eighty µl of ATL buffer and 20 µl of proteinase K was added, vortexed and incubated, with occasional vortexing, at 56°C for three hours to ensure complete lysis. When lysis was complete the mixture was vortexed for 15 seconds and 200µl of buffer AL together with 20 0µl of 96% ethanol was added and mixed thoroughly to yield a homogenous solution. The mixture was pipetted into a DNeasy mini spin column in a 2 ml collection tube and centrifuged at 8 000 rpm for one minute. The flow through in the collection tube was discarded and the spin column transferred to a new 2 ml collection tube. Five hundred µl of buffer AW1 was added to the spin column and centrifuged for another minute at 8 000 rpm. Again the flow through and collection tube was discarded and replaced with a new 2 ml collection tube. Five hundred µl of buffer AW2 was added to the spin column and centrifuged for three minutes at 14 000 rpm to dry the DNeasy membrane. The spin column was carefully removed to avoid contact with the flow through which was discarded. The spin column was transferred to a 1.5 ml microcentrifuge tube and 200 µl of buffer AE was added for elution. The mixture was incubated for one minute at room temperature and centrifuged for one minute at 8 000 rpm. The last step was repeated for maximum DNA yield. The DNA concentration of each isolation was determined using the Nanodrop 1 000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware USA, Fig. 2.3) and DNA was prepared to a working concentration of 25 ng/µl.

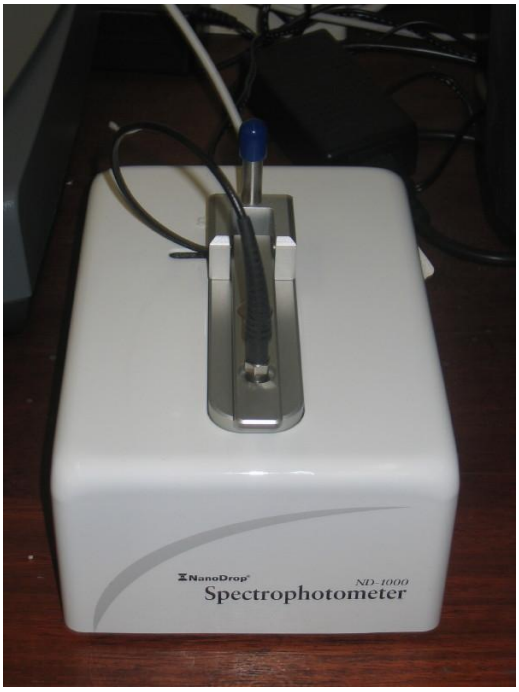


Fig. 2.3 – The Nanodrop 1 000 spectrophotometer used to determine the DNA concentration after DNA isolation (NanoDrop Technologies, Wilmington, Delaware USA).

2.3 PCR AMPLIFICATION FROM DNA EXTRACTION

An Eppendorf Mastercycler (Eppendorf, Germany) as seen in Fig. 2.4 was used for all PCR. PCR was done on the 60 samples from 2004 using the primer set L14723 (5' ACC AAT GAC ATG AAA AAT CAT CGT T 3') and H15915 (5' TCT CCA TTT CTG GTT TAC AAG AC 3'), described in Lecompte *et al.* (2002). Four samples could not be amplified using the primer set L14723 and H15915. Each PCR reaction volume was 25 µl and the reagents consisted of 1X peqGOLD Taq-DNA Polymerase Buffer S containing 100 mM Tris-HCl, 500 mM KCl, 0.1% Tween 20 and 15 mM Magnesium chloride (Peqlab Biotechnologie, GmbH), 0.2 µM of each primer, 0.4 mM deoxynucleotide triphosphate, 0.05U peqGOLD Taq-DNA Polymerase (Peqlab Biotechnologie, GmbH) and 25 ng DNA The reaction cycle conditions for the L14723 and H15915 primer set was as follows: one

minute at 94ºC followed by 35 cycles of 40 seconds at 94ºC, 45 seconds at 57ºC and 40 seconds at 72ºC. A final elongation step of 7 minutes at 72ºC was done. PCR products were screened for success by loading four µl of PCR product combined with 1 µl 6X loading dye into the wells of a 1% agarose gel containing one µl of a 10 mg/ml ethidium bromide solution. The gel was run at 100V for approximately 20-30 minutes. It was visualised and the pictures recorded with the Biorad Gel Doc XR system (Bio-Rad Laboratories). Band size was quantified using the Fermentas O'GeneRuler 1kb DNA ladder (Fig. 2.5) run in conjunction with the samples.

Fig. 2.4 – The Eppendorf Mastercycler used for PCR

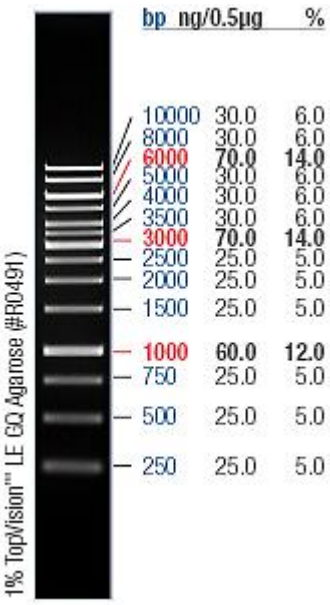


Fig 2.5 – Representation of the band sizes for the Fermentas O'GeneRuler 1kb DNA ladder

The primer set L14723 and H15915 was unable to amplify some samples. New primers designated MasF (5' CAT CAT GAT GAA ACT TCG GCT C 3') and MasR (5' TGT TCT ACT GGT TGG CCT 3') were designed using available Genbank sequences of *Mastomys* spp. Genbank sequences of *Mastomys* spp. cytochrome-*b* consist of only 1140 bp of the 1250 bp that L14723 and H15915 primer set amplify. These sequences therefore do not include the first and last 50 bp sequences, which include the L14723 and H15915 primer sequences. The new primer set MasF and MasR was designed from available cytochrome-*b* sequences and the PCR product was smaller than other primer set. The 30 samples from August 2003, which include the four samples that did not ampligy with L14723 and H15915 primes, were amplified using this new primer set. The protocol was optimised using two different *Taq* DNA polymerase enzymes, namely with U-*Taq* DNA polymerase from SBS Genetech Co and peqGOLD Taq-DNA Polymerase at final magnesium chloride concentrations of 1.5 mM and 2 mM and temperatures (ranging from 54-58°C). It was determined that the reaction best worked with U-*Taq* DNA polymerase from SBS Genetech Co., with a magnesium chloride concentration of 2 mM and an annealing temperature of

57°C. Each PCR reaction volume was 25 µl and the reagents consisted of 1X U – *Taq* DNA Polymerase Buffer containing 100 mM Tris-HCl, 400mM KCl, 15mM $MgCl_2$ (SBS Genetech Co.), 0.2 µM of each primer, 0.4 mM deoxynucleotide triphosphate, 0.05U U- *Taq* DNA Polymerase (SBS Genetech Co.), magnesium chloride was added to give final concentration of 2 mM and 25 ng DNA. The reaction cycle conditions for the MasF and MasR primer set was as follows: one minute at 94ºC followed by 35 cycles of 40 seconds at 94ºC, 45 seconds at 57ºC and 40 seconds at 72ºC. A final elongation step of 7 minutes at 72ºC was done. The PCR products were screened for success as previously described.

2.4 RESTRICTION FRAGMENT LENGTH POLYMORPHISMS FROM PCR AMPLIFICAITON

PCR-RFLP was done as described by Lecompte *et al.* (2005a). Five µl of each positive PCR product from both sample sets were combined with 0.5µl of the Fermentas restriction enzyme *Bsm*AI (*Alw*261, GTCTC(1/5)↓) and 1µl of 10X Buffer Tango™ (Fermentas) in a total volume of 10 µl. Each reaction mixture was incubated at 37°C for one hour and PCR-RFLP profiles was visualised on 1% agarose gel containing ethidium bromide. The species were identified based on the different banding patterns produced, with reference to the banding pattern produced by the control samples. To verify PCR-RFLP patterns produced on the 1% agarose gel, one sample for each banding pattern was chosen and sent to Inqaba Biotechnology, S.A. for DNA sequencing. The BigDye® Terminator v3.1 Cycle Sequencing Kit was used and the product run on the Spectromedix sequencing analyser. A Blast search was done for each DNA sequences to confirm the species identification of the three different RFLP.

Once the samples were identified to species level they were linked to the trap they were caught in as well as the various plant species observed in the vegetation sample plot surrounding the trap. This was done by referencing the trap number (i.e. B1 26) to the plant species classified in the Braun-blanquet table

(MacFadyen, 2007). Statkon, the statistical services at the University of Johannesburg, was consulted for the statistical analysis of the data. Using the SPSS processor, the two rodent species were associated to slope type (A/B/C), vegetation density (1/2/3), specific plant species as well as plant communities and the data interpreted. The preferences of each species to rainfall and altitude were also considered.

2.5. MICROSATELLITES

A set of five microsatellites (Table 2.3), relevant to *M. natalensis* and *M. coucha*, were chosen from Galan *et al.* (2004) and the sequences described in Table 2.3. The PCR for each microsatellite primer set was optimised with one sample of each species to determine the appropriate concentrations of primer, DNA and dNTP's, as well as, the optimal annealing temperature for each primer set (Table 2.4). Each PCR reaction volume was 25 µl and the reaction cycle was as follows: two minutes at 93ºC followed by 35 cycles of 40 seconds at 92ºC, 50 seconds at the specific annealing temperature (Table 2.4) and one minute at 72ºC. A final elongation step of 10 minutes at 72ºC was done. Once optimised the forward primer was 5'-end labeled with a phosphoramidite fluorescent dye (FAM or HEX, Table 2.3).

Table 2.2 – Characteristics of the five microsatellite primers selected

PRIMER	EXPECTED SIZE RANGE (bp)	DYE	FORWARD PRIMER SEQUENCE	REVERSE PRIMER SEQUENCE
MH28	236-264	FAM	5' GGATCTGATGCCCTCCTCTAG 3'	5' AAAGGCTGATGAGTGGTATCC 3'
MH52	81-109	HEX	5' TGGACAGGGAGAGAATTTGG 3'	5' CTTACACACTCAACTCCAAGC 3'
MH60	161-213	FAM	5' GCCAAAAGCCTATAACCTTC 3'	5' CATCTTCCAAGTTCTATTTATGTG 3'
MH141	230-283	FAM	5' TGGAAACAGCCTGTGCCAGC 3'	5' TTGGGCTCCCTCCTGGTTG 3'
MH146	113-159	HEX	5' CAGCAAGTGTCAGGGCGATG 3'	5' GCCATGTTTGCTTAACCAGACC 3'

Table 2.3 – Final reagent concentrations and annealing temperatures in PCR reactions for the five microsatellite primers.

PRIMER	[PRIMER]	[DNA]	[dNTP's]	ANNEALING TEMPERATURE (*M. coucha*)	ANNEALING TEMPERATURE (*M. natalensis*)
MH28	0.2µM	Max 50ng/µl	400µM	56°C	57°C
MH52	0.4µM	Max 100ng/µl	800µM	57°C	58°C
MH60	0.4µM	Max 50ng/µl	400µM	56°C	57°C
MH141	0.4µM	Max 50ng/µl	400µM	57°C	58°C
MH146	0.8µM	Max 100ng/µl	800µM	57°C	58°C

Thirty DNA samples of *M. coucha* from the 2004 sample set (for reliable statistical results) and all six samples of *M. natalensis* with good DNA quality, previously extracted for identification purposes, were randomly chosen for the genetics study. The samples were amplified using each microsatellite primer set under their optimised conditions. Fluorescent labeled PCR products (undiluted) were combined with 0.5 µl of the GeneScan™ 350 ROX™ size standard marker (PE Biosystems) and 8.5 µl of HiDiformamide in 96 well plates and separated on a ABI 3130 Automated Capillary Sequencer (PE Biosystems, Fig. 2.6). The sizes of the alleles amplified with the selected primers for 36 samples, were measured in base pairs from the electrophoretic mobility through the gel, relative to the internal size standard (350 ROX™ size standard marker) as indicated by the GeneScan® AFLP and Genotyping software, version 1.6 (PE Biosysystems). The statistical analysis of the data was executed using BIOSYS-1 (Swofford and Selander, 1981) and RSTCALC (Goodman, 1997). Input files are given in Appendixes A1 and A2. From the F_{st} value obtained the N_{em} value was also calculated (Takahata, 1983). Other values calculated included the allelic diversity and the total genetic diversity. Allelic diversity was calculated using the formula $(1-\sum p_i^2)$, where p_i is the frequency of the *ith* allele (Nei, 1973) and total genetic diversity, (H_{TR}) was

calculate as $[(n)/(n-1)](1-\sum p_i^2)$ where n is the sample size (Nei and Chesser, 1983).

Fig. 2.6 – The ABI 3130 Genetic Analyser (PE Biosystems).

CHAPTER 3

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

3.1. SPECIES IDENTIFICATION

Species identification of 86 rodent samples, which were selected from sample sets collected in Kruger National Park from August 2003 and August 2004, was facilitated by PCR-RFLP profiling methods described by Lecompte *et al.* (2002; 2005a). Four samples from 2004 did not amplify using primer set L14723 and H15915 due to possible mismatch(s) between the template and the primers or DNA degradation from excessive freeze-thaw cycles. The primer set L14723 and H15915 amplified a PCR product of approximately 1200 bp and it was expected that digestion with *Bsm*AI would cut *M. coucha* once at approximately 790 bp (PCR-FRLP profile consisting of 460 bp and 790 bp) and *M. natalensis* three times at approximately 210 bp, 790 bp and 950 bp (PCR-RFLP profile consisting of 160, 210, 300 and 580 bp). These PCR-RFLP profiles were obtained for both species (Fig. 3.1, Appendix F1 and F2). The PCR-RFLP seen in Lecompte *et al.* (2005a) does not correspond to the expected profile and consists of undigested and digested fragments, as well as partially digested profiles for some species. The PCR-RFLP profile image in Lecompte et al. (2005a) is a partial digest because the undigested PCR product, as well as, digested bands are present in the PCR-RFLP image. The PCR-RFLP profile generated in this study is an example of a digested PCR (Fig. 3.1). The PCR product from primer set MasF and MasR yielded a band size of about 300 bp smaller (PCR product ~950 bp) than the other primer set, but the correct PCR-RFLP banding pattern was obtained (results not shown).

Some of the PCR samples amplified with primer set L14723 and H15915 (Fig 3.1, lane 2 and 4) were not cut by the restriction enzyme. This led to the hypothesis that a third species may be present within the Roan Camp, KNP. Selected samples representing each of the restriction map profiles were sent for sequencing by Inqaba Biotechnology, S.A. to confirm the species identification

and determine whether the uncut PCR product was representative of a new species. A Blast search (www.ncbi.nih/nlm.gov/Blast) was done on the DNA sequences and the results confirmed that the samples with PCR-RFLP profiles for *M. coucha* and *M. natalensis* were identified correctly and showed 98% homology to *M. coucha* and *M. natalensis* sequences respectively (Appendixes E1 and E2). With regard to the uncut PCR product, the Blast search revealed that these samples were that of *M. coucha* and not a new species. The above hypothesis was deemed invalid. The uncut PCR products had a point mutation in the *Bsm*AI restriction enzyme (Fig. 3.2, Appendix F3). Due to point mutation in the *Bsm*AI restriction site indicates that the PCR-RFLP is not always reliable and further investigation into another restriction enzyme or molecular technique needs to be investigated.

In total, of the 30 rodent samples identified from August 2003, 28 were *M. coucha* and two were *M. natalensis* and from the 56 samples identified from August 2004, 50 were *M. coucha* and six were *M. natalensis*. Once identification was complete the two species could be statistically associated to the vegetation data obtained by MacFadyen (2007) to gain insight into the habitat preferences of the different species.

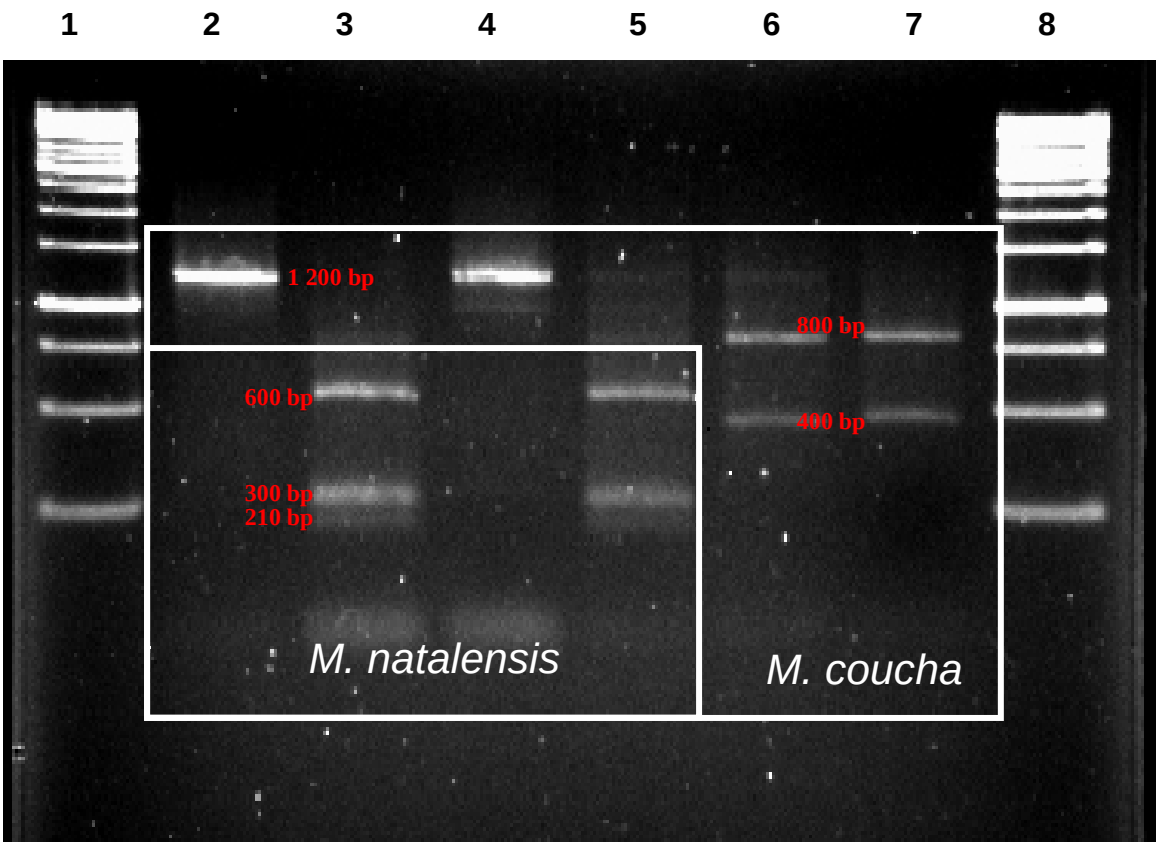


Fig. 3.1 – 1% Agarose gel of the PCR-RFLP fragments obtained after digesting the L14723 and H15915 PCR products with the restriction enzyme *Bsm*AI. The O'GeneRuler 1kb molecular weight marker was deposited in lanes 1 and 8. Lanes 2 and 4 show the uncut PCR-RFLP products amplified using primer sets L14723 and H15915 and digested by the restriction enzyme *Bsm*AI. Lane 3 and 5 show the PCR-RFLP fragments where the *Bsm*AI digest produced fragments of 580, 300, 210 and 160 bp (last two bands not separated on agarose gel) for *M. natalensis* while lanes 6 and 7 show the PCR-RFLP fragments for *M. coucha* with fragment sizes of 790 and 460 bp.

GENBANK NUMBER	SEQUENCE
47834426	5’ ACTAGGAGACCCAG 3’
25814881	5’ ACTAGGAGACCCAG 3’
UNCUT	5’ ACTAGGAGA**T**CCAG 3’

Fig. 3.2 – Section of the alignment of AY554160*, M. coucha* AD115 sample from S.A.: Beitbridge, and AF518334, *M. coucha* MNHN 1999-104 sample from S.A.: Bloemfontein (Lecompte *et al*., 2002), which is aligned with the *Mastomys* sample from S.A.: KNP in April 2003 that remained uncut when digested with *Bsm*AI. The point mutation found in *M. coucha* sample in Roan camp, KNP is indicated in bold and the restriction site of *Bsm*AI is highlighted.

3.2. HABITAT PREFERENCES OF M. COUCHA AND M. NATALENSIS

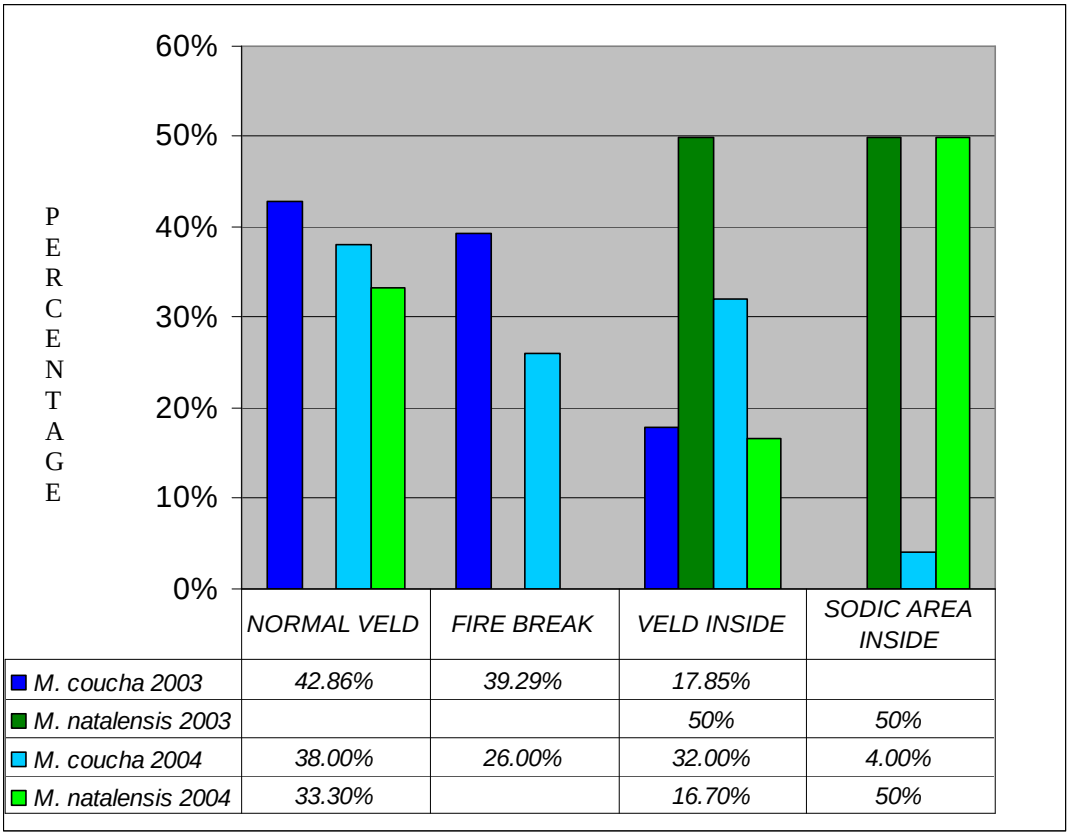
In this study the habitat preferences of the different *Mastomys* species observed within the Roan Camp, KNP where investigated because the most important factor influencing the distribution and abundance of small mammals within their geographic ranges is suitable habitat (Geier and Best, 1980). *Mastomys* is considered to occupy a wide variety of habitats (Stuart and Stuart, 2001) and it is important to relate their abundance and distribution to habitat structure especially in cases of habitat disturbances.

General preferences concerning broad geographical features were noted regarding the higher number of *M. coucha* caught in the Roan Camp, KNP, when compared to that of *M. natalensis* for both sampling periods. Gordon (1984) and Venturi *et al.* (2004) state that *M. coucha* prefers areas with a high altitude and an average rainfall of less than 700 mm per annum whereas *M. natalensis* is abundant in areas where rainfall is more than 600 mm per annum at a low altitude. The Roan Camp has a high altitude of 384 m above sea level and a

moderate average rainfall between 350-450 mm per annum and therefore the results are in accordance to their findings. However *M. natalensis* (9%) was found in the same geographical location as *M. coucha* (91%) which was previously predicted by Venturi *et al.* (2004) to be occupied by *M. coucha* only. Venturi *et al.* (2004) did comment that the two species were sympatric at Satara in the KNP with a possible zone of overlap along the eastern escarpment. Satara has an altitude of 260 m above sea level and an average rainfall of 550mm per annum. These geographical values meet the broad requirements of both species. It is possible for the geographical distribution of *M. natalensis* to extend into the Roan Camp region, with this being the limit of their distribution, considering the relatively close proximity of suitable habitat. These findings therefore coincide with the suggestion of the overlap of species along the eastern escarpment in that *M. coucha* and *M. natalensis* are sympatric throughout a larger area of the KNP.

More specific univariate and multivariate statistical data relating to habitat preferences was obtained for each species, at each sampling time, using the SPSS Processor. Both species were compared to the density of vegetation or habitat type (Fig 1.11) in which each sample was trapped (Fig. 3.3). During August 2003 one individual (50%) of *M. natalensis* were each found in the veld and in the sodic area inside the enclosure (Fig. 3.3). Although the sample size for *M. natalensis* was small, with only two individuals sampled in 2003 and six in 2004, all were captured on the bottom slope (C) of the enclosure site (Fig. 3.4). Similarly during August 2004, 50% of *M. natalensis* were found in the sodic area (33.3% in the normal veld outside the enclosure and 16.7% in the veld inside the enclosure). The areas of veld inside and outside the enclosure, sampled on the bottom slope, are comprised of a vlei that is seasonally flooded. The presence of *M. natalensis* could be associated to non avoidance and the higher moisture content in the general area, because this species is linked to a higher rainfall than *M. coucha* (Gordon, 1984; Venturi *et al.*, 2004).

No *M. natalensis* were found in the fire break either before or after the area was burned in July 2004. In comparison during the 2003 and 2004 sampling periods, 39.29% and 26% of *M. coucha* specimens were captured within the fire break suggesting that this species may be more inclined to make first use of newly sprouted vegetation after fires. Therefore *M. coucha* is more likely to be the pioneer species of the two in the KNP (Fig. 3.3) although this association may be coincidental because of the higher number of *M. coucha* individuals caught compared to that of *M. natalensis*.



	NORMAL VELD	FIRE BREAK	VELD INSIDE	SODIC AREA INSIDE
M. coucha 2003	42.86%	39.29%	17.85%	
M. natalensis 2003			50%	50%
M. coucha 2004	38.00%	26.00%	32.00%	4.00%
M. natalensis 2004	33.30%		16.70%	50%

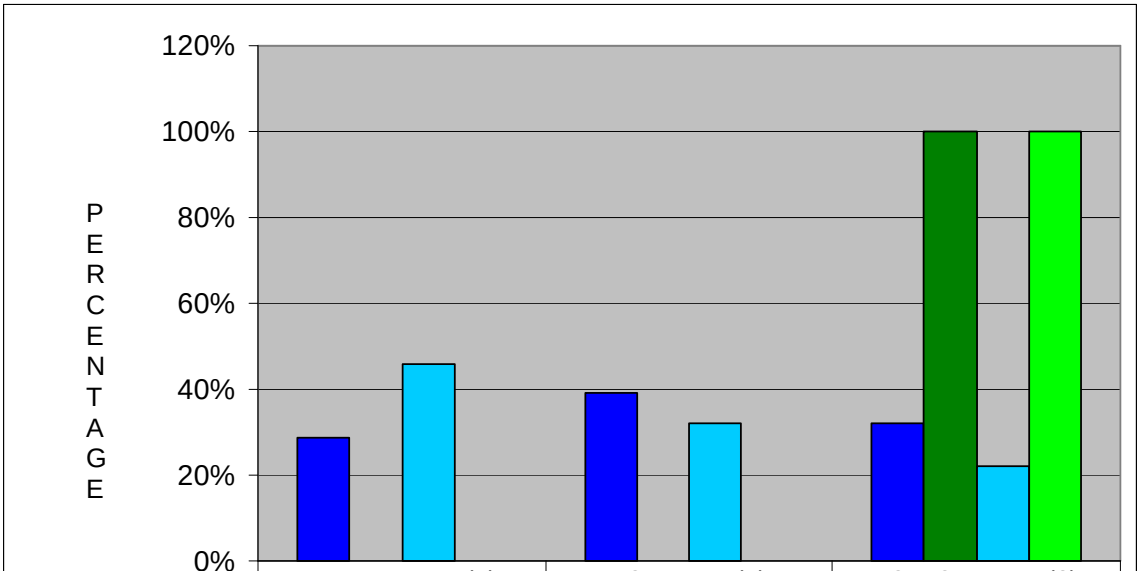
Fig. 3.3 – Percentage distribution of *M. coucha* and *M. natalensis* with regard to the different habitat types sampled inside and around the Roan Camp, KNP.

The habitat type most preferred by *M. coucha* is the normal veld outside the enclosure with 42.86% of the 2003 individuals and 38% of the 2004 individuals captured in this location. This preference was followed by the fire break and the inside veld respectively (Fig. 3.3). The veld inside and outside the enclosure was

relatively similar baring an abundance of grasses and shrubs, except that outside the plant density was less due to disturbances by large herbivores. Both locations provide sufficient cover and food requirements to sustain the population. A slight preference may exist for less dense environments. This could be because foraging may be easier (since the plant cover is more open) and the abundance of specialised rodent species, which compete with *Mastomys*, is decreased due to different requirements. The difference in preference for the veld inside the enclosure between *M. coucha* sampled in 2003 and 2004 is also evident in Fig. 3.2. In 2003 only 17.86% of *M. coucha* individuals were found in the area of veld inside the enclosure compared to 32% in 2004. *Mastomys coucha* is more prevalent in the fire break (39.29%) in 2003 than in 2004 (26%). There is a possibility that the fire break in 2003 provided more cover and food resources than the area would just after burning in 2004, because plant life had re-established from the last burning which was a few years earlier (approximately 1999). The area in 2003 was therefore used not only for foraging but also for shelter. In 2004 it was assumed that enough seed remained in the fire break zone to supply food but that most *Mastomys* individuals took cover in either the normal veld outside the enclosure or the veld inside the enclosure. Most captures in the fire break zone from August 2004 are attributed to animals that were foraging in the area. Foraging may have been easier in this area for the same reasoning as that for the normal veld outside.

Venturi *et al.* (2004) suggested that *M. coucha* is more likely to be affected by drought than *M. natalensis* due to the high potential evaporation rate (double that of *M. natalensis* sampling sites) and lower mean annual temperature of areas where *M. coucha* were located. The present results supports this statement as only 4% of *M. coucha* individuals were found in the sodic area, an area similar in structure to desert conditions. None of the samples from 2003 were sampled in the sodic area. In comparison 50% of *M. natalensis* individuals from both sampling times were captured in the sodic area probably due to this area being in close proximity to the vlei (Fig. 3.3). It must be mentioned however that this trend

of low abundance of *M. coucha* in the sodic area may be one of avoidance rather than preference. The ecological relevance of the difference between avoidance of some areas or preference for others needs to be investigated.



	MIDDLE LAND (A)	TOP LAND (B)	BOTTOM LAND (C)
M. coucha 2003	*28.57%*	*39.29%*	*32.14%*
M. natalensis 2003			*100%*
M. coucha 2004	*46%*	*32%*	*22%*
M. natalensis 2004			*100%*

Fig. 3.4 – Percentage association of *M. coucha* and *M. natalensis* with the slope (A/B/C) of capture (Fig. 1.11) in the Roan camp, KNP.

Mastomys coucha was most prevalent on the top land in 2003 (39.29%) and the middle land in 2004 (46%), although the species was found on each slope type at relatively equal levels (Fig. 3.4). This was attributed to the presence of various grass species which are found on each slope and are considered to provide food (seeds). The presence of *M. natalensis* found only on the bottom slope was discussed earlier.

Comparing the species to slope provided broad information on preference, with *M. coucha* found mostly where *M. natalensis* is not and visa versa. Each species

was therefore associated to the various types of plant species that were within a vegetation sample plot around the trap it was caught in. *Mastomys natalensis* was associated with eight different plant species compared to the 52 different plant species associated with *M. coucha* (Appendix B). Considering that 91% of the specimens were *M. coucha* and caught where literature (Skinner and Smithers, 1990; Venturi *et al*., 2004) expected them to be, the latter species has a wider habitat distribution and tolerance than *M. natalensis* in the Roan Camp. Eight of the 52 plant species associated with *M. coucha* were the same as that for *M. natalensis* although the percentage of *M. coucha* specimens associated with these plant species was lower than the percentage of *M. natalensis* (Fig. 3.5).

Although the sample size for *M. natalensis* was small, Fig. 3.5 shows that *M. natalensis* has the highest association (50% or more) with the following plant species for both sampling periods: *Andropogon* species and *Sporobolus* species while *M. coucha* is associated (60% or more) with *Panicum* species and *Setatia* species. These four genera likely provide food (seeds) to the respective species. It can therefore be assumed that *M. natalensis* and *M. coucha* have different preferences where food resources are concerned but further studies, including sympatric and allopatric areas, should be done to confirm these preferences.

De Graaff (1981) and Veenestra (1985) noted that the *Mastomys* genus was found in primary association with *Colophospermum mopane* scrub and woodland in Botswana and Zimbabwe, respectively. More than 25% *M. coucha* for both sampling periods and more than 25% *M. natalensis* for the 2004 sampling period were associated with *C. mopane* (Fig. 3.5). *Colophospermum mopane* is a tree or shrub used by *Mastomys* as cover, often using the crevices among roots or spaces under fallen logs. *Mastomys natalensis* is however found in high association (50%) with the aquatic sedge, *Cyperus*, and the semi-deciduous leadwood tree, *Combretum imberbe*. *Combretum imberbe* is one of the heaviest woods in the KNP and does not float thereby providing a stable structure in the environment. It together with *Cyprus* is common in low lying areas near streams

and water (Carruthers, 2000). Since *M. natalensis* is associated primarily with the flooded area they may use these plants to walk on to avoid the water. *Mastomys coucha* is associated (below 25%) with the same above mentioned plant species but they are in higher association with *Dalbergia melanoxylon*, the deciduous savannah zebrawood tree or shrub. As for *M. natalensis,* these plants are considered to be cover for *M. coucha* (they do not produce an appropriate food resource). The large difference in the number of plant species and vegetation preferences associated with *M. coucha* compared to *M. natalensis* as well as the difference in sample size leads to considering the population genetics of the two species, to test if factors such as large numbers of individuals, inbreeding, genetic drift or random mating cause more variation.

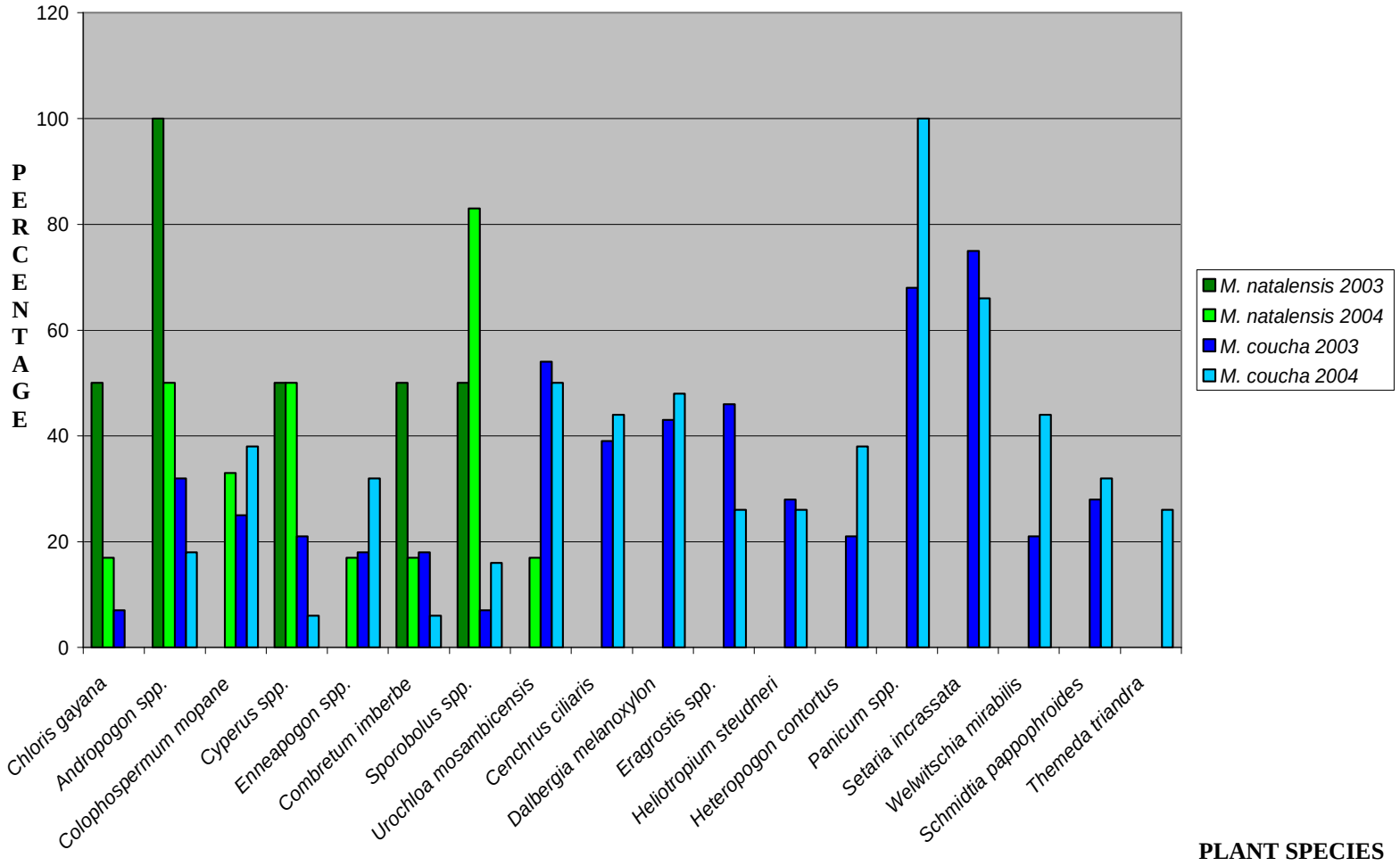


Fig. 3.5 – Bar chart showing the plant types most associated with each species for each sampling period. Each specimen was related to the different plant species found around the trap it was caught in. This table only shows *M. coucha* in relation to the plant species shared with *M. natalensis* as well as those plant species that show over 25% association with *M. coucha* during either sampling period (see Appendix B for a full table).

3.3. POPULATION GENETICS

Genetic variability within and between species are commonly expressed as the mean number of alleles per locus (A), percentage of polymorphic loci (P) and average heterozygosity (H). The genetic differentiation between species was determined with genetic distance (D), F_{st} and the gene flow (N_{em}). D is an estimate of the number of allelic substitutions per locus that have occurred since the two populations diverged and was obtained by using the genetic distances of Nei (1978), whereas F_{st} provides a weighted mean measure of the relatedness of individuals within populations by measuring the amount of differentiation relative to the limiting value for complete fixation according to the observed overall frequency (Wright, 1978). R_{st} is a statistic analogous to F_{st} and is the fraction of the total variance of allele size between populations (Slatkin, 1995). The N_{em} value was calculated from the F_{st} value (Takahata, 1983) and was calculated in RSTCALC using the R_{st} value.

3.3.1. VARIATION

The allele frequencies, heterozygote deficiency (d), Chi-square values (χ^2), degrees of freedom (DF) and expected heterozygosities obtained from the Biosys-1 analysis at each locus for each species are listed in Table 3.1. Deficiencies of heterozygotes were observed at all the loci for *M. coucha* and at three polymorphic loci, excluding MH 052, for *M. natalensis*. The deficiency at MH 146 for *M. coucha* was however very low with an expected Hardy-Weinberg proportion of 0.97. The deficit of heterozygotes in conjunction with the χ^2 values that exceed critical values are indicative of rare alleles present at these loci. Rare or private alleles occurred frequently in *M. coucha* (Table 3.1). Only one locus, MH 052, had a common B allele as the most abundant. At all other loci however, the most abundant alleles were different for each species. For example, at locus MH 141, the most abundant allele for the *M. coucha* population was A with a

frequency of 0.133 while for the *M. natalensis* population, alleles F and J were the most abundant each with a frequency of 0.333. Null alleles were found at each locus for *M. natalensis* but only locus MH 028 for *M. coucha* (Table 3.1). Three loci (MH 141, MH 060 and MH 028) had significant deviations of allele combinations from expected Hardy-Weinberg proportions for both species, as did locus MH 52 for *M. coucha* only. No fixed allele differences were found in the loci but private alleles were found at MH 146, MH 141, MH 052 and MH 060 in *M. coucha* and MH 028 in *M. natalensis*.

The A value for *M. coucha* was more than double that of *M. natalensis* at 9.2 (±2.1) and 4.0 (±1.1) respectively and the allelic diversity indicated a relatively high allelic diversity (0.7427) for the *M. coucha* population and a lower allelic diversity (0.478) for the *M. natalensis* population without adjustment of sample size. With adjustment of sample size the H_{TR} for the *M. coucha* population is 0.767 and 0.575 for the *M. natalensis* population. The P values were 100% and 80% and mean H values were 0.754 (±0.071) and 0.526 (±0.186) for *M. coucha* and *M. natalensis* respectively. As expected there was greater variation when a large number of individuals were studied and as suggested by the presence of more private alleles in *M. coucha*, this species had a greater P value than *M. natalensis.* Chi-square values deviate from the expected Hardy-Weinberg equilibrium and reasons for this may be the small sample size for *M. natalensis*, the presence of null alleles and/or the deficit of heterozygotes. Other factors such as inbreeding, mutation, migration and random genetic drift may also be present and/or mating within the population may not occur at random which contributes to deviations from Hardy-Weinberg equilibrium. The values, obtained from the five microsatellite loci, for A, P and H indicate a lower genetic variation for *M. natalensis* when compared to *M. coucha*. This is possibly due to the factors mentioned above on which Hardy-Weinberg equilibrium is not based. The H values were comparable to those obtained by Thomas *et al*. (2005) who studied two subspecies of house mice, *Mus musculus musculus* (H = 0.80) and *M. m. domesticus* (H = 0.83) as well as the old field mouse, *Peromyscus polionotus* (H =

0.76) studied by Mullen *et al.* (2005). Both studies made use of microsatellite markers, although not the same as the ones used in this study. The H values obtained by Thomas *et al.* (2005) were closer to one another because the same number of individuals per species was studied. Both Thomas *et al*. (2005) and Mullen *et al.* (2005) attributed the deviations from Hardy-Weinberg equilibrium to heterozygote deficiency which suggested the presence of null alleles or other factors as mentioned above.

The lower genetic variation for *M. natalensis* found in this study was opposite to the findings of Smit (2001) who stated that *M. natalensis* possesses more genetic variation than *M. coucha.* Smit's deduction for this trend was based on the greater range of biome types that the former species was captured in or because of the small sample size of one of the *M. coucha* populations. The same reasoning may hold true for this study because *M. natalensis* was captured in small numbers and *M. coucha* were found in a greater variety of locations. Smit (2001) however found less overall variation between species when comparing P and A values than obtained in this study. The P values were 7.3% and 13.1% in Smit (2001) compared to 100% and 80% for *M. coucha* and *M. natalensis* respectively. The A values were similar in each species (*M. natatensis* being 1.14 and *M. coucha* being 1.12) when compared to the measure for *M. coucha* being over double that of *M. natalensis* in this study. This difference was however not unexpected because Smit (2001) used allozyme and isozyme markers (nuclear DNA), which provide less variation.

Table 3.1 – Allele frequencies, heterozygote deficiency (d), χ^2, degrees of freedom (DF) and expected herterozygosity (h) for the five polymorphic loci in *M. coucha* and four polymorphic loci in *M. natalensis.*

LOCI	SPECIES	ALLELE FREQUENCY														d	χ^2	DF	h
		A	B	C	D	E	F	G	H	I	J	K	L	M	N				
MH146	*M. coucha*	0.450	0.550													-0.007	0	1	0.97
	M. natalensis	1.000																	
MH141	*M. coucha*	0.133	0.133	0.017	0.067	0.083	0.050	0.033	0.050	0.117	0.100	0.150	0.017	0.017	0.033	-0.344	223	91	0.00
	M. natalensis	0.083	-	-	0.083	-	0.334	-	-	0.083	0.334	-	-	0.083	-	-0.389	20	15	0.19
MH052	*M. coucha*	0.350	0.400	0.066	0.050	0.083	0.017	0.017	0.017							-0.207	37	28	0.13
	M. natalensis		0.917	-	-	-	0.083	-	-							0.000	0	1	1.00
MH060	*M. coucha*	0.316	0.117	0.083	0.133	0.067	0.100	0.017	0.017	0.050	0.017	0.033	0.033	0.017		-0.571	247	78	0.00
	M. natalensis	0.083	-	0.250	0.167	-	-	-	-	0.500	-	-	-	-		-0.766	19	6	0.00
MH028	*M. coucha*	0.241	0.052	0.378	0.103	0.017	0.034	0.069	0.086	0.017	-	-				-0.120	38	36	0.38
	M. natalensis	0.200	0.200	-	-	-	0.200	0.100	0.100	-	0.100	0.100				-0.357	31	21	0.07

Allelic diversity:

* indicates the most abundant alleles in the *Mastomys* species

* indicates loci where significant deviations of allele combinations from expected Hardy-Weinberg proportions occurred

3.3.2. DIFFERENTIATION

The mean pairwise F_{st} estimate was calculated between the two species. F_{st} is the proportion of total variation that is due to differences between subpopulations. If F_{st} is equal to one then subpopulations have no alleles in common and if it is zero then allele frequencies in all subpopulations are identical. Wright (1978) divided the F_{st} values into four categories each representing a degree of genetic differentiation between compared populations:

1) Values between 0 – 0.05 indicate little genetic differentiation
2) Values between 0.05 – 0.15 indicate moderate genetic differentiation
3) Values between 0.15 - 0.25 indicate great differentiation
4) Values greater than 0.25 indicate very great differentiation

Due to the high mutation rates of microsatellite loci compared to the low mutation rates for allozyme loci, Slatkin (1995) developed the R_{st} value which is interpreted in the same manner as F_{st}. R_{st} provides less biased estimates of "demographic parameters" for a population than that of F_{st} because where F_{st} assumes an infinite number of alleles, R_{st} assumes a generalized stepwise mutation process. The R_{st} value obtained for this study was 0.332 which indicates very great differentiation (P=0.001) between the two *Mastomys* species when compared to the F_{st} value of 0.126 which indicates a moderate genetic differentiation. Considering that R_{st} statistics are more appropriate for the analysis of microsatellite loci, this determination of differentiation will be accepted but F_{st} values were included in this study for comparison with other studies that did not use R_{st} values. Smit (2001) found F_{st} values between two populations of *M. coucha* and two populations of *M. natalensis* to be 0.558 and 0.228 respectively. Considering that species and not conspecific populations were compared in this study the difference was not unexpected. Morgan *et al*. (2003) used microsatellites to determine the population genetics of the house mouse. F_{st} was subdivided into F_{LG} and F_{GT} where F_{LG} corresponds to the probability that two randomly chosen alleles within the same line are identical by descent relative to

the particular selection group where they are nested, and F_{GT} corresponds to the probability that two randomly chosen alleles within the same selection group are identical by descent relative to the base population. Values for F_{LG} and F_{GT} were 0.149 and 0.001. The F_{LG} value obtained by Morgan *et al.* (2003) is comparable to the F_{st} value obtained in this study.

The D value is used to determine how genetically distinct the two species are from one another and can allow for the estimation of crude divergence times. A high D value indicates that the two species separated a long time ago. A value of 0.465 was obtained for the *M. natalensis* and *M. coucha* populations of the Roan Camp. This indicated that the two populations are genetically distinct and have been separated for some time. Literature that provides an indication of divergence times did not use microsatellites for their analyses. Data obtained from DNA-DNA hybridisation experiments (Chevret *et al*., 1994), chromosomal banding patterns (Britton-Davidian *et al*., 1995) and sequence data from the cytochrome-*b* gene and nuclear IRBP gene fragments (Lecompte *et al.,* 2005b) were used to produce phylogenies and the molecular clock hypothesis used to calculate the divergence times. Britton-Davidian *et al*. (1995) stated that the slight morphological divergence of the *Mastomys* genus was evidenced in east African fossil lineages that date back to 3.6 Mya and found that *M. natalensis* split form *M. erythroleucus* and *M. coucha* at Nei's genetic distance of approximately 0.1. Chevret *et al*. (1994) placed the divergence time between *M. natalensis* and *M. erythroleucus* at 0.3 Mya but was uncertain about the relation of *M. coucha.* Lecompte *et al*. (2005) places the divergence time between *M. coucha* and *M. erythroleucus* at 3.2 Mya whereas Chevret *et al*. (1994) positioned the divergence at 1 Mya. Considering that *M. coucha* is the sister group to *M. natalensis* (Britton-Davidian *et al*., 1995) the divergence time in the present study could be considered to be between 1 and 3.2 Mya. It is not uncommon for species belonging to the same genus or sibling species to have low morphological differentiation with high molecular differentiation (Lecompte *et al*., 2005b). Divergence between *Mus-Rattus/Praomys* occurred 10 Mya (Chevret *et al*., 1994; Lecompte *et al.,* 2005b)

and species from these genera are easily identified from one another. It can usually be speculated that the greater the divergence time the greater the morphological differences between species. However, Lecompte *et al.* (2005b) linked cases of low morphological differentiation with high molecular differentiation to savannah species as opposed to forest species which show higher morphological differentiation. *Mastomys natalensis* and *M. coucha* are found in savannah/grassland regions and therefore this factor could be applicable.

In comparison, the F_{st} and D values obtained by Smit (2001) were reversed with his study showing greater genetic variation and distinctness. A F*st* value of 0.68 (very great differentiation) and a D value of 0.123 was obtained by Smit (2001) when biochemical genetic markers were used to determine genetic variation between a *M. natalensis* population from Durban North, S.A. and a M*. coucha* population from Johannesburg, S.A. This may be because a larger variety of genetic markers was used or because the two populations studied by Smit (2001) were sampled from different ecozones, from locations found far apart. The *M. natalensis* and *M. coucha* species from this study were sampled from the same location.

The gene flow or N_{em} value was calculate between the populations using the F_{st} value. This value was 0.434. If a value of more than one was obtained it would mean that at least one individual from the first populations would have changed genes with an individual from the second population per generation and if the value was less than one, the lack of gene flow between populations would be evident. The value indicates that there was no gene flow between the two Roan Camp *Mastomys* populations which was expected as a previous study by Gordon (1984) showed that although hybrids could be produced in captivity none were found in allopatric or sympatric natural populations. The N_{em} value obtained using the R_{st} value was 0.503. This value is comparable to the N_{em} value of 0.434 obtained using the F_{st} value from this study.

CHAPTER 4

CONCLUSIONS AND CONTRIBUTION

CONCLUSIONS AND CONTRIBUTION

The aims of this study were met as addressed in the results and discussion section and the following conclusions can be made:

1. Use of PCR-RFLP identification technique:

The use of the PCR-RFLP based *Mastomys* spp. Identification technique was attained and therefore successful although the primer design and PCR reaction of the DNA isolations required amendment to obtain DNA sequences useful for the PCR-RFLP amplification and analysis. However some *M. coucha* isolates gave uncut PCR products due to a point mutation in the restriction enzyme site of BsmAI. It is therefore necessary to investigate alternative restriction enzymes or an alternative molecular method to more accurately identify the *Mastomys* spp.

2. Relation of *Mastomys* species abundance and distribution to vegetation:

Differences to broad geographic features do exist but both species were found in the same area. A difference in the type of vegetation preferred by each species was found based on their preferences for specific types of grasses. The difference in the type of vegetation structure where *M. natalensis* and *M. coucha* were found may be coincidental considering the robust nature of the results. This difference was likely to be caused by altitude and the amount of water present. For example, *M. natalensis* was caught only in the vlei area compared to 4% of *M. coucha* caught in the vlei area. The study further showed that *M. coucha* was more likely to be the pioneer species within the Roan Camp, KNP. This is the first study to verify that *M. coucha* is possibly the first to pioneer an area. Previous literature could only speculate and attributed *M. natalensis* by default and not science.

3. Population genetics and evolutionary trends

Microsatellites exhibit a wide array of genetic variability with *M. coucha* having more genetic variation when compared to *M. natalensis* within the Roan Camp. A possible reason for the differences in genetic variation is the rapid evolutionary radiation of rodent species. It is thought that a chromosomal mutation in *M. natalensis* gave rise to the younger *M. coucha* which became reproductively isolated (Qumsiyeh *et al*., 1990). The results from this study further indicated the two species had significant differentiation based on R_{st} values and that the two species separated some time ago (approximately 1-3.2 Mya) with a D value of 0.465.

3. The study is the first to:

- determine the positive identification of the two species using ethanol preserved toe clippings,
- resolve which of the two species is the most likely to be the first pioneer in an area after it was disturbed by fire,
- compare the plant species preferences of the two *Mastomys* species using a sympatric population,
- report on private alleles which could possibly be used to identify the species,
- determine genetic variation within *M. natalensis* and *M. coucha* using microsatellite loci for the two species,
- determine the genetic differentiation between the two species using microsatellite loci for the two species, and
- estimate divergence times between *M. natalensis* and *M. coucha* using microsatellite loci for the two species.

The method of identification used in this study proves to be reliable and has the advantages of being easy to do and using tissue samples that are accessible without requiring the death of the specimen. The techniques for the identification

and microsatellite analyses are described herein and will be useful in future field studies. These above mentioned aspects are extremely important due to the medical significance of both species (each carries a different disease pathogen) and most environmental impact studies can aspire, and are now able to, identify the species. *Mastomys natalensis* and *M. coucha* therefore prove to be interesting sibling species for analysis considering their importance and the differences seen in this study and others. Although the population genetics shown here are not conclusive because they are opposite to the report by Smit *et al*. (2001), the study contributed to the global information on species identification, ecology and genetic variation of the genus. This is exciting because these factors are the three pillars on which the Convention of Biological Diversity rest, of which South Africa is a signatory.

Recommendations for further studies should include the genetic comparison of different populations of the same species using microsatellites as well as vegetation surveys to further determine and define the habitat preferences of each species. A location better adapted to *M. natalensis* could also be studied in the same manner to see if the trends in this study are maintained or reversed based on geographical features and different selection pressures. Further research should also be done into the possibility of using the private alleles discovered between the species as a method of identification.

CHAPTER 5

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APPENDIXES

APPENDIXES

APPENDIX A1 – INPUT FILES FOR BIOSYS 1

Genetic differences between different populations of MICE
NOTU=2, NLOC=5,NALL=19,CRT;
(5(1X,A5))
MH146 MH141 MH052 MH060 MH028

STEP DATA:
DATYP=2,NCOL=22,ALPHA;
(6X,22(1X,2A1,1X,I2))
ONE COUCHA
MH146 AA:06 AB:15 BB:09
MH141 AA:02 BB:02 BC:01 BD:01 AE:01 DE:01 DF:01 EF:01 GG:01 BH:01 HH:01 FI:01 II:03 JJ:02 KA:03 EK:02 KJ:02 KK:01 LM:01 BN:01 DN:01
MH052 AA:05 AB:07 BB:06 BC:01 CC:01 BD:01 DD:01 AE:02 BE:02 CE:01 AF:01 BG:01 AH:01
MH060 AA:05 AB:02 BB:02 AC:01 CC:02 AD:02 DD:03 AE:01 EE:01 FF:03 AG:01 AH:01 BI:01 II:01 AJ:01 KK:01 LL:01 EM:01
MH028 AA:04 AB:02 AC:01 CC:05 AD:01 CD:05 CE:01 AF:01 BF:01 CG:02 AH:01 CH:02 GH:02 CI:01
TWO NATALENSIS
MH146 AA:06
MH141 AF:01 DF:01 FF:01 JJ:02 IM:01
MH052 BB:05 BF:01
MH060 AC:01 CC:01 DD:01 II:03
MH028 AA:01 BB:01 FG:01 HJ:01 FK:01
END;
STEP VARIAB:
END;
STEP HDYWBG:
LEVENE;
END;
STEP SIMDIS:
ALLCOEF;
END;
STEP COEFOUT:
BELOW=2;
BELOW=4;
END;
STP HIERARCHY:
NLEVEL=1;
SPECIES
LOCALITY

```
SCHILBE
EUTROPIUS
END;
STP SIMAVE:
COEF=4, COEF=2;
END;
STP DISTRIB:
COEF=11;
END;
STEP FSTAT:
OUTPUT=3;
END;
STP WRIGHT78:
END;
STEP HETXSQ:
END;
TEP CLUSTER:
COEF=2;
COEF=4;
END;
STP DISWAG:
COEF=11, ROOT=1, PLASOUT;
END;
```

APPENDIX A2 – INPUT FILE FOR RSTCALC

```
Christine
5
30
2
30
6
MH1
2
42
MH2
2
39
MH3
2
41
MH4
2
44
MH5
2
42
POP
 101 103  250 250  091 091  159 171  244 252
 101 101  246 260  081 089  187 187  246 256
 103 103  246 246  091 091  169 169  244 244
 101 101  252 272  081 091  171 171  246 256
 101 103  258 272  081 081  167 167  240 246
 101 103  238 238  081 093  193 193  252 260
 101 103  250 272  081 081  159 159  244 246
 101 103  242 246  081 081  193 193  246 254
 101 103  248 248  091 091  159 179  246 262
 101 103  248 248  089 089  159 159  244 260
 101 103  254 254  091 097  159 159  246 254
 101 103  244 244  089 097  149 159  246 256
 101 103  244 254  091 097  159 169  242 246
 103 103  244 272  093 093  169 169  246 246
 101 103  244 272  091 091  193 193  244 256
 101 103  244 254  091 101  159 169  244 244
 101 103  246 252  083 091  159 167  244 262
 101 101  240 262  091 081  171 183  244 244
 101 103  248 248  091 091  159 181  244 244
 101 103  248 258  081 081  159 159  246 256
```

103 103 246 256 081 091 183 183 246 246
103 103 242 242 081 091 171 171 246 246
103 103 252 258 081 081 173 173 246 246
101 101 244 250 081 091 176 176 246 262
103 103 244 250 081 091 187 187 000 000
103 103 254 254 081 097 169 169 246 256
101 101 252 256 081 097 159 171 246 246
101 101 250 250 081 081 159 159 254 262
103 103 244 250 081 091 159 187 244 252
103 103 246 246 081 099 167 180 254 262
POP
101 101 258 258 081 083 183 183 254 260
101 101 254 254 081 081 183 183 262 266
101 101 252 258 081 081 169 169 252 252
101 101 248 262 081 081 187 187 250 260
101 101 250 258 081 081 159 187 000 000
101 101 254 254 081 081 183 183 244 244

APPENDIX B – PLANT SPECIES ASSOCIATION

Table A -1 – Plant species associated to *M. natalensis* and *M. coucha*

	% MICE ASSOCIATED WITH PLANT SPECIES			
	M. natalensis 2003	*M. natalensis 2004*	*M. coucha 2003*	*M. coucha 2004*
PLANT SPECIES				
Acacia nigrescens	0	0	4	6
Albizia harveyi	0	0	0	6
Andropogon spp	100	50	32	18
Aristida spp	0	0	29	26
Asparagus spp	0	0	4	4
Aspilia spp	0	0	4	8
Blepharis spp	0	0	4	2
Capparis spp	0	0	0	12
Cenchris ciliaris	0	0	39	44
Ceratotheca triloba	0	0	18	4
Chamaecrista spp	0	0	0	2
Chloris gayana	50	17	7	0
Clerodendrum ternatum	0	0	0	2
Colophospermum mopane	0	33	25	38
Combretum spp	50	17	18	10
Commicarpus spp	0	0	4	4
Cyperus spp	50	50	21	18
Dalbergia melanoxylon	0	0	42	48
Dichrostachys cinerea	0	0	4	6
Ehretia amoena	0	0	7	4
Enneapogon spp	0	17	18	32
Eragrostis spp	0	0	46	26
Grewia monticola	0	0	0	2
Helichrysum kraussii	0	0	0	4
Heliotropium steuderi	0	0	29	26
Hermannia boraginiflor	0	0	7	12
Heteropogon contortus	0	0	21	38
Indigofera spp	0	0	4	4
Kyphocarpa angustifoli	0	0	4	18
Lonchocarpus capassa	0	0	21	24
Maytenus spp	0	0	4	4
Merremia palmata	0	0	18	14
Mesembryanthemum	0	0	4	2

spp				
Oenothera biennis	0	0	0	2
Ormocarpum trichocarpu	0	0	7	2
Panicum spp	0	0	68	100
Phullanthus spp	0	0	0	2
Poa spp	0	0	0	6
Rhynchosia spp	0	0	18	20
Schmidia pappaphoroides	0	0	29	32
Sclerocarya birrea	0	0	0	2
Sesbania spp	0	0	25	12
Setaria spp	0	0	75	70
Soanum panduriforme	0	0	0	2
Sorgum spp	0	0	0	2
Sporobolus spp	50	83	7	14
Thecacoris trichogyne	0	0	0	6
Themeda triandra	0	0	14	26
Tragia spp	0	0	4	8
Urochloa mosambicensis	0	17	54	50
Vernonia poskeana	0	0	21	16
Welwitschia mirabilis	0	0	21	44

APPENDIX C – STATISTICAL TABLES FOR HABITAT ASSOCIATION

Table A-2 – Statistical analysis using the SPSS processor associating the mouse species to different habitat types sampled inside and around the Roan Camp, KNP

		2003		2004	
		COUCHA	***NATALENSIS***	**COUCHA**	***NATALENSIS***
NORMAL VELD	COUNT	12	0	19	2
	% WITHIN SPP	42.86%	0%	38.00%	33.00%
FIRE BREAK	COUNT	11	0	13	0
	% WITHIN SPP	39.29%	0%	26.00%	0%
VELD INSIDE	COUNT	5	1	16	1
	% WITHIN SPP	17.86%	50%	32.00%	16.70%
SODIC AREA INSIDE	COUNT	0	1	2	3
	% WITHIN SPP	0%	50%	4.00%	50%
	TOTAL	28	2	50	6

Table A-3 – Statistical analysis using the SPSS processor associating the mouse species to the slope type (A/B/C) at the Roan Camp, KNP

		2003		2004	
		COUCHA	***NATALENSIS***	**COUCHA**	***NATALENSIS***
MIDDLE LAND (A)	COUNT	8	0	23	0
	% WITHIN SPP	28.57%	0%	46%	0%
TOP LAND (B)	COUNT	11	0	16	0
	% WITHIN SPP	39.29%	0%	32%	0%
BOTTOM LAND (C)	COUNT	9	2	11	6
	% WITHIN SPP	32.14%	100%	22%	100%
	TOTAL	28	2	50	6

APPENDIX D – MICROSATELLITE ANALYSIS

Table A-4 – Analysis of the Microsatellite fragment profiles. Numbers represent the length of the fragment.

		LOCUS									
		MH146		**MH141**		**MH52**		**MH60**		**MH28**	
SAMPLE	**MC5**	101	103	250	250	91	91	159	171	244	252
	MC9	101	101	246	260	81	89	187	187	246	256
	MC10	103	103	246	246	91	91	169	169	244	244
	MC13	101	101	252	272	81	91	171	171	246	256
	MC15	101	103	258	272	81	81	167	167	240	246
	MC18	101	103	238	238	81	93	193	193	252	260
	MC22	101	103	250	272	81	81	159	159	244	246
	MC24	101	103	242	246	81	81	193	193	246	254
	MC26	101	103	248	248	91	91	159	179	246	262
	MC31	101	103	248	248	89	89	159	159	244	260
	MC33	101	103	254	254	91	97	159	159	246	254
	MC35	101	103	244	244	89	97	149	159	246	256
	MC37	101	103	244	254	91	97	159	169	242	246
	MC43	103	103	244	272	93	93	169	169	246	246
	MC44	101	103	244	272	91	91	193	193	244	256
	MC48	101	103	244	254	91	101	159	169	244	244
	MC51	101	103	246	252	83	91	159	167	244	262
	MC54	101	101	240	262	91	81	171	183	244	244
	MC55	101	103	248	248	91	91	159	181	244	244
	MC60	101	103	248	258	81	81	159	159	246	256
	MC4	103	103	246	256	81	91	183	183	246	246
	MC16	103	103	242	242	81	91	171	171	246	246
	MC17	103	103	252	258	81	81	173	173	246	246
	MC25	101	101	244	250	81	91	176	176	246	262
	MC29	103	103	244	250	81	91	187	187		
	MC39	103	103	254	254	81	97	169	169	246	256
	MC42	101	101	252	256	81	97	159	171	246	246
	MC46	101	101	250	250	81	81	159	159	254	262
	MC49	103	103	244	250	81	91	159	187	244	252
	MC58	103	103	246	246	81	99	167	180	254	262
	MN7	101	101	258	258	81	83	183	183	254	260
	MN12	101	101	254	254	81	81	183	183	262	266
	MN28	101	101	252	258	81	81	169	169	252	252
	MN34	101	101	248	262	81	81	187	187	250	260
	MN38	101	101	250	258	81	81	159	187		
	MN53	101	101	254	254	81	81	183	183	244	244

APPENDIX E1 – BLAST SEARCH FOR MASTOMYS COUCHA SAMPLE

BLAST search of sequenced *Mastomys* sample collected in Roan camp that was identified as *M. coucha* using PCR-RFLP (indicated as Query). Highest homology to *M. coucha* AD115 (AY554160, collected in Beitbridge, S.A.; indicated as Sbjct).

Mastomys coucha voucher AD115 cytochrome b gene, partial cds;
Mitochondrial
Length=1070

Score = 1293 bits (700), Expect = 0.0
Identities = 728/741 (98%), Gaps = 3/741 (0%)
Strand=Plus/Minus

```
Query  11    AGTAGACTACTCTTCGATGATTCCTGAGATAGGTATTAAGATGAGAATAATAGAGAAGTA  70
             ||||||  || ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1070  AGTAGATTA-TCTTCGATGATTCCTGAGATAGGTATTAAGATGAGAATAATAGAGAAGTA  1012

Query  71    ACTGATTGATGCTAGTTGGCCGATAATGATGAATGGGTGTTCTACTGGTTGGCCTCCAAT  130
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1011  ACTGATTGATGCTAGTTGGCCGATAATGATGAATGGGTGTTCTACTGGTTGGCCTCCAAT  952

Query  131   TCAGGTTAGTACTAGTAGGTTTGCTACTAAGATTCAGTAAAGAATTTGTGTGATTGGGCG  190
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  951   TCAGGTTAGTACTAGTAGGTTTGCTACTAAGATTCAGTAAAGAATTTGTGTGATTGGGCG  892

Query  191   AAATGTGAGGCTTCGTTGTTTTGAGGTGTGGAGTAATGGTATTAGTGCTAGGATTAGGAT  250
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  891   AAATGTGAGGCTTCGTTGTTTTGAGGTGTGGAGTAATGGTATTAGTGCTAGGATTAGGAT  832

Query  251   TGAAAGCGATCAGGGCTAGCACTCCTCCTAGTTTGTTAGGGATAGATCGTAGGATGGCGT  310
             ||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  831   TGAAAG-GATCAGGGCTAGCACTCCTCCTAGTTTGTTAGGGATAGATCGTAGGATGGCGT  773

Query  311   AGGCAAAGAGGAAATATCATTCTGGTTTAATATGGGGAGGGGTATTAAGTGGGTTAGCTG  370
             |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  772   AGGCAAAGAGGAAATATCATTCTGGTTTAATATGGGGAGGGGTATTTAGTGGGTTAGCTG  713

Query  371   GTGTATAATTGTCTGGGTCTCCTAGTAAGTCTGGGAAAAATAGTACTAGTGTCATTAGGA  430
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  712   GTGTATAATTGTCTGGGTCTCCTAGTAAGTCTGGGAAAAATAGTACTAGTGTCATTAGGA  653

Query  431   ATACAATTATTATGATAATTCCGAGAATGTCTTTAATTGTGTAGTACGGGTGAAATGGAA  490
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  652   ATACAATTATTATGATAATTCCGAGAATGTCTTTAATTGTGTAGTACGGGTGAAATGGAA  593

Query  491   TTTTGTCTGAGTCAGAGTTTAGGCCTGTTGGGTAATTGGATCCTGTTTCATGGAGGAATA  550
             |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  592   TTTTGTCTGAGTCAGAGTTTAGGCCTGTTGGGTTATTGGATCCTGTTTCATGGAGGAATA  533

Query  551   AAAGGTGGACGATGACTAGGGCTGCGATATATAAATGGTAGGATGAAGTGGAATGCGAAG  610
             |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  532   AAAGGTGGACGATGACTAGGGCTGCGATA-ATAAATGGTAGGATGAAGTGGAATGCGAAG  474

Query  611   AAGCGTGTTAGTGTTGCTTTATCTCCTGAGAATCCTCCTCAGATTCATTCTACTAGTGAA  670
             |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||| |
Sbjct  473   AAGCGTGTTAGTGTTGCTTTATCTACTGAGAATCCTCCTCAGATTCATTCTACTAGTGTA  414

Query  671   GTTCCAATGTAAGGGATTGCTGAGAGAAAGTTTGAAATGACTGAAGCCCCTCAGAATGAT  730
             |||||||||||||||||||||||| ||||||| ||||||||  ||||||||||||||||
Sbjct  413   GTTCCAATGTAAGGGATTGCTGAGAGTAAGTTTGTAATGACTGTTGCCCCTCAGAATGAT  354

Query  731   TTTTGTCCTCATGGAAGTACG  751
              ||||||||||||||||||||
Sbjct  353   ATTTGTCCTCATGGAAGTACG  333
```

APPENDIX E2 – BLAST SEARCH FOR MASTOMYS NATALENSIS SAMPLE

BLAST search of sequenced *Mastomys* sample collected in Roan camp that was identified as *M. natalensis* using PCR-RFLP (indicated as Query). Highest homology to *M. natalensis* AD65 (AY554149, collected in Richards Bay, S.A.; indicated as Sbjct)

Mastomys natalensis voucher AD65 cytochrome b gene, partial cds; mitochondrial
Length=1070

Score = 1336 bits (723), Expect = 0.0
Identities = 740/748 (98%), Gaps = 2/748 (0%)
Strand=Plus/Minus

```
Query  5     GTTGTCCTTCGAATAATTCCTGAGATAGGTATCAGGATAAGGATAATAGAGAAGTAACTG  64
             ||||| ||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1065  GTTGT-CTTCG-ATAATTCCTGAGATAGGTATCAGGATAAGGATAATAGAGAAGTAACTG  1008

Query  65    ATTGATGCTAGTTGGCCAATAATGATAAATGGATGTTCTACTGGTTGGCCTCCGATTCAA  124
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1007  ATTGATGCTAGTTGGCCAATAATGATAAATGGATGTTCTACTGGTTGGCCTCCGATTCAA  948

Query  125   GTTAGTACTAGTAGGTTTGCTACTAAGATTCAGTAAAGAATTTGTGTGATTGGACGGAAT  184
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  947   GTTAGTACTAGTAGGTTTGCTACTAAGATTCAGTAAAGAATTTGTGTGATTGGACGGAAT  888

Query  185   GTGAGACTTCGTTGTTTTGAGGTGTGGAGTAGGGGTATTAGTGCTAGGATAAGGATGGAA  244
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  887   GTGAGACTTCGTTGTTTTGAGGTGTGGAGTAGGGGTATTAGTGCTAGGATAAGGATGGAA  828

Query  245   AGGATTAGGGCTAGGACTCCTCCTAGTTTATTAGGGATGGATCGTAGGATGGCATAGGCA  304
             ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  827   AGGATTAGGGCTAGGACTCCTCCCAGTTTATTAGGGATGGATCGTAGGATGGCATAGGCA  768

Query  305   AAGAGGAAATATCATTCTGGTTTGATATGGGGAGGTGTGTTGAGTGGGTTGGCTGGTGTA  364
             |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  767   AAGAGGAAGTATCATTCTGGTTTGATATGGGGAGGTGTGTTGAGTGGGTTGGCTGGTGTA  708

Query  365   TAGTTGTCGGGGTCTCCTAGTAAGTCTGGAAAAAATAGTACTAGAATTATTAGGAGGGCA  424
             ||||||||||||||||||||||| ||||||||||||||||||||||||||||||| |||
Sbjct  707   TAGTTGTCGGGGTCTCCTAGTAAATCTGGAAAAAATAGTACTAGAATTATTAGGAGAGCA  648

Query  425   ATTATTAAGATAATTCCAAGAATGTCTTTAATTGTATAGTACGGGTGGAATGGGATTTTA  484
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  647   ATTATTAAGATGATTCCAAGAATGTCTTTAATTGTATAGTACGGGTGGAATGGGATTTTG  588

Query  485   TCTGAGTCAGAGTTTAAGCCCGTTGGGTTATTAGAGCCCGTTTCGTGAAGAAATAGAAGG  544
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  587   TCTGAGTCAGAGTTTAAGCCCGTTGGGTTATTAGAGCCCGTTTCGTGAAGAAATAGAAGG  528

Query  545   TGGACGATGACTAGGGCTGCGATAATGAATGGGAGGATGAAGTGAAATGCGAAAAAGCGT  604
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  527   TGGACGATGACTAGGGCTGCGATAATGAATGGGAGGATGAAGTGAAATGCGAAAAAGCGT  468

Query  605   GTTAGTGTTGCTTTATCTACTGAGAATCCTCCTCAGATTCATTCTACTAATGTAGTTCCA  664
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  467   GTTAGTGTTGCTTTATCTACTGAGAATCCTCCTCAGATTCATTCTACTAATGTAGTTCCA  408

Query  665   ATGTATGGGACTGCTGAGAGTAAGTTTGTAATGACTGTTGCTCCTCAGAATGATATTTGT  724
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  407   ATGTATGGGACTGCTGAGAGTAAGTTTGTAATGACTGTTGCTCCTCAGAATGATATTTGT  348

Query  725   CCTCATGGAAGTACATAGCCTATGAATG  752
             |||||||||||||||||||||||||||
Sbjct  347   CCTCATGGAAGTACATAGCCTATGAATG  320
```

APPENDIX F1 – ALIGNMENT OF TWO MASTOMYS COUCHA SPECIMENS FROM GENBANK AND THE MASTOMYS COUCHA SAMPLE USING CLUSTALW

(Mismatches must be verified)

Multiple alignment of AF518334.1, *M. coucha* MNHN 1999-104 sample from S.A.: Bloemfontein (Lecompte *et al*., 2002), and AY554160.1, *M. coucha* AD115 sample from S.A.: Beitbridge, which is aligned with *Mastomys* sample from S.A.: KNP that was identified as *M. coucha* using PCR-RFLPs. The asterisks (*) indicated identical nucleotide at specific position of all three samples. The *Bsm*AI restriction site is indicated with green highlight.

```
CLUSTAL W (1.83) multiple sequence alignment


gi|25814881|gb|AF518334.1|      ATGACAAACATCNNAAAAACTCACCCACTAGTCAAAATTATTAACCACTC
gi|47834426|gb|AY554160.1|      --------------------------------------------------
Mastomys coucha sample          --------------------------------------------------


gi|25814881|gb|AF518334.1|      ATTCATCGACTTACCTGCCCCATCCAATATTTCATCATGATGAAACTTCG
gi|47834426|gb|AY554160.1|      ----------TTACCTGCCCCATCCAATATTTCATCATGATGAAACTTCG
Mastomys coucha sample          --------------------------------------------------


gi|25814881|gb|AF518334.1|      GCTCACTATTAGGAATCTGCCTAATAATTCAAATCATCACAGGCCTATTC
gi|47834426|gb|AY554160.1|      GCTCACTATTAGGAATCTGCCTAATAATTCAAATCACCACAGGCCTATTC
Mastomys coucha sample          --------------------------------------------------


gi|25814881|gb|AF518334.1|      CTAGCAATACACTACACATCAGATACTATAACAGCATTCTCATCAGTAAC
gi|47834426|gb|AY554160.1|      CTAGCAATACACTACACATCAGATACTATAACAGCATTCTCATCAGTAAC
Mastomys coucha sample          --------------------------------------------------


gi|25814881|gb|AF518334.1|      CCATATCTGCCGAGATGTAAACTACGGATGAATAATCCGATATATACACG
gi|47834426|gb|AY554160.1|      CCATATCTGCCGAGATGTAAACTATGGATGAATAATCCGATATATACACG
Mastomys coucha sample          --------------------------------------------------


gi|25814881|gb|AF518334.1|      CAAATGGAGCATCAATATTTTTTATCTGCTTGTTCCTTCACGTAGGACGG
gi|47834426|gb|AY554160.1|      CAAACGGAGCATCAATATTTTTTATCTGCTTGTTCCTTCACGTAGGACGG
Mastomys coucha sample          --------------------------------------------------


gi|25814881|gb|AF518334.1|      GGAATATATTACGGATCCTACACATTCACAGAAACATGAAATATTGGAGT
gi|47834426|gb|AY554160.1|      GGGATATATTACGGATCCTACACATTCACAGAAACATGAAATATTGGAGT
Mastomys coucha sample          --------------------------------------------------


gi|25814881|gb|AF518334.1|      AGTCTTACTTTTCGCAGTAATAGCAACCGCATTCATAGGCTACGTACTTC
gi|47834426|gb|AY554160.1|      AGTCTTACTTTTCGCAGTAATAGCAACCGCATTCATAGGCTACGTACTTC
Mastomys coucha sample          -------------------------------------------CGTACTTC
                                                                            ********


gi|25814881|gb|AF518334.1|      CATGAGGACAAATATCATTCTGAGGGGCAACAGTCATTACAAACTTACTC
gi|47834426|gb|AY554160.1|      CATGAGGACAAATATCATTCTGAGGGGCAACAGTCATTACAAACTTACTC
Mastomys coucha sample          CATGAGGACAAAAATCATTCTGAGGGGCTTCAGTCATTTCAAACTTTCTC
                                ************ ***************  ******** ******* ***


gi|25814881|gb|AF518334.1|      TCAGCAATCCCTTACATTGGAACTACACTAGTAGAATGAATCTGAGGAGG
gi|47834426|gb|AY554160.1|      TCAGCAATCCCTTACATTGGAACTACACTAGTAGAATGAATCTGAGGAGG
Mastomys coucha sample          TCAGCAATCCCTTACATTGGAACTTCACTAGTAGAATGAATCTGAGGAGG
                                ************************ ************************
```

```
gi|25814881|gb|AF518334.1|      ATTCTCAGTAGATAAAGCAACACTAACACGCTTCTTCGCATTCCACTTCA
gi|47834426|gb|AY554160.1|      ATTCTCAGTAGATAAAGCAACACTAACACGCTTCTTCGCATTCCACTTCA
Mastomys coucha sample          ATTCTCAGGAGATAAAGCAACACTAACACGCTTCTTCGCATTCCACTTCA
                                ******** ****************************************

gi|25814881|gb|AF518334.1|      TCCTACCATTTAT-TATCGCAGCCCTAGTCATCGTCCACCTTTTATTCCT
gi|47834426|gb|AY554160.1|      TCCTACCATTTAT-TATCGCAGCCCTAGTCATCGTCCACCTTTTATTCCT
Mastomys coucha sample          TCCTACCATTTATATATCGCAGCCCTAGTCATCGTCCACCTTTTATTCCT
                                ************* ***********************************

gi|25814881|gb|AF518334.1|      CCATGAAACAGGATCCAATAACCCAACAGGCCTAAACTCTGACTCAGACA
gi|47834426|gb|AY554160.1|      CCATGAAACAGGATCCAATAACCCAACAGGCCTAAACTCTGACTCAGACA
Mastomys coucha sample          CCATGAAACAGGATCCAATTACCCAACAGGCCTAAACTCTGACTCAGACA
                                ******************* ****************************

gi|25814881|gb|AF518334.1|      AAATTCCATTTCACCCGTACTACACAATTAAAGACATTCTCGGAATTATC
gi|47834426|gb|AY554160.1|      AAATTCCATTTCACCCGTACTACACAATTAAAGACATTCTCGGAATTATC
Mastomys coucha sample          AAATTCCATTTCACCCGTACTACACAATTAAAGACATTCTCGGAATTATC
                                **************************************************

gi|25814881|gb|AF518334.1|      ATAATAATTGTATTCCTAATGACACTAGTACTATTTTTCCCAGACTTACT
gi|47834426|gb|AY554160.1|      ATAATAATTGTATTCCTAATGACACTAGTACTATTTTTCCCAGACTTACT
Mastomys coucha sample          ATAATAATTGTATTCCTAATGACACTAGTACTATTTTTCCCAGACTTACT
                                **************************************************

gi|25814881|gb|AF518334.1|      AGGAGACCCAGACAATTATACACCAGCTAACCCACTAAATACCCCTCCCC
gi|47834426|gb|AY554160.1|      AGGAGACCCAGACAATTATACACCAGCTAACCCACTAAATACCCCTCCCC
Mastomys coucha sample          AGGAGACCCAGACAATTATACACCAGCTAACCCACTTAATACCCCTCCCC
                                ********************************** *************

gi|25814881|gb|AF518334.1|      ATATTAAACCAGAATGATATTTCCTCTTTGCCTACGCCATCCTACGATCT
gi|47834426|gb|AY554160.1|      ATATTAAACCAGAATGATATTTCCTCTTTGCCTACGCCATCCTACGATCT
Mastomys coucha sample          ATATTAAACCAGAATGATATTTCCTCTTTGCCTACGCCATCCTACGATCT
                                **************************************************

gi|25814881|gb|AF518334.1|      ATCCCTAACAAACTAGGAGGAGTGCTAGCCCTGATC-CTTTCAATCCTAA
gi|47834426|gb|AY554160.1|      ATCCCTAACAAACTAGGAGGAGTGCTAGCCCTGATC-CTTTCAATCCTAA
Mastomys coucha sample          ATCCCTAACAAACTAGGAGGAGTGCTAGCCCTGATCGCTTTCAATCCTAA
                                ********************************** *************

gi|25814881|gb|AF518334.1|      TCCTAGCACTAATACCATTACTCCACACCTCAAAACAACGAAGCCTCACA
gi|47834426|gb|AY554160.1|      TCCTAGCACTAATACCATTACTCCACACCTCAAAACAACGAAGCCTCACA
Mastomys coucha sample          TCCTAGCACTAATACCATTACTCCACACCTCAAAACAACGAAGCCTCACA
                                **************************************************

gi|25814881|gb|AF518334.1|      TTTCGCCCAATCACACAAATTCTTTACTGAATCTTAGTAGCAAACCTACT
gi|47834426|gb|AY554160.1|      TTTCGCCCAATCACACAAATTCTTTACTGAATCTTAGTAGCAAACCTACT
Mastomys coucha sample          TTTCGCCCAATCACACAAATTCTTTACTGAATCTTAGTAGCAAACCTACT
                                **************************************************

gi|25814881|gb|AF518334.1|      AGTACTAACCTGAATTGGAGGCCAACCAGTAGAACACCCATTCATCATTA
gi|47834426|gb|AY554160.1|      AGTACTAACCTGAATTGGAGGCCAACCAGTAGAACACCCATTCATCATTA
Mastomys coucha sample          AGTACTAACCTGAATTGGAGGCCAACCAGTAGAACACCCATTCATCATTA
                                **************************************************

gi|25814881|gb|AF518334.1|      TCGGCCAACTAGCATCAATCAGTTACTTCTCTATNATTCTCATCTTAATA
gi|47834426|gb|AY554160.1|      TCGGCCAACTAGCATCAATCAGTTACTTCTCTATTATTCTCATCTTAATA
Mastomys coucha sample          TCGGCCAACTAGCATCAATCAGTTACTTCTCTATTATTCTCATCTTAATA
                                ********************************** ***************

gi|25814881|gb|AF518334.1|      CCTATCTCAGGAATCATCGAAGA-TAATCTACTCAAATGAAA-
gi|47834426|gb|AY554160.1|      CCTATCTCAGGAATCATCGAAGA-TAATCTACT----------
Mastomys coucha sample          CCTATCTCAGGAATCATCGAAGAGTAGTCTACTCTCACAATGA
                                ********************** ** ******
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APPENDIX F2 – ALIGNMENT OF A MASTOMYS NATALENSIS SPECIMEN FROM GENBANK AND THE MASTOMYS NATALENSIS SAMPLE USING CLUSTALW

(Mismatches must be verified)

Multiple alignment of AY751296.1, *M. natalensis* isolate 1999-342 from Tanzania: Berega (Lecompte *et al.,* 2005a), which is aligned with *Mastomys* sample from S.A.: KNP that was identified as *M. natalensis* using PCR-RFLPs. The asterisks (*) indicated identical nucleotide at specific position of both samples (however mismatches must be verified by repeating the sequencing). The *Bsm*AI restriction site is indicated with green highlight.

```
gi|58614535|gb|AY751296.1|      ATGACAAACATACGAAAAACTCACCCACTACTCAAAATTATCAACCACTC
Mastomys natalensis sample      --------------------------------------------------

gi|58614535|gb|AY751296.1|      ATTCATTGACTTACCTGCCCCATCCAATATCTCATCATGATGAAACTTCG
Mastomys natalensis sample      --------------------------------------------------

gi|58614535|gb|AY751296.1|      GCTCTCTATTAGGAATTTGCCTAATAATCCAAATCATCACAGGTTTATTC
Mastomys natalensis sample      --------------------------------------------------

gi|58614535|gb|AY751296.1|      CTGGCAATACACTACACATCAGACACTATAACAGCATTCTCATCAGTAAC
Mastomys natalensis sample      --------------------------------------------------

gi|58614535|gb|AY751296.1|      ACATATCTGCCGAGACGTAAATTATGGATGAGTAATCCGATATATACACG
Mastomys natalensis sample      --------------------------------------------------

gi|58614535|gb|AY751296.1|      CAAATGGAGCATCAATATTTTTTATTTGCTTATTCCTTCATGTAGGACGA
Mastomys natalensis sample      --------------------------------------------------

gi|58614535|gb|AY751296.1|      GGAATATACTATGGATCCTACACATTTACAGAAACATGAAATATTGGAGT
Mastomys natalensis sample      --------------------------------------------------

gi|58614535|gb|AY751296.1|      GGTCCTACTCTTCGCAGTTATAGCAACCGCATTCATAGGCTATGTACTTC
Mastomys natalensis sample      ------------------------------CATTCATAGGCTATGTACTTC
                                                              **********************

gi|58614535|gb|AY751296.1|      CATGAGGGCAAATATCATTCTGAGGAGCAACAGTCATTACAAACTTACTC
Mastomys natalensis sample      CATGAGGACAAATATCATTCTGAGGAGCAACAGTCATTACAAACTTACTC
                                ******* ****************************************

gi|58614535|gb|AY751296.1|      TCAGCAATCCCATACATTGGAACTACATTAGTAGAATGGATCTGAGGAGG
Mastomys natalensis sample      TCAGCAGTCCCATACATTGGAACTACATTAGTAGAATGAATCTGAGGAGG
                                ****** ******************************** ***********

gi|58614535|gb|AY751296.1|      ATTCTCAGTAGATAAAGCAACACTAACACGCTTTTTCGCATTTCACTTCA
Mastomys natalensis sample      ATTCTCAGTAGATAAAGCAACACTAACACGCTTTTTCGCATTTCACTTCA
                                **************************************************

gi|58614535|gb|AY751296.1|      TCCTCCCATTCATTATTGCAGCCCTAGCCATCGTCCACCTTCTATTTCTT
Mastomys natalensis sample      TCCTCCCATTCATTATCGCAGCCCTAGTCATCGTCCACCTTCTATTTCTT
                                *************** ********** **********************

gi|58614535|gb|AY751296.1|      CACGAAACGGGCTCTAACAACCCAACGGGCTTAAACTCTGACTCAGATAA
Mastomys natalensis sample      CACGAAACGGGCTCTAATAACCCAACGGGCTTAAACTCTGACTCAGATAA
                                **************** ******************************
```

```
gi|58614535|gb|AY751296.1|      AATTCCATTCCACCCGTACTATACAATTAAAGACATTCTTGGAATCATCA
Mastomys natalensis sample      AATCCCATTCCACCCGTACTATACAATTAAAGACATTCTTGGAATTATCT
                                *** ****************************************** ***

gi|58614535|gb|AY751296.1|      TAATAATTGCTCTCCTAATGATTCTAGTTCTATTCTTTCCAGATTTACTA
Mastomys natalensis sample      TAATAATTGCCCTCCTAATAATTCTAGTACTATTTTTTCCAGACTTACTA
                                ********** ******** ******** ***** ******** ******

gi|58614535|gb|AY751296.1|      GGAGACCCAGACAACTATACACCAGCCAACCCACTCAACACACCTCCCCA
Mastomys natalensis sample      GGAGACCCCGACAACTATACACCAGCCAACCCACTCAACACACCTCCCCA
                                ******** ****************************************

gi|58614535|gb|AY751296.1|      TATCAAACCAGAATGATATTTCCTCTTTGCCTATGCCATCTTACGATCTA
Mastomys natalensis sample      TATCAAACCAGAATGATATTTCCTCTTTGCCTATGCCATCCTACGATCCA
                                ************************************** ******* *

gi|58614535|gb|AY751296.1|      TCCCTAACAAACTGGGAGGAGTCCTAGCCCTAATCCTTTCCATCCTAATC
Mastomys natalensis sample      TCCCTAATAAACTAGGAGGAGTCCTAGCCCTAATCCTTTCCATCCTTATC
                                ******* ***** ******************************** ***

gi|58614535|gb|AY751296.1|      TTAGCACTAATACCCCTACTCCACACCTCAAAACAACGAAGTCTCACATT
Mastomys natalensis sample      CTAGCACTAATACCCCTACTCCACACCTCAAAACAACGAAGTCTCACATT
                                 ************************************************

gi|58614535|gb|AY751296.1|      CCGTCCAATCACACAAATTCTTTACTGAATCTTAGTAGCAAACCTACTAG
Mastomys natalensis sample      CCGTCCAATCACACAAATTCTTTACTGAATCTTAGTAGCAAACCTACTAG
                                ************************************************

gi|58614535|gb|AY751296.1|      TACTAACTTGAATCGGAGGCCAACCAGTAGAACATCCATTTATCATTATT
Mastomys natalensis sample      TACTAACTTGAATCGGAGGCCAACCAGTAGAACATCCATTTATCATTATT
                                ************************************************

gi|58614535|gb|AY751296.1|      GGCCAATTAGCATCTATCAGTTACTTCTCTATTATCCTTATCCTGATGCC
Mastomys natalensis sample      GGCCAACTAGCATCAATCAGTTACTTCTCTATTATCCTTATCCTGATACC
                                ****** ******* ******************************** **

gi|58614535|gb|AY751296.1|      TATCTCAGGAATTATC--GAAGACAACCTACTCAAATGAAAC
Mastomys natalensis sample      TATCTCAGGAATTATTCGAAGGACAACTCAC-----------
                                ***************    * ******  **
```

APPENDIX F3 – ALIGNMENT OF TWO MASTOMYS COUCHA SPECIMENS FROM GENBANK AND THE UNCUT PCR-RFLP OBTAINED CLUSTALW

(Mismatches must be verified)

Multiple alignment of AY554160.1*, M. coucha* AD115 sample from S.A.: Beitbridge, and AF518334.1, *M. coucha* MNHN 1999-104 sample from S.A.: Bloemfontein (Lecompte *et al*., 2002), which is aligned with *Mastomys* sample from S.A.: KNP that remained uncut when digested with *Bsm*AI. The asterisks (*) indicated identical nucleotide at specific position of all three samples. The *Bsm*AI restriction site is indicated with green highlight.

```
gi|47834426|gb|AY554160.1|      --------------------------------------------------
gi|25814881|gb|AF518334.1|      ATGACAAACATCNNAAAAACTCACCCACTAGTCAAAATTATTAACCACTC
UNCUT                           -------TCCGGAGAGAAACTCACCCACTAATCAAAATTATTAACCACTC


gi|47834426|gb|AY554160.1|      ----------TTACCTGCCCCATCCAATATTTCATCATGATGAAACTTCG
gi|25814881|gb|AF518334.1|      ATTCATCGACTTACCTGCCCCATCCAATATTTCATCATGATGAAACTTCG
UNCUT                           ATTCATCGACTTACCTGCCCCATCCAATATTTCATCATGATGAAACTTCG
                                          ****************************************

gi|47834426|gb|AY554160.1|      GCTCACTATTAGGAATCTGCCTAATAATTCAAATCACCACAGGCCTATTC
gi|25814881|gb|AF518334.1|      GCTCACTATTAGGAATCTGCCTAATAATTCAAATCATCACAGGCCTATTC
UNCUT                           GCTCACTATTAGGAATCTGCCTAATAATTCAAATCATCACAGGCCTATTC
                                ********************************** *************

gi|47834426|gb|AY554160.1|      CTAGCAATACACTACACATCAGATACTATAACAGCATTCTCATCAGTAAC
gi|25814881|gb|AF518334.1|      CTAGCAATACACTACACATCAGATACTATAACAGCATTCTCATCAGTAAC
UNCUT                           CTAGCAATACACTACACATCAGATACTATAACAGCATTCTCATCAGTAAC
                                ************************************************

gi|47834426|gb|AY554160.1|      CCATATCTGCCGAGATGTAAACTATGGATGAATAATCCGATATATACACG
gi|25814881|gb|AF518334.1|      CCATATCTGCCGAGATGTAAACTACGGATGAATAATCCGATATATACACG
UNCUT                           CCATATCTGCCGAGATGTAAACTACGGATGAATAATCCGATATATACACG
                                ********************** ************************

gi|47834426|gb|AY554160.1|      CAAACGGAGCATCAATATTTTTTATCTGCTTGTTCCTTCACGTAGGACGG
gi|25814881|gb|AF518334.1|      CAAATGGAGCATCAATATTTTTTATCTGCTTGTTCCTTCACGTAGGACGG
UNCUT                           CAAATGGAGCATCAATATTTTTTATCTGCTTGTTCCTTCACGTAGGACGG
                                **** ********************************************

gi|47834426|gb|AY554160.1|      GGGATATATTACGGATCCTACACATTCACAGAAACATGAAATATTGGAGT
gi|25814881|gb|AF518334.1|      GGAATATATTACGGATCCTACACATTCACAGAAACATGAAATATTGGAGT
UNCUT                           GGGATATATTACGGATCCTACACATTCACAGAAACATGAAATATTGGAGT
                                ** ********************************************

gi|47834426|gb|AY554160.1|      AGTCTTACTTTTCGCAGTAATA-GCAACCGCATTCATAGGCTACGTACTT
gi|25814881|gb|AF518334.1|      AGTCTTACTTTTCGCAGTAATA-GCAACCGCATTCATAGGCTACGTACTT
UNCUT                           AGTCTTACTTTTCGCAGCAATACGCAACCGCATTCATAGGCTACGTACTT
                                ***************** **** **************************

gi|47834426|gb|AY554160.1|      CCATGAGGACAAATATCATTCTGAGGGGCAACAGTCATTACAAACTTACT
gi|25814881|gb|AF518334.1|      CCATGAGGACAAATATCATTCTGAGGGGCAACAGTCATTACAAACTTACT
UNCUT                           CCATGAGGACAAATATCATTCTGAGGGGCAACAGTCATTACTAACTTACT
                                ************************************** ********

gi|47834426|gb|AY554160.1|      CTCAGCAATCCCTTACATTGGAACTACACTAGTAGAATGAATCTGAGGAG
gi|25814881|gb|AF518334.1|      CTCAGCAATCCCTTACATTGGAACTACACTAGTAGAATGAATCTGAGGAG
UNCUT                           CTCAGCAATCCCTTACATTGGAACTACACTAGKAGAATGAATCTGAGGAG
                                ******************************** ****************
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gi|47834426|gb|AY554160.1|      GATTCTCAGTAGATAAAGCAACACTAACACGCTTCTTCGCATTCCACTTC
gi|25814881|gb|AF518334.1|      GATTCTCAGTAGATAAAGCAACACTAACACGCTTCTTCGCATTCCACTTC
UNCUT                           GATTTTCAGTAGATAAAGCAACACTAACACGCTTCTTCGCATTCCACTTC
                                **** ******************************************

gi|47834426|gb|AY554160.1|      ATCCTACCATTTATTATCGCAGCCCTAGTCATCGTCCACCTTTTATTCCT
gi|25814881|gb|AF518334.1|      ATCCTACCATTTATTATCGCAGCCCTAGTCATCGTCCACCTTTTATTCCT
UNCUT                           ATCCTACCATTTATTATCGCAGCCCTAGTCATCGTCCACCTTTTATTCCT
                                **************************************************

gi|47834426|gb|AY554160.1|      CCATGAAACAGGATCCAATAACCCAACAGGCCTAAACTCTGACTCAGACA
gi|25814881|gb|AF518334.1|      CCATGAAACAGGATCCAATAACCCAACAGGCCTAAACTCTGACTCAGACA
UNCUT                           CCATGAAACAGGATCCAATAACCCAACAGGCCTAAACTCTGACTCAGACA
                                **************************************************

gi|47834426|gb|AY554160.1|      AAATTCCATTTCACCCGTACTACACAATTAAAGACATTCTCGGAATTATC
gi|25814881|gb|AF518334.1|      AAATTCCATTTCACCCGTACTACACAATTAAAGACATTCTCGGAATTATC
UNCUT                           AAATTCCATTTCACCCGTACTACACAATTAAAGACATTCTCGGAATTATC
                                **************************************************

gi|47834426|gb|AY554160.1|      ATAATAATTGTATTCCTAATGACACTAGTACTATTTTTCCCAGACTTACT
gi|25814881|gb|AF518334.1|      ATAATAATTGTATTCCTAATGACACTAGTACTATTTTTCCCAGACTTACT
UNCUT                           ATAATAATTGTATTCCTAATGACACTAGTACTATTTTTCCCAGACTTACT
                                **************************************************

gi|47834426|gb|AY554160.1|      AGGAGACCCAGACAATTATACACCAGCTAACCCACTAAATACCCCTCCCC
gi|25814881|gb|AF518334.1|      AGGAGACCCAGACAATTATACACCAGCTAACCCACTAAATACCCCTCCCC
UNCUT                           AGGAGATCCAGACAATTATACACCAGCTAACCCACTAAATACCCCTCCCC
                                ****** *****************************************

gi|47834426|gb|AY554160.1|      ATATTAAACCAGAATGATATTTCCTCTTTGCCTACGCCATCCTACGATCT
gi|25814881|gb|AF518334.1|      ATATTAAACCAGAATGATATTTCCTCTTTGCCTACGCCATCCTACGATCT
UNCUT                           ATATTAAACCAGAATGATATTTCCTCTTTGCCTACGCCATCCTACGATCT
                                **************************************************

gi|47834426|gb|AY554160.1|      ATCCCTAACAAACTAGGAGGAGTGCTAGCCCTGATCCTTTCAATCCTAAT
gi|25814881|gb|AF518334.1|      ATCCCTAACAAACTAGGAGGAGTGCTAGCCCTGATCCTTTCAATCCTAAT
UNCUT                           ATCCCTAACAAACTAGGAGGAGTGCTAGCCCTGATCCTTTCAATCCTAAT
                                **************************************************

gi|47834426|gb|AY554160.1|      CCTAGCACTAATACCATTACTCCACACCTCAAAACAACGAAGCCTCACAT
gi|25814881|gb|AF518334.1|      CCTAGCACTAATACCATTACTCCACACCTCAAAACAACGAAGCCTCACAT
UNCUT                           CCTAGCACTAATACCATTACTCCACACCTCAAAACAACGAAGCCTCACAT
                                **************************************************

gi|47834426|gb|AY554160.1|      TTCGCCCAATCACACAAATTCTTTACTGAATCTTAGTAGCAAACCTACTA
gi|25814881|gb|AF518334.1|      TTCGCCCAATCACACAAATTCTTTACTGAATCTTAGTAGCAAACCTACTA
UNCUT                           TTCGCCCAATCACACAAATTCTTTACTGAATCTTAGTAGCAAACCTACTA
                                **************************************************

gi|47834426|gb|AY554160.1|      GTACTAACCTGAATTGGAGGCCAACCAGTAGAACACCCATTCATCATTAT
gi|25814881|gb|AF518334.1|      GTACTAACCTGAATTGGAGGCCAACCAGTAGAACACCCATTCATCATTAT
UNCUT                           GTACTAACCTGAATTGGAGGCCAACCAGTAGAACACCCATTCATCATTAT
                                **************************************************

gi|47834426|gb|AY554160.1|      CGGCCAACTAGCATCAATCAGTTACTTCTCTATTATTCTCATCTTAATAC
gi|25814881|gb|AF518334.1|      CGGCCAACTAGCATCAATCAGTTACTTCTCTATNATTCTCATCTTAATAC
UNCUT                           CGGCCAACTAGCATCAATCAGTTACTTCTCTATTATTCTCATCTTAATAC
                                ********************************* ****************

gi|47834426|gb|AY554160.1|      CTATCTCAGGAATCATCGAAGATAATCTACT---------
gi|25814881|gb|AF518334.1|      CTATCTCAGGAATCATCGAAGATAATCTACTCAAATGAAA
UNCUT                           CTATCTCAGGAATCATCGAAGATAATCTACTCAAA-----
                                ******************************
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