Molecular prevalence and diversity of Anaplasmataceae and Bartonellaceae in indigenous Muridae from South Africa

By

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Vir mamma en pappa.

Dankie vir alles!

General Abstract

The main aim of the current study was to determine the prevalence and diversity of potentially zoonotic bacterial genera in accurately identified indigenous rodents from South Africa. Bacterial prevalence and diversity were determined by PCR amplification and sequence analyses. Rodents were molecularly identified by amplification and sequence analysis of the mitochondrial cytochrome *b* gene region. Three species (*Aethomys ineptus, Mastomys coucha* and *Otomys angoniensis*) belonging to murid species complexes were identified. Furthermore, phylogenetic analyses revealed that both the proposed subspecies (*R. dilectus dilectus* and *R. d. chakae*) within the recently erected *Rhabdomys dilectus* occur in Hammanskraal and at the University of Pretoria Experimental farm, both in the Gauteng Province of South Africa. An overall bacterial prevalence of 38.6 % was observed in kidney samples of commensal and natural indigenous rodents after molecular screening with broad range 16S rRNA gene primers. Nucleotide sequence analyses identified a diverse range of bacterial genera namely, *Bartonella, Anaplasma, Helicobacter, Burkholderia, Streptococcus, Aerococcus* and *Lactobacillus.* Some members of these genera have been identified as causative agents of human and animal diseases, being transmitted either through environmental contamination or through haematophagous arthropod vectors. Subsequent genus-specific bacterial screening focussed on vector-borne genera identified in the commensal and natural rodent populations sampled. *Bartonella* prevalence and genetic diversity was compared between a natural and commensal population of the southern multimammate mouse (*M. coucha*) using two gene regions (Citrate synthase gene and NADH dehydrogenase gamma subunit gene). A significantly higher infection prevalence was detected in the commensal population (92.9 %) as compared to the natural population (56.9 %). No differences however, were detected between infection status and the ectoparasite loads

calculated for both rodent populations. Apart from several novel *Bartonella* strains identified in both *M. coucha* populations, phylogenetic analyses also identified a species of known zoonotic potential (*B. elizabethae*) in both populations. The present study represents one of the first to screen indigenous rodents for tick-borne members of the bacterial family Anaplasmataceae. *Anaplasma bovis*-like DNA was detected in five of the six rodent species sampled (*A. ineptus*, *Lemniscomys rosalia*, *M. coucha*, *O. angoniensis* and *R. dilectus*) at an overall prevalence of 39.2 %. The potentially zoonotic *Ehrlichia ewingii* was detected in *M. coucha* samples only at a prevalence of 5.3 %. The diverse bacterial genera detected in commensal and natural populations of indigenous rodents comprise members of zoonotic potential and agricultural significance, highlighting the importance of continuous disease surveillance of indigenous rodents.

Keywords: Indigenous rodents, South Africa, zoonotic diseases, bacteria, *Aethomys ineptus*, *Lemniscomys rosalia*, *Mastomys coucha*, *Otomys angoniensis*, *Rhabdomys dilectus*, *Saccostomus campestris*, *Bartonella*, *Anaplasma*, *Ehrlichia*

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Declaration

I, the undersigned hereby declare that the dissertation, which I hereby submit for the degree Master of Science (Zoology) at the University of Pretoria is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature:

Date:

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Disclaimer

This dissertation consists of a series of chapters that have been prepared as stand-alone manuscripts for subsequent submission for publication purposes. Consequently, unavoidable overlaps and/or repetitions may occur between chapters.

"...But Mousie, thou art no thy lane,
In proving foresight may be vain:
The best-laid schemes o' mice an' men
Gang aft agley,
An' lea'e us nought but grief an' pain,
For promis'd joy!.."

-Robert Burns-

Chapter 1

General Introduction

Rodents represent nearly 40 % of all mammal species and have a worldwide distribution (Huchon *et al.* 2002). The largest and most successful of the rodent families is the Muridae (Alderton 1996). This family includes familiar members such as rats, mice, hamsters and gerbils and are present on almost all the continents. The house mouse (*Mus musculus*) has even managed to invade the sub-Antarctic (Alderton 1996). Murid rodents inhabit diverse habitats and often occur in close association with humans (De Graaf 1981). For instance, members of the genus *Rattus* have been associated with human settlements since 1500 BC (Atkinson 1985). This long association between rats and humans was recently exploited to trace human migration and settlement patterns (Matisoo-Smith and Robins 2004). These commensal species can also be important in spreading diseases, causing damage to agricultural crops and food stores, and in causing damage to infrastructure such as electrical installations (Drummond 1975; Willan 1992).

Economic importance

Rodents are among the most important global pests in both urban and agricultural settings (Skonhoft *et al.* 2006). In cities, they can cause serious structural damage or even fires due to gnawed electrical cables (Pimentel *et al*. 2000; Skonhoft *et al.* 2006). As agricultural pests they cause serious damage to a wide variety of crops, pre- and post-harvest (Stenseth *et al.* 2003; Skonhoft *et al.* 2006). In the United States, it is

estimated that losses due to rodent damage of stored grain can be as high as US$19 billion per annum (Pimentel *et al*. 2000).

Agricultural losses are often more serious in developing countries where rodents are literally in direct competition with people for food (Meerburg *et al.* 2009a). For instance, in Asia rats annually consume enough crops to feed approximately 200 million people for an entire year (Stenseth *et al.* 2003). In India, Ahmad *et al.* (1995) reported annual grain losses of 3 % due to rodent consumption and contamination.

In Africa, losses are equally devastating, with 15 % to 40 % of crops lost to rodent damage, worsening the already critical food situation on the continent (Skonhoft *et al.* 2006; Meerburg *et al.* 2009a). In Africa, more than 70 rodent pest species have been reported, with the multimammate mouse (*Mastomys natalensis*) being one of the most important (Skonhoft *et al.* 2006). This species undergoes irregular and frequently unpredictable population eruptions leading to large agricultural losses (Leirs *et al*. 1996). In Kenya, over 50 % of crop losses are due to the multimammate mouse and in Tanzania rodent damage to maize by this species has been estimated to be as high as US$60 million per annum (Stenseth *et al.* 2001; Skonhoft *et al.* 2006). Several *Mastomys* species, including *M. natalensis*, have also been implicated in the spread of zoonotic diseases (Gratz 1997).

Apart from the indirect cost through reduced yield, the direct costs of controlling rodent pests are just as high. For example, in early 1998 about US$16 million was spent on rodent control by the Tanzanian government (Mwanjabe *et al.* 2002). The cost of rodent-borne diseases of humans and livestock also add to the

burden of these developing countries, however, the economic impact of these diseases is poorly documented and difficult to estimate (Meerburg *et al.* 2009a).

Although rodents often compete directly with humans, some species contribute to the economy. Certain species such as the beaver and chinchilla are highly prized for their exquisite fur, and the beaver fur trade even led to the development of major cities (Alderton 1996; Spotorno *et al* 2004). A wide range of rodents are also hunted for food. Dormice (*Glis glis*) are eaten in France and other parts of Europe (Alderton 1996). In the Mekong Delta of Vietnam, rat meat markets process about 77-480 kg of rat meat per day and provide an important source of income for many poor local farmers (Khiem *et al.* 2003). In Ghana, it is estimated that as much as two metric tons of cane rat (*Thryonomys swinderianus*) meat is eaten annually and commercial cane rat production is becoming a viable animal production activity in many African countries (Jori and Chardonnet 2001).

Environmental importance

Invasive mammals are a major cause of extinction and ecosystem change worldwide (Howald *et al.* 2009). Invasive rodents play a big part in this problem. Due to their small size, nocturnal nature and inherent cautiousness rodents easily escape detection and, therefore, can easily be introduced as alien species (Alderton 1996). In the past, the shipping industry was a significant cause of rodent invasion, most notably spreading members of the genera *Rattus* and *Mus* around the world (Perry *et al.* 2006). The black rat (*Rattus rattus*) has been called one of the world's worst invasive aliens, especially in island ecosystems causing the extinction or decline of various insular plants, invertebrates and vertebrates (Harris and Macdonald 2007; Howald *et*

al. 2009). Circumstantial evidence suggests that *R. rattus* has played a leading role in the extinctions of eight of the 12 endemic rodent species on the Galápagos (Harris and Macdonald 2007). Invasive species are not just detrimental to biodiversity but also lead to significant economic costs. In 2009, the U.S. governments spent US$1.8 million to eradicate *R. rattus* from the Ancapa Island in California (Howald *et al.* 2009).

Invasions by these rodent species is an on-going problem exacerbated by increased international trade and transport, however, the exotic pet trade has provided a new opportunity for the spread of invasive rodents (Perry *et al.* 2006). Recently, Perry *et al.* (2006) demonstrated that an escaped exotic rodent pet, the Gambian rat (*Cricetomys gambianus*), poses a serious and potentially long-term threat to the indigenous ecological communities within the Florida Keys in the U.S.A.

Invasive rodents frequently lead to a decrease in the diversity of indigenous fauna by the introduction of new pathogens and parasites to naïve indigenous populations (Konečný *et al*. 2013). The recent discovery of the Asian house rat (*R. tanezumi*) in South Africa has fuelled renewed interest in the study of invasive *Rattus* species in Africa (Bastos *et al.* 2011). The last decade, several studies have shown that invasive *Rattus* species are host to a number of pathogens and parasites, potentially spreading them to native rodents and humans (Bastos 2007; Taylor *et al*. 2008; Mostert 2009; Brettschneider 2010; Archer *et al*. 2011).

The pest status of rodents often makes us forget that natural populations of rodents play an important role in their native environments. Small mammals

determine the habitat structure of many ecosystems ranging from African savannahs to deserts (Keesing 2000; Eldridge *et al.* 2009). Rodents are also important seed-dispersers and pollinators (Forget 1997; Biccard and Midgley 2009). Zoochory is essential, for instance in the maintenance of bush-pockets in the threatened coastal dune forest habitats of South Africa (Castley *et al.* 2001). Finally, rodents also form the staple diet of many predators, including mammals, birds, and reptiles (Malan and Crowe 1996; Perrin and Bodbijl 2001).

Medical importance

Many important medical and biological discoveries have been made possible with the help of rodents, which have become established laboratory animals (De Graaf 1981). According to the American Association for Laboratory Animal Science, of the more than 20 million animals used in biomedical research each year, over 90 % are rodents (www.aalas.org). Rats specifically have been used as model organisms in nearly all fields of biological research and have even been sent into space (Alderton 1996). Although laboratory animals are far removed from the image of plague infested sewer rats, one of the biggest problems caused by rodents is still the spread of disease to humans and other animals (Meerburg *et al.* 2009b).

Rodents have been linked to at least 60 zoonotic pathogens including viruses, bacteria, helminths and protozoa, many of which are considered emerging or re-emerging diseases (Taylor *et al.* 2008). More than half of the emerging infectious disease events recorded over the last six decades were caused by bacterial pathogens (Jones *et al.* 2008). Rodent-borne bacteria (Table 1) can be transmitted directly through bites or scratches as in the case of rat bite fever, indirectly through

Table 1. Major bacterial pathogens associated with rodents, the mode of transmission, prevalence rate in rodents and geographical distribution.

Disease	Agent	Transmission	Prevalence in Rodents	Geographic Distribution	Reference
Human granulocytic anaplasmosis	*Anaplasma phagocytophilum*	Vector-borne	Moderate	Asia, Europe, North America, South America	Rar and Golovljova 2011
Bartonellosis	*Bartonella* spp.	Direct/Vector-borne	High	Worldwide	Brettschneider 2010
Lyme disease	*Borrelia burgdorferi*	Vector-borne	Moderate	Europe, North Amierica	Ferquel *et al* . 2006
Tick-borne Relapsing fever	*Borrelia* spp.	Vector-borne	Low	Worldwide	Tissot-Dupont *et al.* 1997
Gastroenteritis	*Campylobacter* spp.	Food-borne	Low	Worldwide	Meerburg and Kijlstra 2007
Q-fever	*Coxiella burnetii.*	Vector-borne	High	Worldwide	Comer *et al.* 2001
Tularemia	*Francisella tularensis*	Direct/Food-borne/Vector-borne	Moderate	Europe, North Amierica	Christova and Gladnishka 2005
Gastritis, Peptic ulcers	*Helicobacter* spp.	Environmental	Moderate	Worldwide	Comunian *et al* . 2006
Leptospirosis	*Leptospira* spp.	Environmental	Low to high	Worldwide	Meerburg *et al.* 2009b
Listeriosis	*Listeria monocytogenes*	Food-borne	Low	Worldwide	Zaytseva *et al.* 2007
Pasteurellosis	*Pasteurella pneumotropica*	Direct/Environmental	Low to high	Worldwide	Frebourg *et al.* 2002
Scrub typhus	*Orientia tsutsugamushi*	Vector-borne	Moderate	Asia, Australia	Meerburg *et al.* 2009b
Spotted fevers/Typhus fevers/Tick-bite fevers	*Rickettsia* spp.	Vector-borne	High	Worldwide	Comer *et al.* 2001
Gastroenteritis	*Salmonella* spp.	Food-borne	Moderate	Worldwide	Meerburg and Kijlstra 2007
Staphylococcal bacteremia	*Staphylococcus* spp.	Environmental	Low to high	Worldwide	Hauschild *et al.* 2010
Rat-bite fever	*Streptobacillus moniliformis* / *Spirillum minus*	Direct/Food-borne	Low to high	Worldwide	Gaastra *et al.* 2009
Pneumococcal diseases	*Streptococcus* spp.	Direct/Environmental	High	Worldwide	Van der Linden *et al.* 2009
Plague	*Yersinia pestis*	Vector-borne	Low to high	Worldwide	Neerinckx *et al.* 2010
Yersinosis	*Yersinia enterocolitica* *Y. pseudotuberculosis*	Food-borne	Low to moderate	Wordwide	Neubauer *et al.* 2000

environmental contamination with urine or faeces as in the case of food borne pathogens or by ectoparasites in the case of vector-borne diseases such as plague and Lyme disease (Meerburg *et al*. 2009b).

Furthermore, it is estimated that vector-borne diseases are responsible for almost 30 % of the emerging infectious diseases reported in the last decade (Jones *et al.* 2008). Rodents play host to a wide variety of ectoparasites that can act as vectors of a number of pathogens (Morand *et al*. 2006). The ecology, development, behaviour, and survival of these arthropod vectors are influenced by both on- and off-host factors (Gubler *et al*. 2001, Krasnov *et al.* 2004). Epidemiological studies of vector-borne diseases are therefore often complicated by a complex interplay of factors affecting the arthropod vectors.

Rodent-borne zoonoses in Africa

Rodent-borne zoonotic diseases are responsible for a large proportion of morbidity and mortality in Africa, however, limited prevalence data are available (Gratz 1997). One of the most well-known rodent-borne bacterial infections, the plague, occurs endemically in many African countries (Neerinckx *et al*. 2010). Plague is caused by the bacteria *Yersinia pestis* and is transmitted amongst rodent reservoirs and to humans by fleas from the genus *Xenopsylla* (Butler *et al.* 1982). The swift clinical course, rapid spread and high mortality rate of plague makes it a significant threat especially if left untreated (Stenseth *et al.* 2008). Several indigenous murid species have been implicated as natural reservoirs for plague in Africa (Davis 1953; Isaäcson *et al.* 1981; Matthee *et al.* 2007). Plague remains an important threat in Africa and almost 90 % of all cases reported each year occur on the continent with outbreaks reported as recently as 2008 (Makundi *et al*. 2008).

Another group of bacterial diseases that has a long history in Africa is rickettsioses (Tissot-Dupont *et al.* 1995). Murine typhus, a rodent-borne rickettsial infection, is often under-reported and is thought to have a high prevalence throughout Africa (Azad and Beard 1998). *Rickettsia typhi* is transmitted mainly by several flea species including *Xenopsylla cheopis*. However, fleas are usually only vectors, with commensal rodents acting as the primary reservoirs (Raoult and Roux 1997). Although invasive rodents such as *R. rattus, R. norvegicus, Mus musculus* and their ectoparasites are mostly implicated, the importance of this disease in indigenous murid rodents from Africa has not been fully investigated (Gratz 1999).

A recent study of invasive *Rattus* species in South Africa revealed the presence of *Rickettsia felis* in 0.9 % of animals tested (Julius 2013). *Rickettsia felis* is a flea-borne spotted fever that has been implicated in human diseases across Europe, the Americas and Africa (Parola 2011). Flea-borne spotted fevers frequently present as non-specific febrile illnesses accompanied by a maculopapular rash (Parola *et al.* 2005). The recently reported high prevalence of *R. felis* found in fleas from small African mammals suggests that this rickettsia may be endemic in Africa, however, due to the non-specific symptoms it remains undetected and under reported (Khaldi *et al.* 2012).

An expanding human population, globalisation and climate change is leading to the emergence or re-emergence of diseases at an unparalleled rate (Brown 2004). Nowhere are emerging diseases more important than in developing countries where infrastructure is usually lacking (Gratz 1999). One group of emerging bacterial diseases that has received increasing attention in southern Africa belongs to the genus *Bartonella*. Of the 33 named

Bartonella species, more than half are hosted by rodent species and at least six of these have been implicated in human diseases (Buffet *et al.* 2013).

Rodent-borne *Bartonella* species cause a diverse array of symptoms including fever, myocarditis, endocarditis, lymphadenitis and hepatitis (Kaiser *et al*. 2011). Clinical symptoms associated with *Bartonella* infections are highly variable, especially in immune-compromised individuals (Frean *et al.* 2002). The bacterium is generally transmitted between hosts by haematophagous arthropod vectors such as Ixodid ticks and fleas of the genus *Xenopsylla* (Breitschwerdt and Kordick 2000; Bai *et al.* 2008). *Bartonella* infections have been found to be highly prevalent in a wide range of rodent species worldwide, with rates of more than 50 % in some species (Bai *et al.* 2008; Buffet *et al*. 2013).

Several studies have recently reported on the prevalence of *Bartonella* infections in rodents in South Africa (Pretorius *et al.* 2004; Bastos 2007; Mostert 2009; Brettschneider 2010; Brettschneider *et al*. 2012). These studies have demonstrated that indigenous murid rodents harbour a diverse collection of known and potentially novel *Bartonella* strains at prevalence rates as high as 96 % in some species (Pretorius *et al.* 2004; Bastos 2007; Brettschneider 2010; Brettschneider *et al*. 2012). From these studies it also appears that commensal rodents in South Africa may have higher incidences of *Bartonella* infection than wild populations (Pretorius *et al.* 2004; Bastos 2007). Although *Bartonella* has received attention, focussed host species-specific studies are generally lacking and many aspects of disease maintenance and transmission remain under-studied. Furthermore, the observation that rodents carry several pathogenic *Bartonella* species, when considered in light of the high HIV infection rates in South Africa, makes it important to study *Bartonella* in rodent species that occur commensally with humans (Bastos 2007).

Although some diseases have received less attention in the literature, they nevertheless remain plausible threats in Africa. Tick-borne relapsing fever is a disease caused by numerous species of the genus *Borrelia* and is transmitted from rodent reservoirs to humans by soft-bodied ticks of the genus *Ornithodoros* (Meerburg *et al* 2009b). West African tick-borne relapsing fever is caused by the spirochete *Borrelia crocidurae*, and cases have been reported from Senegal, Morocco, Libya, Egypt, Iran, and Turkey (Rebaudet and Parola 2006). A survey of small mammals in Senegal demonstrated that the African grass rat (*Arvicanthis niloticus*) and Hubert's multimammate mouse (*Mastomys huberti*) serve as reservoir hosts for the pathogen (Godeluck *et al.* 1994). If left untreated, *B. crocidurae* infections can have mortality rates of up to 5 % (Meerburg *et al* 2009b). Lyme disease caused by *B. burgdoferi* has also been reported from urban areas in South Africa and rats were once again implicated as important reservoirs (Gratz 1999).

It was recently also demonstrated that, apart from the brown rat (*R. norvegicus*), the multimammate mouse (*M. natalensis*) is a competent reservoir host for leptospirosis and it is thought that indigenous commensal rodents are the most important source of the infection (Holt *et al.* 2006). Apart from the above mentioned accounts, African rodents are associated with several other zoonotic pathogens, however, relatively little information is available on the prevalence and dynamics of bacterial rodent-borne diseases in indigenous African rodents.

Southern African murids

In southern Africa, there are more than 20 genera of murid rodents encompassing about 50 species (Skinner and Chimimba 2005). Several species within this family are widespread and

often occur commensally with humans and can become pest species leading to damaged crops and stored food (De Graaff 1981). Some of the most commonly implicated pest species belong to the genera *Mastomys*, *Aethomys*, *Tatera* and *Rhabdomys* (Bastos *et al.* 2005).

Mastomys contains some of Africa's most notable pest species and they are often of great importance in the cycle of many zoonotic diseases (Leirs *et al.* 1996). Specifically, members of this genus have been implicated in the spread of plague, leptospirosis and the haemorrhagic virus causing Lassa fever (Isaäcson *et al.* 1981, Stenseth *et al.* 2001). They are opportunistic omnivores that are well-adapted to disturbed environments (Wallen 1992). They are capable of very high reproductive rates, with litter sizes reaching as high as 24, and are, therefore, prone to population eruptions (Lima *et al.* 2003). At least four *Mastomys* species are morphologically indistinguishable sibling species and often two or three of these species occur sympatrically (Lecompte *et al.* 2005).

Rhabdomys is a widespread murid genus endemic to Africa that is generally found in grassland (Skinner and Chimimba 2005). It is a habitat generalist that has successfully adapted to urban environments and because of their frequent population eruptions they are also considered to be pests (Matthee *et al.* 2007). The taxonomy of the genus *Rhabdomys* has been problematic (Castiglia *et al.* 2012). It has historically been considered to be a monotypic genus containing only one species, *R. pumilio*, with a varying number of subspecies (Skinner and Chimimba 2005). However, recent cytogenetic and molecular studies have suggested that in southern Africa this genus contains at least two species, namely *R. pumilio* and the newly erected *R. dilectus* (Rambau *et al.* 2003; Castiglia *et al.* 2012). *Rhabdomys* has also been linked to the spread of zoonotic diseases such as plague (Drummond 1975). Widely distributed species of the genus *Aethomys*, also undergo sporadic population eruptions and

have also been associated with zoonotic diseases such as rift valley fever, plague and schistosomiasis (Muteka *et al.* 2006).

Correct host identification is crucial for understanding disease dynamics during epidemiological studies (Mills and Childs 1998). However, correct species identification is often a problem amongst African rodents, as several cryptic species complexes have been identified over the last 30 years (Meester *et al.* 1986; Bronner *et al.* 2003; Skinner and Chimimba 2005). The lack of distinguishable external morphological characteristics between cryptic rodent species can seriously compromise the study of the transmission of certain rodent-borne diseases (Lecompte *et al.* 2005). For instance, comparative studies have shown that two cryptic *Mastomys* species (*M. coucha* and *M. natalensis*) differ in their susceptibility to experimental infection with *Yersinia pestis* (Isaäcson *et al.* 1981; Arntzen *et al.* 1991). Experimental evidence suggests that different *Rhabdomys* lineages may also display differences in susceptibility to plague infection (Shepherd *et al.* 1986). This highlights possible problems with previous epidemiological research involving members of cryptic species complexes, as constituent species may have very different roles in disease transmission.

In developing African countries such as South Africa, one or more of these rodent species are often extremely abundant in anthropogenic habitats such as large cities and informal settlements (Fichet-Calvet *et al*. 2007). In these situations, the combination of poor sanitation, inadequate health care and rodent abundance increases the threat of disease transmission between these reservoir hosts and humans (Drummond 1975; Taylor et al. 2008). This situation is frequently exacerbated by the large number of undernourished and immune-compromised people in these communities (Gratz 1999). Despite this threat there are

very few detailed studies on these commensal rodents (Taylor *et al.* 2008). Although the prevalence of rodent-associated bacterial species of medical importance has been evaluated in South Africa in recent years, many of these studies have focused on invasive species rather than on indigenous species alone (Bastos 2007; Taylor *et al.* 2008; Mostert 2009).

Justification

By the year 2025 more than half of the world's human population will live in urban areas and most growth will occur in the developing countries such as South Africa (Gratz 1999). These urban human population eruptions often occur in areas with inadequate sanitation and infrastructure and unavoidably favour commensal rodents, which will inevitably lead to an increased risk of zoonotic diseases (Gratz 1999). Nowhere is this more important than in South African communities where there are a large number of immune-compromised individuals and overwhelmed healthcare systems (Taylor *et al.* 2008). Furthermore changes in climate and human behaviour are placing humans in increasing contact with wild rodents that could be harbouring emerging and re-emerging infectious diseases (McMichael 2004). This study attempts to provide valuable information on the prevalence of various bacterial diseases of health concern and the potential risk of infection to humans due to indigenous, commensal and wild murid rodents and their ectoparasites. Additionally, the present study attempts to expand the limited information available on ectoparasite loads of indigenous African rodents. Many of the murid rodent species that pose a disease risk to humans belong to cryptic species complexes, and the sibling species often have very different roles in the transfer of diseases. Therefore, the genetic identification of animals in this study will lead to the correct identification of hosts species and a better understanding of disease dynamics.

Aim

Given the above background, the aim of the present study was to investigate the prevalence and diversity of bacterial pathogens associated with commensal and wild indigenous rodents. In order to accurately assess their role as hosts of the identified pathogens, the rodent species were molecularly identified to species level. Subsequently two of the identified bacterial families were selected to further explore the role of indigenous rodents as reservoirs of potential zoonotic diseases. Firstly, *Bartonella* prevalence and diversity was compared between commensal and wild populations of *Mastomys coucha*. Secondly, the prevalence and diversity of the tick-borne Anaplasmataceae family members was determined in commensal indigenous rodents.

Research Questions

More specifically, the following research questions were addressed in the present study:

1. What cryptic species belonging to the murid genera *Aethomys, Mastomys, Otomys* and *Rhabdomys* are present in the present sampling localities in South Africa?

2. What is the prevalence, diversity and zoonotic potential of bacteria, potentially transmitted through environmental contamination, associated with indigenous commensal and wild rodents in South Africa?

3. What is the *Bartonella* prevalence and diversity in commensal and wild populations of *Mastomys coucha* in South Africa?

4. What is the prevalence and diversity of Anaplasmataceae bacteria in indigenous commensal rodents in South Africa?

Research hypotheses

Given the above, the following research hypotheses will be tested in the present study:

Hypothesis 1

Null hypothesis (H_0): Both cryptic sibling species of the murid genera *Aethomys* (*A. chrysophilus* and *A. ineptus*), *Mastomys* (*M. coucha* and *M. natalensis*), *Otomys* (*O. angoniensis* and *O. irroratus*) and *Rhabdomys* (*R. dilectus* and *R. pumilio*) are present in Hammanskraal and the University of Pretoria Experimental farm, Gauteng province, South Africa.

Alternative hypothesis (H_A): Only one of the cryptic sibling species of the murid genera *Aethomys* (*A. chrysophilus* or *A. ineptus*), *Mastomys* (*M. coucha* or *M. natalensis*), *Otomys* (*O. angoniensis* or *O. irroratus*) and *Rhabdomys* (*R. dilectus* or *R. pumilio*) is present in Hammanskraal and the University of Pretoria Experimental farm, Gauteng province, South Africa.

Hypothesis 2

Null hypothesis (H_0): There are no bacteria of zoonotic potential that are potentially transmitted through environmental contamination, associated with indigenous commensal and wild rodents in South Africa.

Alternative hypothesis (H_A): There are bacteria of zoonotic potential that are potentially transmitted through environmental contamination, associated with indigenous commensal and wild rodents in South Africa.

Hypothesis 3

Null hypothesis (H_0): There is no *Bartonella* prevalence and diversity in commensal and wild populations of *Mastomys coucha* in South Africa.

Alternative hypothesis 1(H_{A1}): There is *Bartonella* present in commensal and wild populations of *Mastomys coucha* in South Africa and the *Bartonella* prevalence and diversity is the same in commensal and wild populations of *Mastomys coucha* in South Africa.

Alternative hypothesis 2 (H_{A2}): There is *Bartonella* present in commensal and wild populations of *Mastomys coucha* in South Africa and there is a difference in *Bartonella* prevalence and diversity between commensal and wild populations of *Mastomys coucha* in South Africa.

Hypothesis 4

Null hypothesis (H_0): There is no prevalence and diversity of Anaplasmataceae bacteria in indigenous commensal rodents in South Africa.

Alternative hypothesis (H_A): There is a prevalence and diversity of Anaplasmataceae bacteria in indigenous commensal rodents in South Africa.

Study approach

Sample collection

Commensal rodents were sampled at 4-6 week intervals between August 2010 and July 2011 from two localities in the Gauteng Province (South Africa) as authorised by a valid collection permit (Permit number CPF6-0032) issued by the Gauteng Department of Agriculture Conservation and Environment and with permission from land owners. The two sampling localities included Hammanskraal, north of Pretoria (25°42´60´´S, 28°27´20´´E, 1111 meters above sea level) and the University of Pretoria Experimental farm, Hatfield, Pretoria (25°75´11´´S, 28°26´29´´E, 1375 m.a.s.l.). The sampling was conducted largely in semi-urban areas including informal settlements, office buildings, school grounds and small

holdings (see Figure 1 in Chapter 2). Wild rodents were sampled from the Kruger National Park (Mpumalanga Province, South Africa) in a previous study (Brettschneider 2010) and were selected to represent a rodent population with little human contact. Sampling localities were situated between the Lower Sabie and Tshokwane Rest Camps (24°48´02´´S, 31°52´45´´E to 24°89´77´´S, 39°12´33´´E, 200-400 m.a.s.l) and were sampled with permission from the South African National Parks Scientific Services. Sampling was conducted bi-annually (in April during the wet season and in September during the dry season) over a two year period from September 2008 to April 2010. Animals were live-trapped using Sherman traps (H.B. Sherman Traps Inc. Florida, U.S.A.) and housed in polyurethane cages with wood shavings as bedding, with mouse pellets and water provided *ad libitum*, as per the guidelines of the American Society of Mammalogists (ASM, www.mammalogy.org/committees/index.asp; Animal Care and Use Committee 1998).

Sample processing

Sample processing was conducted in a biosafety level 2 (BCL 2) facility and all research on potentially infective material was done with permission from the Department of Agriculture, Fisheries and Forestry (Ref no. 12/11/1/1/8). Animals were euthanized using halothane inhalation as approved by the Animal Ethics Committee of the University of Pretoria, Pretoria, South Africa (Ethics Clearance number: EC023-08 and EC026-10). Standard external measurements were recorded and all visible ectoparasites were removed through combing of the animal's coat and stored in 96 % ethanol. All removed ectoparasites were subsequently sorted into ticks (Acari), mites (Acari), fleas (Siphonaptera) and lice (Phthiraptera) and counted. Oral and rectal swabs were taken and stored in a 1:1 mixture of glycerol and phosphate buffered saline at -20^{o} C. Heart, lung, liver, kidney, gastrointestinal tract and reproductive organ tissues were removed and stored at -20^{o} C for further analyses.

Voucher specimens of all samples were prepared using standard natural history museum procedures for mammal specimens and deposited in the Ditsong National Museum of Natural History (former Transvaal Museum) of the Northern Flagship Institute (NFI), Pretoria, South Africa.

Laboratory procedures

Total genomic DNA was extracted from kidney tissue of all sampled rodents using the High Pure PCR template preparation kit (Roche) according to the prescribed manufacturer's protocol. Kidney tissue DNA extracts were used for rodent host identification (Chapter 2), to determine bacterial species associated with commensal and wild rodents possibly shed in the environment by urine and faeces (Chapter 3) and for the detection of *Anaplasma* in commensal species (Chapter 5). Total genomic DNA was also extracted from *Mastomys coucha* heart tissue using the High Pure PCR template preparation kit (Roche) according to the prescribed manufacturer's protocol. Heart extracts were used to screen commensal and wild *M. coucha* samples for *Bartonella* prevalence (Chapter 4).

Rodent host detection

Cryptic rodent species were identified molecularly by means of cytochrome *b* (cyt *b*) gene amplification and sequence analyses (Bastos *et al*. 2005). Correct species identification is especially important during epidemiological studies, where primary reservoir identification is crucial for understanding disease dynamics (Mills and Childs 1998). The mitochondrial cytochrome *b* (cyt *b*) gene is a protein coding gene that is highly conserved amongst mammals (Irwin *et al*. 1991). The rate of evolution of the cytochrome *b* gene is well-known and therefore, the level of sequence divergence associated with species-level distinction has been well-documented (Irwin *et al*. 1991; Bradley and Baker 2001). As such cytochrome *b*

has been used extensively in recent years to address the phylogeny of rodents (Ducroz *et al.* 2001).

Bacterial detection

Traditionally, bacterial species were detected and identified using culture, biochemical or immunochemical techniques (Amman *et al.* 1995). However, these methods often require expensive reagents that are not commonly available in standard laboratories or can involve lengthy processes, especially in the case of culturing of fastidious bacterial species (Doern 2000). Molecular methods have become increasingly popular in the detection of bacteria as they overcome many of the limitations associated with traditional methods (Clarridge 2004). Therefore molecular methods, including PCR and DNA sequencing, were chosen during this study to rapidly and accurately identify bacterial species.

Different gene regions were used for the detection of bacterial species during the present study. A broad range bacterial primer set was used to determine what bacteria are associated with the kidneys of commensal and wild rodents (Chapter 3). This broad range primer set amplifies a 1,500bp region of the bacterial 16S rRNA gene (Hauben *et al.* 1997). The 16S rRNA gene is highly conserved amongst all bacterial taxa and is extensively used in the phylogenetic study of bacteria (Clarridge 2004). By screening kidney tissue one can determine what bacterial genera are possibly being spread to the environment through rodent urine and faeces.

After preliminary primer assessment, a primer set that amplifies a partial fragment of the 16S rRNA gene was also chosen to investigate the prevalence and diversity of Anaplasmataceae bacteria (Chapter 5). The selected primer set is frequently cited in the

literature and has been shown to have a high sensitivity and specificity (Parola *et al.* 2000). This primer amplified a 305 bp fragment of the 16S rRNA gene region of members of the family Anaplasmataceae (Parola *et al.* 2000).

Bartonella prevalence and diversity was investigated using primer sets targeting two gene regions namely, the citrate synthase (*gltA*) and the NADH dehydrogenase gamma subunit (*nuoG*) genes (Chapter 3). The citrate synthase gene has been confirmed to provide good resolution at the species level for *Bartonella* and has been extensively used in the phylogenetic analyses of novel *Bartonella* strains (Birtles and Raoult 1996; La Scola *et al.* 2003). Recently the NADH dehydrogenase gamma subunit gene was shown to detect a wide diversity of *Bartonella* species, especially closely related species, with minimal mammalian host genome cross-reactivity (Colborn *et al.* 2010). As such, *nuoG* is a useful gene region to use in complement with the use of *gltA*.

Study outline

The objective of chapter 2 was to accurately identify all rodent species sampled using molecular methods in order to correctly assess their role in disease transmission. This chapter also aimed to identify which *Rhabdomys* mitochondrial lineages are present in the current sampling localities in an attempt to refine distributional data for the proposed species and subspecies within this previously monotypic genus. Chapter 3 investigated the diversity of bacteria associated with indigenous rodents. In this chapter, kidney tissues of commensal and wild rodent populations were screened with a broad range bacterial primer to identify bacterial species with potential to be spread by environmental contamination. In chapter 3 *Bartonella* was identified in both commensal and wild rodent populations at high prevalences. Chapter 4 compared the *Bartonella* prevalence and diversity between wild and

commensal populations of *M. coucha*. The ectoparasite loads were also determined for both *M. coucha* populations. As *Bartonella* contains vector-borne diseases with zoonotic potential, this chapter also assessed whether ectoparasite loads influence *Bartonella* prevalence. Chapter 5 determined the prevalence and diversity of tick-borne bacteria of the family Anaplasmataceae within and among commensal rodent species. In addition the tick load was determined for commensal rodent species in an attempt to evaluate how tick load influences bacterial prevalence. Chapter 6 presents a general discussion and conclusions of the findings of the present study as well as highlighting its limitations and recommendations for future studies.

References

Ahmad, E., Hussain, I. and Brooks, J.E. 1995. Losses of stored foods due to rats at grain markets in Pakistan. *International Biodeterioration & Biodegradation* **95**: 125-133.

Alderton, D. 1996. *Rodents of the world*. Blandford, London.

Amann, R.I., Ludwig, W. and Schleifer, K. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews* **59**: 143-169.

American Association for Laboratory Animal Science. Use of Animals in Biomedical Research: Understanding the Issues. Available at www.aalas.org/pdf/08-00007 [accessed on 25 February 2014].

Animal Care and Use Committee. 1998. Guidelines for the capture, handling and care of mammals as approved by the American Society of Mammalogists. *Journal of Mammalogy* **79**: 1416-1431.

Archer, C.E., Appleton, C.C., Mukaratirwa, S. and Hope, K.J. 2011. The rat lung-worm *Angiostrongylus cantonensis*: A first report in South Africa. *South African Medical Journal* **101**: 174-175.

Arntzen, L.,Wadee, A.A. and Isaäcson, M. 1991. Immune responses of two *Mastomys* sibling species to *Yersinia pestis*. *Infection and Immunity* **59**: 1966-1971.

Atkinson, I.A.E. 1985. Spread of commensal *Rattus* to oceanic islands and their effect on island avifaunas. In: Conservation of island birds: case studies for the management of threatened island species, (ed.) P.J. Moors. International Council for Bird Preservation. *Technical Publication* **3**: 35-81.

Azad, A.F. and Beard, C.B. 1998. Rickettsial pathogens and their arthropod vectors. *Emerging Infectious Diseases* **4**: 179-186.

Bai, Y., Kosoy, M.Y., Ray, C., Brinkerhoff, R.J. and Collinge, S.K. 2008. Temporal and spatial patterns of *Bartonella* infection in Black-tailed prairie dogs (*Cynomys ludovicianus*). *Microbial Ecology* **56**: 373-382.

Bastos, A.D.S., Chimimba, C.T., von Maltitz, E., Kirsten, F. and Belmain, S. 2005. Identification of rodent species that play a role in disease transmission to humans in South Africa. *Proceedings of the Southern African Society for Veterinary Epidemiology and Preventive Medicine*, pp 78-83.

Bastos, A.D.S. 2007. *Bartonella* incidence and diversity in endemic South African murid rodents occurring commensally with humans. *Proceedings of the Southern African Society for Veterinary Epidemiology and Preventive Medicine*, pp 76-81.

Bastos, A.D.S., Nair, D., Taylor, P.J., Brettschneider, H., Kirsten, F., Mostert, E., Von Maltitz, E., Lamb, J.M., Van Hooft, P., Belmain, S.R., Contrafatto, G., Downs, S. and Chimimba, C.T. 2011. Genetic monitoring detects an overlooked cryptic species and reveals the diversity and distribution of three invasive *Rattus* congeners in South Africa. *BMC Genetics* **12**: 26.

Biccard, A. and Midgley, J.J. 2009. Rodent pollination in *Protea nana*. *South African Journal of Botany* **75**: 720-725.

Birtles, R.J. and Raoult, D. 1996. Comparison of partial Citrate Synthase gene (*gltA*) sequences for phylogenetic analysis of *Bartonella* species. *International Journal of Systematic Bacteriology* **46**: 891-897.

Bradley, R.D. and Baker, R.J. 2001. A test of the genetic species concept: Cytochrome *b* sequences and mammals. *Journal of Mammalogy* **82**: 60-973.

Breitschwerdt, E.B. and Kordick, D.L. 2000. *Bartonella* infection in animals: carriership, reservoir potential, pathogenicity, and zoonotic potential for human infection. *Clinical Microbiology Reviews* **13**: 428-438.

Brettschneider, H. 2010. *Bartonella* dynamics in indigenous and invasive murid rodents from South Africa: Prevalence, diversity and mathematical modelling. PhD Thesis, University of Pretoria, Pretoria, South Africa.

Brettschneider, H., Bennett, N.C., Chimimba, C.T. and Bastos, A.D.S. 2012. Bartonellae of the Namaqua rock mouse, *Micaelamys namaquensis* (Rodentia: Muridae) from South Africa. *Veterinary Microbiology* **157**: 132-136.

Bronner, G.N., Hoffman, M., Taylor, P.J., Chimimba, C.T., Best, P.B., Matthee, C.A. and Robinson, T.J. 2003. *A revised systematic checklist of the extant mammals of the southern African subregion*. *Durban Museum Novitates* **28**: 56-106.

Brown, C. 2004. Emerging zoonoses and pathogens of public health significance - an overview. *Revue Scientifique et Technique Office International des Epizooties* **23**: 435-442.

Buffet, J., Kosoy, M. and Vayssier-Taussat, M. 2013. Natural history of *Bartonella*-infecting rodents in light of new knowledge on genomics, diversity and evolution. *Future Microbiology* **8**: 1117-1128.

Butler, T., Fu, Y., Furman, L., Almeida, C. and Almeida, A. 1982. Experimental *Yersinia pestis* infection in rodents after intragastric inoculation and ingestion of bacteria. Infection and Immunity **36**:1160-1167.

Castiglia, R., Solano, E., Makundi, R.H., Hulselmans, J., Verheyen, E. and Colangelo, P. 2012. Rapid chromosomal evolution in the mesic four-striped grass rat *Rhabdomys*

dilectus (Rodentia, Muridae) revealed by mtDNA phylogeographic analysis. *Journal of Zoological Systematics and Evolutionary Research* **50**: 165-172.

Castley, J. G., Bruton, J.S., Kerley, G. I. H. and McLachlan, A. 2001. The importance of seed dispersal in the Alexandria coastal dunefield, South Africa. *Journal of Coastal Conservation* **7**: 57-70.

Christova, I. and Gladnishka, T. 2005. Prevalence of infection with *Francisella tularensis, Borrelia burgdorferi* sensu lato and *Anaplasma phagocytophilum* in rodents from an endemic focus of Tularemia in Bulgaria. *Annals of Agriculture and Environmental Medicine* **12**: 149-152.

Clarridge, J.E. 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews* **17**: 840-862.

Colborn, J.M., Kosoy, M.Y., Motin, V.L., Telepnev, M.V., Valbuena, G., Myint, K.S., Fofanov, Y., Putonti, C., Feng, C. and Peruski, L. 2010. Improved detection of *Bartonella* DNA in mammalian hosts and arthropod vectors using the NADH dehydrogenase gamma subunit (*nuoG*) by real-time PCR. *Journal of Clinical Microbiology* **48**: 4630-4633.

Comer, J.A., Paddock, C.D. and Childs, J.E. 2001. Urban zoonoses caused *by Bartonella, Coxiella, Ehrlichia* and *Rickettsia* species. *Vector Borne and Zoonotic Diseases* **1**: 91-118.

Comunian, L.B., Moura, S.B., Paglia, A.P., Nicoli, J.R., Guerra, J.B., Rocha, G.A. and Queiroz, D.M.M. 2006. Detection of *Helicobacter* species in the gastrointestinal tract of wild rodents from Brazil. *Current Microbiology* **53**: 370-373.

Davis, D.H.S. 1953. Plague in South Africa: a study of the epizootic cycle in gerbils (*Tatera brantsi*) in the northern Orange Free State. *The Journal of Hygiene* **51**: 427-449.

De Graaff, G. 1981. *The rodents of Southern Africa*. Butterworth, Durban.

Doern, G.V. 2000. Detection of selected fastidious bacteria. *Clinical Infectious Diseases* **30**: 166-173.

Ducroz, J-F., Volobouev, V. and Granjon, L. 2001. An assessment of the systematics of Arvicanthine rodents using mitochondrial DNA sequences: evolutionary and biogeographical implications. *Journal of Mammalian Evolution* **8**: 173-206.

Drummond, D.C. 1975. Rodent problems in developing countries. *Ecological Bulletin* **19**: 65-71.

Eldridge, D.J., Whitford, W.G. and Duval, B.D. 2009. Animal disturbances promote shrub maintenance in a desertified grassland. *Journal of Ecology* **97**: 1302-1310.

Ferquel, E., Garnier, M., Marie, J., `de-Bauduin, C.B., Baranton, G., Perez-Eid, C. and `le Postic, D. 2006. Prevalence of *Borrelia burgdorferi* sensu lato and Anaplasmataceae members in *Ixodes ricinus* ticks in Alsace, a focus of Lyme borreliosis endemicity in France. *Applied and Environmental Microbiology* **72**: 3074-3078.

Fichet-Calvet, E., Lecompte, E., Koivogui, L., Soropogui, B., Doré, A., Kourouma, F., Sylla, O., Daffis, S., Koulémou, K. Ter meulen, J. 2007. Fluctuation of abundance and Lassa virus prevalence in *Mastomys natalensis* in Guinea, West Africa. *Vector-borne and Zoonotic Diseases* **7**: 119-128.

Forget, P. 1997. Effect of microhabitat on seed fate and seedling performance in two rodent-dispersed tree species in rain forest in French Guiana. *Journal of Ecology* **85**: 693-703.

Frean, J., Arndt, S. and Spencer, D. 2002. High rate of *Bartonella henselae* infection in HIV-positive outpatients in Johannesburg, South Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **96**: 549-550.

Frebourg, N.B., Berthelot, G., Hocq, R., Chibani, A. and Lemeland, J. 2002. Septicemia due to *Pasteurella pneumotropica*: 16S rRNA sequencing for diagnosis confirmation. *Journal of Clinical Microbiology* **40**: 687-689.

Gaastra, W., Boot, R., Ho, H.T.K. and Lipman, L.J.A. 2009. Rat bite fever. *Veterinary Microbiology* **133**: 211-228.

Godeluck, B., Duplantier, J., Ba, K. and Trape, J. 1994. A Longitudinal survey of *Borrelia crocidurae* prevalence in rodents and insectivores in Senegal. *American Journal of Tropical Medicine and Hygiene* **50**: 165-168.

Gratz, N.G. 1997. The burden of rodent-borne diseases in Africa south of the Sahara. *Belgian Journal of Zoology* **127**: 71-84.

Gratz, N.G. 1999. Urbanization, arthropod and rodent pests and human health. In: *Proceedings of the 3rd International Conference on Urban Pests*, (eds) W.H. Robinson, F. Rettich and G.W. Rambo. pp 51-59.

Gubler, D.J., Reiter, P., Ebi, K.L., Yap, W., Nasci, R. and Patz, J.A. 2001. Climate variability and change in the United States: potential impacts on vector- and rodent-borne diseases. *Environmental Health Perspectives* **109**: 223-233.

Hauben, L., Vauterin, L., Swings, J. and Moore, E.R.B. 1997. Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. *International Journal of Systematic Bacteriology* **47**: 328-335.

Harris, D.B. and Macdonald, D.W. 2007. Interference competition between introduced black rats and endemic Galápagos rice rats. *Ecology* **88**:2330-234.

Hauschild, T., Slizewskia, P. and Masiewicz, P. 2010. Species distribution of staphylococci from small wild mammals. *Systematic and Applied Microbiology* **33***:* 457-460

Heisch, R.B. 1956. Zoonoses as a study in ecology: with special reference to plague, relapsing fever, and Leishmaniasis. *The British Medical Journal* **2**: 669-673.

Holt, J., Davis, S. and Leirs, H. 2006. A model of Leptospirosis infection in an African rodent to determine risk to humans: Seasonal fluctuations and the impact of rodent control. *Acta Tropica* **99**: 218-225.

Howald, G., Donlan, C.J., Faulkner, K.R., Ortega, S., Gellerman, H., Croll, D.A. and Tershy, B.R. 2009. Eradication of black rats *Rattus rattus* from Anacapa Island. *Oryx* **44**: 30-40.

Huchon, D., Madsen, O., Sibbald, M.J.J.B., Ament, K., Stanhope, M.J., Catzefis, F., De Jong, W.W. and Douzery, E.J.P. Rodent phylogeny and a timescale for the evolution of glires: Evidence from an extensive taxon sampling using tree nuclear genes. *Molecular Biology and Evolution* **19**: 1053-1065.

Irwin, D.M., Kocher, T.D. and Wilson, A.C. 1991. Evolution of the Cytochrome *b* gene of mammals. *Journal of Molecular Evolution* **32**: 128-144.

Isaäcson, M., Arntzen, L. and Taylor, P. 1981. Susceptibility of members of the *Mastomys natalensis* species complex to experimental infection with *Yersinia pestis*. *Journal of Infectious Diseases* **144**: 80.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L. and Daszak, P. 2008. Global trends in emerging infectious diseases. *Nature* **451**: 990-993.

Jori, F. and Chardonnet, P. 2001. Cane rat farming in Gabon. Status and perspective. 5th International Wildlife Ranching Symposium, Pretoria, South Africa.

Julius, R.S. 2013. Molecular prevalence and diversity of zoonotic bacteria of invasive *Rattus* from South Africa, with emphasis on the genera *Rickettsia* and *Streptobacillus*. MSc Thesis, University of Pretoria, Pretoria, South Africa.

Kaiser, P.O., Riess, T., O'Rourke, F., Linke, D., Kempf, V.A.J., 2011. *Bartonella* spp.: Throwing light on uncommon human infections. *International Journal of Medical Microbiology* **301**: 7-15.

Keesing, F. 2000. Cryptic consumers and the ecology of an African Savanna. *Bioscience* **50**: 205-215.

Khaldi, M., Socolovschi, C., Benyettou, M., Barech, G., Biche, M., Kernif, T., Raoult, D. and Parola, P. 2012. Rickettsiae in arthropods collected from the North African hedgehog (*Atelerix algirus*) and the desert hedgehog (*Paraechinus aethiopicus*) in Algeria. *Comparative Immunology, Microbiology and Infectious Diseases* **35**: 117-122.

Khiem, N.T., Cuong, L.Q. and Chien, H.V. 2003. Market study of meat from field rats in the Mekong delta. In: *Rats, mice and people: rodent biology and management*, (eds) G.R. Singleton, L.A. Hinds, C.J. Krebs and Spratt, D.M. pp. 543-547. Australian Centre for International Agricultural Research, Canberra, Australia.

Konečný, A., Estoup, A., Duplantier, J., Bryja, J., Khalilou, B.Â., Galan, M., Tatard, C. and Cosson, J. 2013. Invasion genetics of the introduced black rat (*Rattus rattus*) in Senegal, West Africa. *Molecular Ecology* **22**: 286-300.

Krasnov, B.R., Shenbrot, G.I., Khokhlova, I.S. and Degen, A.A. 2004. Flea species richness and parameters of host body, host geography and host 'milieu'. *Journal of Animal Ecology* **73**: 1121-1128.

La Scola, B., Zeaiter, Z., Khamis, A. and Raoult, D. 2003. Gene-sequence-based criteria for species definition in bacteriology: the *Bartonella* paradigm. *Trends in Microbiology* **11**: 318-321.

Lecompte, E., Brouat, C., Duplantier, J., Galan, M., Granjon, L., Loiseau, A., Mouline, K. and Cosson, J. 2005. Molecular identification of four cryptic species of *Mastomys* (Rodentia, Murinae). *Biochemical Systematics and Ecology* **33**: 681-689.

Leirs, H., Verhagen, R., Verheyen, W., Mwanjabe, P. and Mbise, T. 1996. Forecasting rodent outbreaks in Africa: an ecological basis for Mastomys control in Tanzania. *Journal of Applied Ecology* **33**: 937-943.

Lima, M., Stenseth, N.C., Leirs, H. and Jaksic, F.M. 2003. Population dynamics of small mammals in semi-arid regions: a comparative study of demographic variability in two rodent species. *Proceedings: Biological Sciences* **270**: 1997-2007.

Makundi, R.H., Massawe, A.W., Mulungu, L.S., Katakeba, A., Mbisi, T.J. and Mgode, G. 2008. Potential mammalian reservoirs in a bubonic plague outbreak focus in Mbulu district, Northern Tanzania, in 2007. *Mammalia* **72**: 253-257.

Malan, G. and Crowe, T.M. 1996. The diet and conservation of monogamous and polyandrous pale chanting goshawks in the Little Karoo, South Africa. *South African Journal of Wildlife Research* **26**: 1-10.

Matisoo-Smith, E. and Robins, J.H. 2004. Origins and dispersals of Pacific peoples: evidence from mtDNA phylogenies of the Pacific rat. *Proceedings of the National Academy of Science* **101**: 9167-9172.

Matthee, S., Horak, I.G., Beaucournu, J., Durden, L.A., Ueckermann, E.A. and McGeoch , M.A. 2007. Epifaunistic arthropod parasites of the four-striped mouse, *Rhabdomys pumilio*, in the Western Cape Province, South Africa. *Journal of Parasitology* **93**: 47-59.

McMichael, A. J. 2004. Environmental and social influences on emerging infectious diseases: past, present and future. *Philosophical Transactions: Biological Sciences* **359**: 1049-1058.

Meerburg, B.G. and Kijlstra, A. 2007. Role of rodents in transmission of *Salmonella* and *Campylobacter*. *Journal of the Science of Food and Agriculture* **87**: 2774-2781.

Meerburg, B.G., Singleton, G.R. and Leirs, H. 2009a. The Year of the Rat ends - time to fight hunger! *Pest Management Science* **65**: 351-352.

Meerburg, B.G., Singleton, G.R. and Kijlstra, A. 2009b. Rodent-borne diseases and their risks for public health. *Critical Reviews in Microbiology* **35**: 221-270.

Meester, J.A.J., Rautenbach, I.L. and Dippenaar, N.J. and Baker, C.M. 1986. Classification of southern African mammals. *Transvaal Museum Monograph* **5**: 1-359.

Mills, J.N. and Childs, J.E. 1998. Ecologic studies of rodent reservoirs: their relevance for human health. *Emerging Infectious Diseases* **4**: 529-537.

Morand, S. Krasnov, B.R. and Poulin, R. 2006. *Micromammals and Macroparasites: From Evolutionary Ecology to Management*. Springer-Verlag, Tokyo.

Mostert, M.E. 2009. Molecular and morphological assessment of invasive, inland *Rattus* (Rodentia: Muridae) congenerics in South Africa and their reservoir host potential with respect to *Helicobacter* and *Bartonella*. MSc Thesis, University of Pretoria, Pretoria, South Africa.

Muteka, S.P., Chimimba, C.T. and Bennett, N.C. 2006. Reproductive seasonality in *Aethomys namaquensis* (Rodentia: Muridae) from Southern Africa. *Journal of Mammalogy* **87**: 67-74.

Mwanjabe, P.S., Sirima, F.B. and Lusingu, J. 2002. Crop losses due to outbreaks of *Mastomys natalensis* (Smith 1834) Muridae, Rodentia, in the Lindi Region of Tanzania. *International Biodeterioration & Biodegradation* **49**: 133-137.

Neerinckx, S., Bertherat, E. and Leirs, H. 2010. Human plague occurrences in Africa: an overview from 1877 to 2008. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **104**: 97-103.

Neubauer, H., Rahalison, L., Brooks, T.J., Aleksic, S., Chanteau, S. and Splettstösser, W.D. 2000. Serodiagnosis of human plague by an Anti-F1 capsular antigen specific IgG/IgM ELISA and immunoblot. *Epidemiology and Infection* **125**: 593-597.

Parola, P., Roux, V., Camicas, J.L., Baradji, I., Brouqui, P. and Raoult, D. 2000. Detection of ehrlichiae in African ticks by PCR. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **94**: 707-708.

Parola, P., Davoust, B. and Raoult, D. 2005. Tick- and flea-borne rickettsial emerging zoonoses. *Veterinary Research* **36**: 469-492.

Parola, P. 2011. *Rickettsia felis*: from a rare disease in the USA to a common cause of fever in sub-Saharan Africa. *Clinical Microbiology and Infection* **17**: 996-1000.

Perrin, M.R. and Bodbijl, T. 2001. Diet and prey selection of the Gaboon adder in Zululand (KwaZulu-Natal), South Africa. *South African Journal of Wildlife Research* **31**: 127-134.

Perry, N.D., Hanson, B., Hobgood, W., Lopez, R.L., Okraska, C.R., Karem, K., Damon, I.K. and Carroll, D.S. 2006. New invasive species in Southern Florida: Gambian Rat (*Cricetomys gambianus*). *Journal of Mammalogy* **87**: 262-264.

Pimentel, D., Lach, L., Zuniga, R. and Morrison, D. 2000. Environmental nd economic costs of non-indigenous species in the United States. *BioScience* **50**: 53-65.

Pretorius, A., Beati, L. and Birtles, R.J. 2004. Diversity of Bartonellae associated with small mammals inhabiting Free State province, South Africa. *International Journal of Systematic and Evolutionary Microbiology* **54**: 1959-1967.

Rambau, R.V., Robinson, T.J. and Stanyo, R. 2003. Molecular genetics of *Rhabdomys pumilio* subspecies boundaries: mtDNA phylogeography and karyotypic analysis by fluorescence in situ hybridization. *Molecular Phylogenetics and Evolution* **28**: 564-575.

Raoult, D. and Roux, V. 1997. Rickettsioses as paradigms of new or emerging infectious diseases. *Clinical Microbiology Reviews* **10**: 694-719.

Rar, V.A. and Golovljova, I. 2011. *Anaplasma*, *Ehrlichia*, and ‘‘Candidatus *Neoehrlichia’’* bacteria: Pathogenicity, biodiversity, and molecular genetic characteristics, a review. *Infections Genetics and Evolution* **11**: 1842-1861.

Rebaudet, S. and Parola, P. 2006. Epidemiology of relapsing fever borreliosis in Europe. *FEMS Immunology and Medical Microbiology* **48**: 11-15.

Shepherd, A. J., Hummitzsch, D. E., Leman, P. A., Swanepoel, R. and Searle, L.A. 1986. Comparative tests for detection of plague antigen and antibody in experimentally infected wild rodents. *Journal of clinical microbiology* **24**: 1075-1078.

Skinner, J.D. and Chimimba, C.T. 2005. *The Mammals of the Southern African Subregion*. Cambridge University Press, Cape Town.

Skonhoft, A., Leirs, H., Andreassen, H.P., Mulungu, L.S. and Stenseth, N.C. 2006. The bioeconomics of controlling an African rodent pest species. *Environment and Development Economics* **11**: 453-475.

Spotorno, A.E., Zuleta, V.A., Valladares, J.P., Deane, A.L. and Jiménez, J.E. 2004. *Chinchilla laniger*. *Mammalian Species* **758**: 1-9.

Stenseth, N.C., Leirs, H., Mercelis, S. and Mwanjabe, P. 2001. Comparing strategies for controlling an African pest rodent: An empirically based theoretical study. *Journal of Applied Ecology* **38**: 1020-1031.

Stenseth, N.C., Leirs, H., Skonhoft, A., Davies, S.A., Pech, R.P., Andreassen, H.P., Singleton, G.R., Lima, M., Machangu, R.M., Makundi, R.H., Zhang, Z., Brown, P.B., Shi, D. and Wan, X. 2003. Mice, rats and people: the bioeconomics of agricultural rodent pests. *Frontiers in Ecology and the Environment* **1**: 367-375.

Stenseth, N.C., Atshabar, B.B., Begon, M., Belmain, S.R., Bertherat, E.C., Carniel, E., Gage, K.L, Leirs, H. and Rahalison, L. 2008. Plague: Past, present, and future. *PLoS Medicine* **5**: 9-14.

Taylor, P.J., Arntzen, L., Hayter, M., Iles, M., Frean, J and Belmain, S. 2008. Understanding and managing sanitary risks due to rodent zoonoses in an African city: beyond the Boston Model. *Integrative Zoology* **3**: 38-50.

Tissot-Dupont, H.T., Brouqui, P., Faugere, B. and Raoult, D. 1995. Prevalence of antibodies to *Coxiella burnetii*, *Rickettsia conorii*, and *Rickettsia typhi* in seven African countries. *Clinical Infectious Diseases* **21**: 1126-1133.

Tissot-Dupont, H., La Scola, B., Williams, R. and Raoult, D. 1997. A focus of tick-borne relapsing fever in southern Zaire. *Clinical Infectious Diseases* **25**: 139-144.

Van Der Linden, M., Al-Lahham, A., Nicklas, W. Reinert, R.R. 2009. Molecular characterization of pneumococcal isolates from pets and laboratory animals. *Plos One* **4**: e8286.

Willan, K. 1992. *Problem rodents and their control: A handbook for South African farmers, foresters and smallholders*. University of Natal, Durban.

Zaytseva, E., Ermolaeva, S. and Somov, G.P. 2007. Low genetic diversity and epidemiological significance of *Listeria monocytogenes* isolated from wild animals in the far east of Russia. *Infection Genetics and Evolution* **7**: 736-746.

Chapter 2

Molecular identification of cryptic murid rodent species from South Africa: implications for zoonotic disease epidemiology

Abstract

Cryptic species lack distinguishable external morphological characteristics and often occur sympatrically. Cryptic species can seriously hamper epidemiological studies, where host identification is crucial for understanding disease dynamics. The aim of the present study was firstly to correctly identify cryptic indigenous murid rodent species occurring commensally in the Gauteng Province in South Africa, in order to accurately assess their role in potential disease transmission. Furthermore, a number of recent studies have described the presence of several mitochondrial lineages within *Rhabdomys* which was previously considered to be a monotypic genus. Therefore, the second aim of the study was to identify which *Rhabdomys* mitochondrial lineages are present in the current sampling localities. Partial cytochrome *b* sequences were generated for all rodent specimens collected. The third aim of the present study was to confirm species-specific multiplex PCR specificity for rapid differentiation between two cryptic species of the genus *Mastomys*, namely *M. natalensis* and *M. coucha*, without the need for further sequencing. Phylogenetic analyses revealed the presence of three murid species belonging to cryptic species complexes (*Mastomys coucha*, *Aethomys ineptus* and *Otomys angoniensis*). Cytochrome *b* phylogenetic analyses identified four *Rhabdomys* samples corresponding to the proposed subspecies *Rhabdomys dilectus chakae* and the

remaining 32 belonging to the proposed subspecies *R. d. dilectus*. Both proposed subspecies within *R. dilectus* were sampled from Hammanskraal and from the University of Pretoria Experimental farm, providing tentative evidence of a contact zone. The species-specific multiplex PCR primers identified all cryptic *Mastomys* samples to species level accurately, without the need for further sequencing. The present study not only contributes to the refinement of rodent distributional maps but also to future zoonotic epidemiological research in southern Africa.

Keywords: Cryptic rodents, *Aethomys*, *Mastomys*, *Otomys*, *Rhabdomys*, cytochrome *b*, Muridae

Introduction

Rodents of the family Muridae have for centuries had a massive impact on humankind (Meerburg *et al.* 2009a). As pest species, they have not only been associated with the spread of zoonotic diseases but also with damage to food crops, stored food and native ecosystems (Stenseth *et al.* 2003; Howald *et al.* 2009; Meerburg *et al.* 2009b). The family Muridae belongs to a species-rich group of mammals, the superfamily Muroidea (Musser and Carleton 2005). Murid rodents constitute the largest mammalian family in the world and have a worldwide distribution, with more than 50 species represented in southern Africa (Skinner and Chimimba 2005). There is, however, uncertainty about the taxonomic status of many members within the family (Skinner and Chimimba 2005). This is a particular concern among the African members of this family, where several cryptic species complexes have been identified over the last 30 years (Meester *et al.* 1986; Bronner *et al.* 2003; Skinner and Chimimba 2005).

These species complexes usually consist of cryptic species that are morphologically indistinguishable and often occur sympatrically (Lecompte *et al.* 2005; Russo *et al.* 2006). The most well-known example of a species complex in southern Africa is that of the genus *Mastomys* (Taylor 2000). Green *et al.* (1980) recognized the presence of two sibling chromosomal species, having different diploid numbers (2*n*), within what was traditionally considered to be a single species. This discovery subsequently led to the recognition of two distinct species, namely, *M. natalensis* (2*n* = 32) and *M. coucha* (2*n* = 36) (Green *et al.* 1980; Gordon and Watson 1986).

Recently, species complexes have also been identified in other southern African murid genera (Bronner *et al.* 2003; Skinner and Chimimba 2005). Several studies found that

Aethomys chrysophilus comprised two morphologically identical but cytogenetically distinct species (Gordon and Rautenbach 1980; Gordon and Watson 1986). In a subsequent systematic revision based on morphometric and qualitative morphological data and the examination of type specimens; two distinct species, *A. ineptus* (2*n* = 44) and *A. chrysophilus* (2*n* = 50), were recognised (Chimimba 1998; Chimimba *et al.* 1999). This delimitation was subsequently confirmed by molecular data (Russo *et al.* 2006).

Otomyines represent another widely distributed taxonomically uncertain group of African rodents, however, their phylogenetic relationships have only recently been studied (Maree 2002; Engelbrecht *et al*. 2011). Within this group of rodents, *Otomys angoeniensis* (2*n* = 56) and *O. irroratus* (2*n* = 23-32) are often confused when identified based on morphological characteristics alone (Bronner and Meester 1988). This is potentially problematic given the recent identification of at least five morphologically similar but distinct cytotypes within *O. irroratus* (Taylor 2000; Maree 2002).

The genus *Rhabdomys* represents yet another African group of rodents whose taxonomy has been re-assessed in the last decade. *Rhabdomys* is a widespread murid genus endemic to Africa that is generally found in grassland (Skinner and Chimimba 2005). This genus is grouped among Arvicanthine rodents, together with the genera *Arvicanthis, Desmomys, Lemniscomys, Mylomys and Pelomys* (Ducroz *et al.* 2001; Lecompte *et al.* 2008). *Rhabdomys* occurs throughout southern Africa, including most of Namibia, Botswana, Zimbabwe, Mozambique, Swaziland, Lesotho, and South Africa but has a limited range in eastern Africa where it is restricted to small areas in Zambia, Malawi, Tanzania, Democratic Republic of Congo (DRC) and Kenya (Musser and Carleton 2005).

The taxonomy of the genus *Rhabdomys* has been problematic (Castiglia *et al.* 2012). It has historically been considered to be a monotypic genus containing only one species *R. pumilio*, with a varying number of subspecies (Skinner and Chimimba 2005). As early as 1905, four distinct groups were identified within the genus and were referred to as subspecies, a view that was accepted by several authors (Musser and Carleton 1993). Based on pelage colour, body size and morphological characteristics, such as tail length; Roberts (1951) described 20 subspecies from southern Africa. However, some authors disputed the number of subspecies described (Skinner and Chimimba 2005). De Graaf (1981) suggested the possibility of two subspecies, whereas Misonne (1974) maintained *Rhabdomys* to be a monotypic genus. In a subsequent review only seven of the subspecies proposed by Roberts (1951) were retained (Meester *et al.* 1986). Karyotypic and allozyme studies revealed the presence of two different karyotypes ($2n = 46$ and $2n = 48$) and restricted gene flow between widely separated populations in southern Africa (Ducroz *et al.* 1999; Mahida 1999; Taylor 2000). Breeding and behavioural studies were also not able to provide conclusive information on specific and subspecific boundaries (Pillay 2000a; b; Pillay *et al.* 2006).

Subsequent molecular phylogenetic analysis of cytochrome *b* DNA sequences revealed the presence of two distinct mitochondrial lineages within *Rhabdomys* which suggest that in southern Africa this genus contains at least two species, namely *R. pumilio* and the newly erected *R. dilectus* (Rambau *et al.* 2003). Lighter coloured animals with a diploid number of $2n = 48$ from the drier western and southern central regions of South Africa, Namibia and Botswana to south-western Angola were assigned to *R. pumilio*; whereas darker individuals with diploid numbers of either $2n = 48$ or $2n = 46$ from the more mesic regions of southern and eastern Africa were assigned to *R. dilectus* (Rambau *et al.* 2003; Musser and Carleton 2005). Based on the two chromosomal forms and mtDNA subclades within *R. dilectus*, this

taxon was provisionally sub-divided into two subspecies. *Rhabdomys dilectus dilectus* (2*n* = 46) that occurs from eastern to southern Africa and *R. d. chakae* (2*n* = 48) that is restricted to South Africa (Rambau *et al.* 2003). Recent studies have also confirmed some of these mitochondrial lineages by nuclear DNA and have shown that both *R. pumilio* and *R. dilectus* are even more diverse both consisting of at least three distinct mtDNA lineages (Castiglia *et al.* 2012; du Toit *et al.* 2012). Although the above-mentioned studies included samples from a wide geographic range in southern and eastern Africa, the exact specific and subspecific distributional limits and possible contact zones are still not well-understood (Skinner and Chimimba 2005). This taxonomic uncertainty has implications for epidemiological studies and therefore a comprehensive taxonomic revision of this genus is needed.

The lack of distinguishable external morphological characteristics between cryptic species not only hinders taxonomic and population studies of a species but also other comparative biological studies (Russo *et al.* 2006). Correct species identification is especially important during epidemiological studies, where host identification is crucial for understanding disease dynamics (Bastos *et al.* 2005). Comparative studies have shown two cryptic species, *M. coucha* and *M. natalensis* to differ in their susceptibility to experimental infection with the well-known plague bacterium, *Yersinia pestis* (Isaäcson *et al.* 1981; 1983; Arntzen *et al.* 1991). These studies found *M. natalensis* to be more resistant, while *M. coucha* was sensitive to experimental infection with *Y. pestis* (Arntzen *et al.* 1991). Experimental evidence suggests that different *Rhabdomys* lineages may also display differences in susceptibility to plague infection. *Rhabdomys* sampled from geographically distant populations in South Africa exhibited different levels of susceptibility to experimental plague infection (Sheppard *et al.* 1986). These studies highlight possible problems with previous epidemiological research involving members of cryptic species complexes, such as members

of the genera *Aethomys* and *Otomys*, which have been implicated in the transmission of zoonotic diseases (Davis *et al.* 1968; Hallet *et al.* 1970). Consequently, the importance of resolving the taxonomy and systematics of these cryptic species complexes surpasses the needs of pure systematics (Peppers and Bradley 2000).

In the past, protein electrophoresis, karyology and morphometrics were used to distinguish between these cryptic rodent species. However, these methods have several limitations. Firstly, they often require either fresh or frozen material and cannot be used for ethanol-fixed or museum samples and secondly, they involve invasive sampling and time-consuming laboratory analyses (Lecompte *et al.* 2005). They are therefore impractical for studies involving a large number of samples. Advances in molecular genetics and DNA sequencing during the last 15 years have provided an alternative method of distinguishing between these morphologically indistinguishable cryptic taxa (Castiglia *et al.* 2003).

The mitochondrial cytochrome *b* (cyt *b*) gene has been particularly useful in making species-level distinctions (Bradley and Baker 2001). Cytochrome *b* has been used to address the phylogeny of a wide range of taxa including rodents (Cantatore *et al.* 1994; Ducroz *et al.* 2001). This protein coding gene has evolved over the period spanning the origin of mammalian orders, it evolves more rapidly than nuclear genes and its rate of evolution is well-known (Irwin *et al.* 1991). Furthermore, the level of cytochrome *b* gene sequence divergence associated with species-level distinction has been well-documented (Bradley and Baker 2001). The aforementioned features and availability of cytochrome *b* data, makes this gene particularly useful for phylogenetic studies (Martin *et al.* 2000). Recently, the development of species-specific multiplex PCR reactions based on the cytochrome *b* gene

region have provided a cost- and time-effective alternative to sequencing, in identifying cryptic species (Bastos *et al.* 2005; Lecompt *et al.* 2005; van Sandwyk 2014).

On-going initiatives to assess the role of commensal rodents in the spread of zoonotic diseases in Gauteng Province in South Africa have mainly focussed on invasive, commensal species of the genus *Rattus*, namely *R. rattus*, *R. norvegicus* and *R. tanezumi* (Mostert 2009; Brettschneider 2010; Julius 2013). The present study therefore used phylogenetic analysis of cytochrome *b* sequences to genetically identify cryptic indigenous murid rodent species occurring commensally in the Gauteng Province in order to accurately assess their potential role in zoonotic disease transmission. Furthermore, the present study aimed to identify which *Rhabdomys* mitochondrial lineages are present in the current sampling localities in an attempt to refine distributional data for the proposed species and subspecies within the genus. Lastly, the study aimed to test the efficacy of a species-specific multiplex PCR reaction recently developed to quickly and cost-effectively distinguish between two cryptic *Mastomys* species, namely *M. natalensis* and *M. coucha* without the need for further sequencing (van Sandwyk 2014).

Materials and Methods

Sample collection

Commensal rodent samples were collected at 4-6 week intervals between August 2010 and July 2011 in Hammanskraal (25°42′60′′S, 28°27′20′′E, 1111 meters above sea level) and the University of Pretoria (UP) Experimental farm (25°75′11′′S, 28°26′29′′E, 1375 m.a.s.l., Figure 1). Animals were live-trapped using Sherman live traps (H.B. Sherman Traps Inc. Florida; U.S.A.) baited with a mixture of peanut butter, oatmeal and fish oil and processed as detailed in Chapter 1.

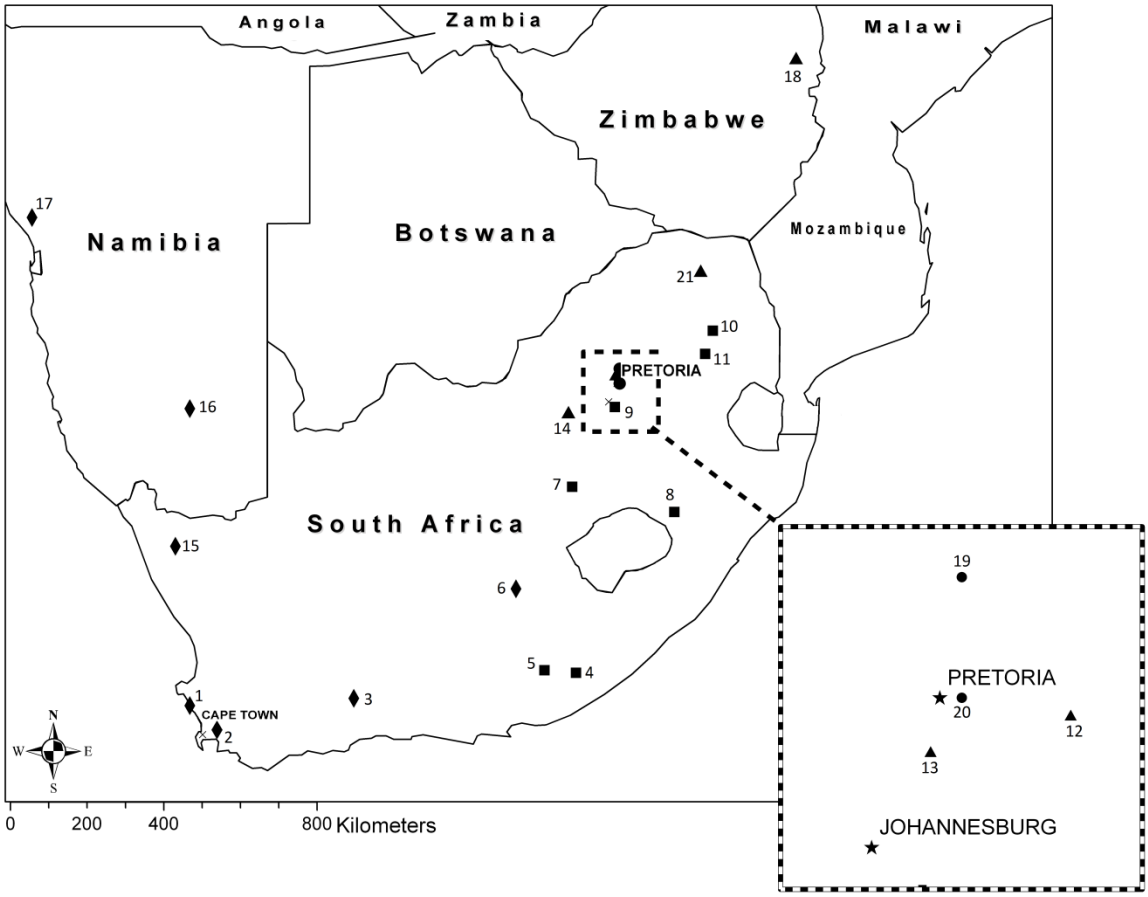

Figure 1. Sampling localities of all commensal rodents sampled in the current study (no: 19, 20; ●). Additional *Rhabdomys* samples collected in previous studies are also indicated (no: 1-18; Rambau *et al.* 2003; Castiglia *et al.* 2012; for detailed species composition and sampling locality data see Appendix I). *Rhabdomys* species and subspecies are represented by the following symbols: *R. pumilio* (♦), *R. d. chakae* (■), *R. d. dilectus* (▲).

Molecular techniques

Total genomic DNA was extracted from kidney tissue using the High Pure PCR template preparation kit (Roche) according to the prescribed manufacturer's protocol. Primers L14724 (GAYATGAAAAAYCATCGTTG) and H15915 (CATTTCAGGTTTACAAGAC) were used to target a 1200 base pair (bp) region of the mitochondrial genome, amplifying the entire cytochrome *b* gene (Bastos *et al.* 2005). PCRs were performed in a final reaction volume of 50 µl containing 1x Buffer (Fermentas), 0.25 µM dNTPs (Fermentas), 0.4 µM of L14724 and H15915 (IDT), 1U of DreamTaq™ DNA polymerase and 100-200 ng DNA template. A

touchdown PCR program was used with an initial denaturation of 20 s at 96° C, followed by two cycles of 12 s at 96° C, 25 s at 49° C and 60 s at 72° C. This was followed by three cycles of 12 s at 96° C, 20 s at 47° C and 55 s at 72° C. Subsequently, an additional 35 cycles of 12 s at 96° C, 15 s at 45° C and 50 s at 72° C was followed by a final extension of 60 s at 72° C. The resulting amplicon was purified directly from the tube using the High Pure PCR product purification kit (Roche) according to manufacturer's specifications. Nucleotide sequences were determined with L14724 and H15915 at an annealing temperature of 48° C by cycle sequencing with Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) v3.1. Sequences were precipitated and run on the ABI PRISM™ 3100 Analyser (Applied Biosystems). Nucleotide sequences were viewed and aligned in Mega5 (Tamura *et al.* 2011).

Molecular identification of cryptic *Mastomys* samples was done by specific multiplex PCR identification targeting the cytochrome *b* gene region in order to test the efficacy of a species-specific multiplex PCR reaction developed to distinguish between two cryptic *Mastomys* species, namely *M. natalensis* and *M. coucha* without the need for further sequencing (van Sandwyk 2014). Additionally, cytochrome *b* sequences were generated from a subset of 17 *Mastomys* samples with primers L14724 and H15915 (as detailed above) to verify species-specific multiplex results.

Phylogenetic analysis

Phylogenetic analyses were performed on two separate datasets. Dataset 1 included sequences generated from murid genera containing known cryptic species complexes (*Mastomys*, *Aethomys* and *Otomys*) and dataset 2 included samples of the genus *Rhabdomys* only. To correctly identify species belonging to the genera *Mastomys*, *Aethomys* and *Otomys*,

published cytochrome *b* reference sequences from the National Centre for Biotechnology Information database (GenBank; www.ncbi.nm.nih.gov/genbank) were incorporated in dataset 1 sequence alignments and phylogenetic analyses (Accession Numbers: *Aethomys chrysophilus*, AJ604526, AY585663; *Aethomys ineptus*, AY585667; *Otomys irroratus*, AY224677; *Otomys angoniensis*, AF492711, AM408343; *Mastomys natalensis*, AY554149, *Mastomys coucha*, AY 554159, AY554160) (Castiglia *et al.* 2003; Maree 2002; McKenzie *et al.* 2003; Michaux *et al.* 2007; Russo *et al.* 2006). For phylogenetic analyses of the *Rhabdomys* samples in dataset 2, the 36 sequences generated from the current study were aligned with all available cytochrome *b* sequences of *Rhabdomys* downloaded from GenBank (Accession Numbers AF141214, AF533083-AF533116, FR837633-FR837651) (Ducroz *et al.* 2001; Rambau *et al.* 2003; Castiglia *et al.* 2012). Sequences of *Rattus norvegicus* (NC001665) and *Mus musculus* (EF108340) were used as outgroups.

For both datasets 1 and 2, Neighbour Joining (NJ) analyses were performed in MEGA5 (Tamura *et al.* 2011), Maximum Likelihood (ML) analyses in PhyML (Guidon and Gascuel 2003) and Bayesian Inference (BI) with MrBayes (Huelsenbeck and Ronquist 2001) using the best-fit model and parameters identified under the Akaike Information Criterion (AIC), chosen with jModelTest (Posada 2008). For BI, four Markov Monte Carlo chains were run for 10,000,000 generations using default heating and swap settings, and were sampled every 100 generations. Trace files were viewed using Tracer version 1.5 (http://beast.bio.ed.ac.uk/) after which 20 % of the runs were discarded as burn-in. Additionally a Maximum Parsimony (MP) analysis was performed on dataset 2 (*Rhabdomys* dataset) using PAUP4.0b10 (Swofford 2000) and based on heuristic search and tree-bisection-reconnection (TBR) and random addition of sequences (10 replicates). Successive (*a posteriori*) weighting with the rescaled consistency (RC) was performed in order to reduce

the contribution of homoplasious characters in the parsimony analysis. Nodal support was determined for both datasets by 10,000 and 1,000 bootstrap replicates for NJ and ML, respectively and from Bayesian posterior probabilities (BPP). Nodal support for MP analyses performed on dataset 2 was determined by 1,000 bootstrap replicates. Pairwise uncorrected p-distances were determined in MEGA5 (Tamura *et al.* 2011).

Results

A total of 175 rodents from six genera (*Mastomys*, *Otomys*, *Aethomys*, *Rhabdomys*, *Lemniscomys* and *Saccostomus*) belonging to two families (Muridae and Nesomyidae) were sampled during the current study (Table 1). The species-specific multiplex PCR identified all 84 *Mastomys* samples as *M. coucha*.

Table 1. Number of rodent species sampled from Hammanskraal and the University of Pretoria Experimental farm, Gauteng Province, South Africa during present study. F = number of females, M = number of males, N = Total sample size (see Appendix I for detailed species composition and sampling locality data)

Family	Species	Hammanskraal			Experimental farm		
		F	M	N	F	M	N
Muridae	*Aethomys ineptus*	12	17	29			
	Lemniscomys rosalia		2	2			
	Mastomys coucha	13	53	66	4	14	18
	Otomys angoniensis	3	11	14	4	4	8
	Rhabdomys dilectus chakae	1	1	2	1	1	2
	R. d. dilectus	8	22	30	1	1	2
Nesomyidae	*Saccostomus campestris*		2	2			

Despite several attempts, usable cytochrome *b* sequences were not obtained from the two *Lemniscomys* and two *Saccostomus* samples and therefore they were not included in any phylogenetic analyses. Dataset 1 (*Mastomys*, *Aethomys* and *Otomys* cytochrome *b* dataset)

included 68 sequences from the current study and 10 reference sequences downloaded from GenBank. The 68 sequences generated from the current study were shortened to 370-bp to allow their comparison with reference sequences. This partial cyrochrome *b* dataset contained 104 variable and 84 parsimony-informative sites and had an average nucleotide composition of A = 0.332, C = 0.261, G = 0.141, T = 0.267. The TrN+G model (G = 0.224) model of sequence evolution was selected as the best fit model under the AIC in jModelTest (Posada 2008). The ML, BI and NJ analyses recovered the same tree topology, in terms of species clustering, and support indices are summarised on relevant nodes of the NJ tree (Figure 2).

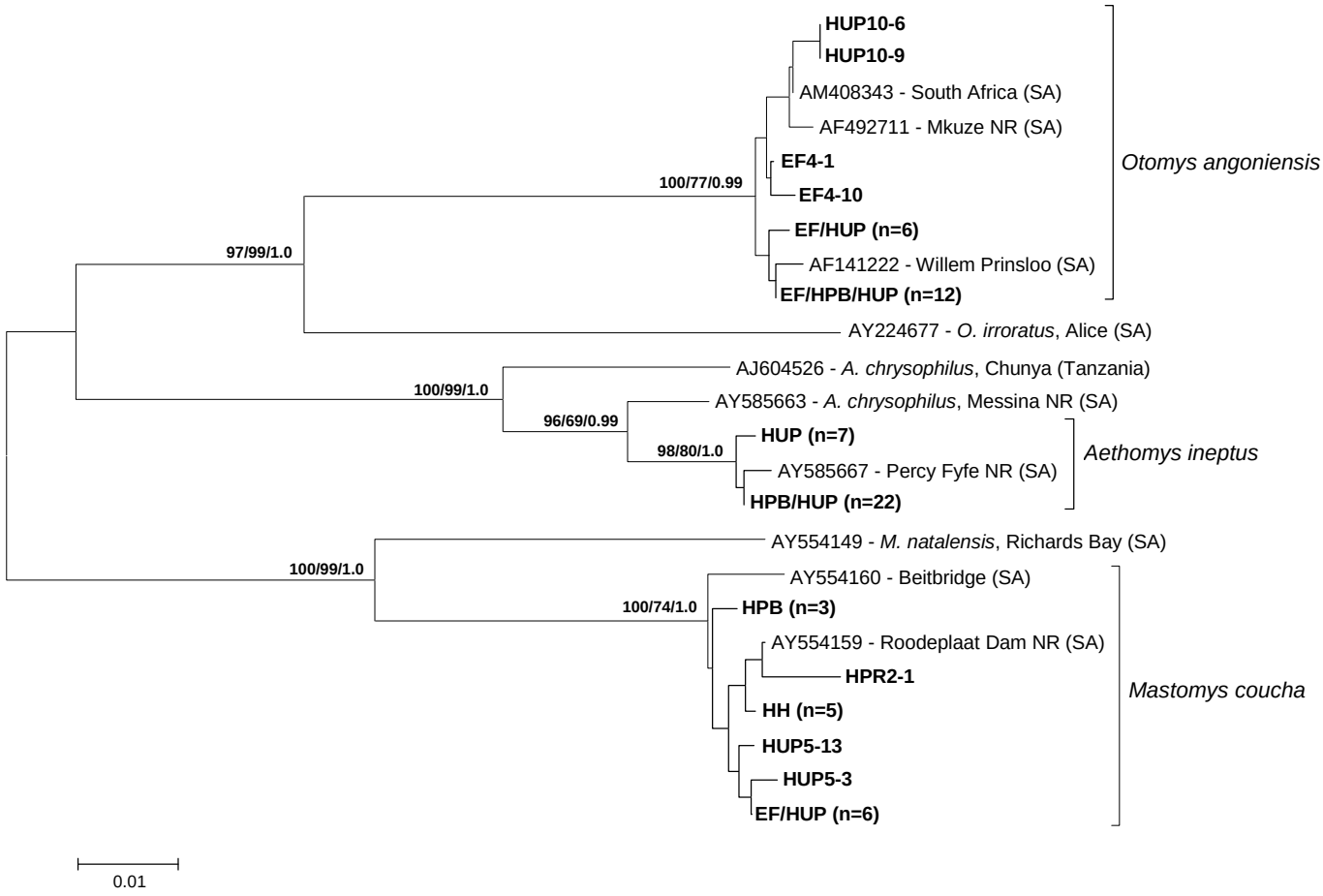

Figure 2. Neighbour Joining (NJ) tree inferred from partial cytochrome *b* gene sequence data (370 bp) for commensal *Mastomys*, *Otomys* and *Aethomys* sampled from Gauteng Province in South Africa from 2010-2011 (Dataset 1). Bootstrap support (> 70 %) and Bayesian posterior probabilities (BPP) (> 0.90) are indicated at relevant nodes (NJ/ML/BPP). Samples indicated in bold represent samples from the current study and are prefixed with EF/HUP/HPB/HH/HPR. Samples not in bold indicate accession numbers of the sequences downloaded from GenBank.

The partial cytochrome *b* gene tree (Figure 2) identified all *Aethomys* samples from this study as *A. ineptus* (NJ = 98 %, ML = 80 %). Of interest is the paraphyly of the Tanzanian (AJ604526) and southern African (AY585663) *A. chrysophilus* reference sequences. All the *Otomys* samples were identified as *O. angoniensis* based on 100 % and 77 % nodal support for NJ and ML analysis, respectively. Furthermore, based on 99 % (NJ) and 74 % (ML) nodal support, the partial gene tree confirms the species-specific PCR results, identifying all *Mastomys* samples from this study as *M. coucha*. Between-species distances were 3.3 % for the two cryptic species of *Aethomys* (*A. ineptus* and *A. chrysophilus*), 10 % for the two species of *Otomys* (*O. irroratus* and *O. angoniensis*) and 7.5 % for the two species of *Mastomys* (*M. coucha* and *M. natalensis*). However, within-species variation was low, ranging from 0.1- 0.7 %.

Dataset 2 (*Rhabdomys* cytochrome *b* dataset) contained 95 taxa, including reference sequences. This 869 bp dataset contained 338 variable and 251 parsimony-informative sites, with average nucleotide frequencies of A = 0.313, C = 0.294, G = 0.116, T = 0.277. As would be expected for a coding gene, the proportion of base position mutations was $3^{rd} > 1^{st} > 2^{nd}$, with 233 (69 %) of the mutations occurring in the 3^{rd} base position, 74 (22 %) in the 1^{st} base position and the remaining 32 (9 %) being attributed to 2^{nd} base position mutations. The TPM1uf+I+G (I=0.512, G = 2.274) model of sequence evolution was selected as the best-fit model under the AIC in jModelTest. The parsimony analysis saved 108 equally parsimonious trees (Tree Length (TL) = 644 steps, Consistency Index (CI) = 0.52, Retention Index (RI) = 0.89, Rescaled Consistency Index (RC) = 0.46). Reweighting with the RC resulted in 18 equally parsimonious trees (TL = 231 steps) and the CI (0.69), RI (0.93) and RC (0.62) values were higher. The MP, ML, BI and NJ analyses recovered the same tree topology and support indices are summarised on relevant nodes of the NJ tree (Figure 3).

All the analyses returned the same tree topology recovering two distinct monophyletic groups corresponding to *R. pumilio* and *R. dilectus* (Figure 3). The *R. pumilio* group is well-supported by all analyses (NJ: 94 %; ML: 75 %; MP: 88 %; BPP: 0.99). The *R. dilectus* group although not well-supported by ML and BI analyses had high levels of support in the NJ (99 %) and MP (87 %) analyses. The average genetic distance between these two species, is relatively high (11.6 %). The *R. pumilio* group includes samples from south-western South Africa and Namibia only ($2n$ = 48; Rambau *et al.* 2003) and showed high (8.5 %) within species variation. None of the samples from the current study fell within this group.

Within the *R. dilectus* group are two monophyletic groups corresponding to the proposed subspecies *R. d. chakae* and *R. d. dilectus* (Figure 3). The *R. d. chakae* grouping is well-supported by all analyses (NJ: 99 %; ML: 97 %; MP: 100 %; BPP: 1.0). This group contains samples from south-eastern South Africa, as well as four samples from the current study (Hammanskraal and UP Experimental farm). The genetic distance between the two proposed subspecies is 5.2 %. Karyotyped *R. d. chakae* samples from a previous study showed a diploid number of $2n$ = 48 (Rambau *et al.* 2003).

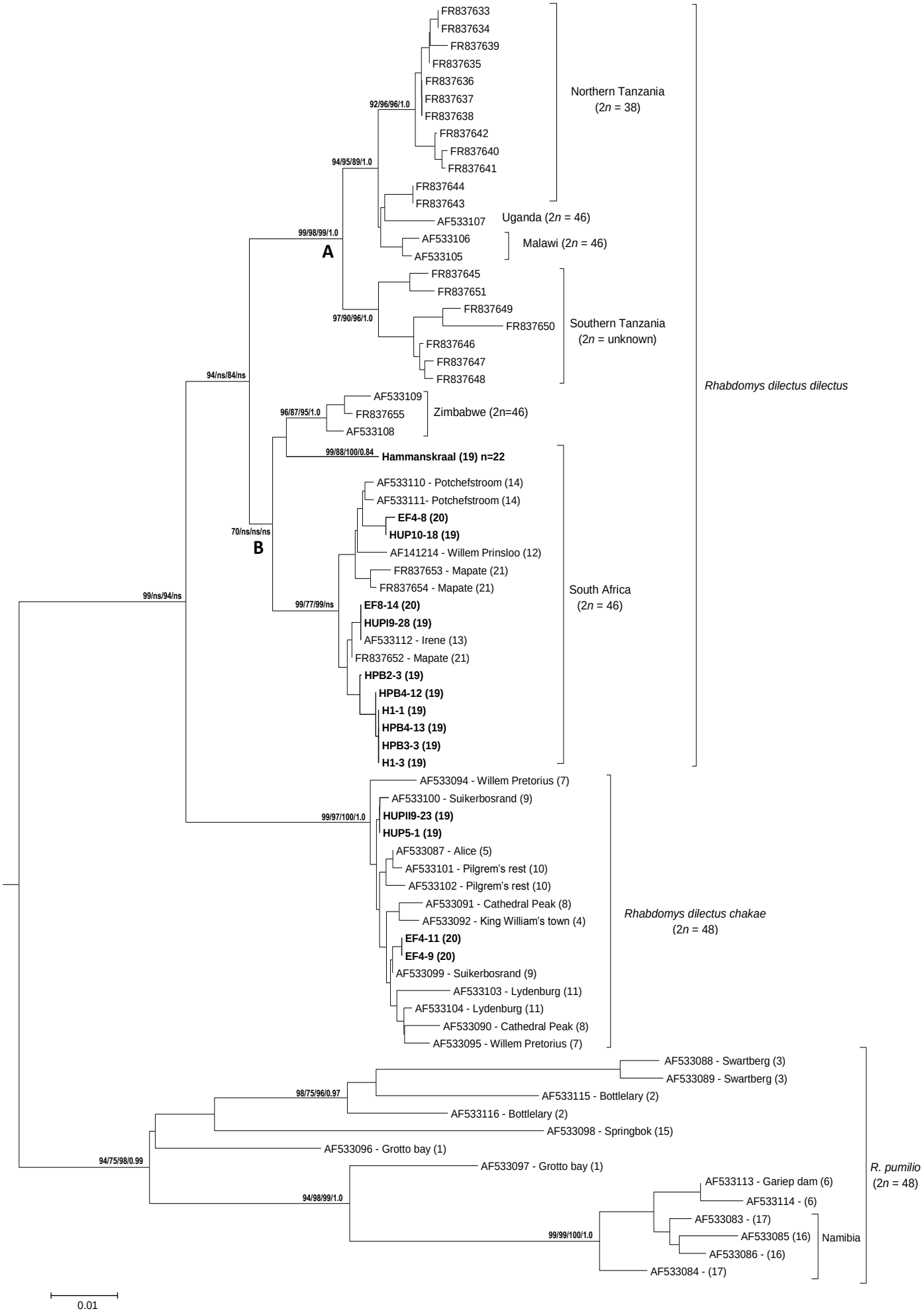

Figure 3. Neighbour Joining (NJ) tree inferred from partial cytochrome *b* gene sequence data (869 bp) of *Rhabdomys* sampled from Gauteng Province in South Africa during 2010-2011 (Dataset 2). Bootstrap support (> 70 %) and Bayesian posterior probabilities (> 0.90) are indicated at relevant nodes (NJ/ML/MP/BPP). Samples indicated in bold represent samples from the current study and are prefixed with EF/HUP/HPB/HH. Samples not in bold indicate accession numbers of sequences from previous studies downloaded from Genbank. Number in parentheses corresponds with sampling locations on map (Figure 1).

The remaining samples from this study fall within the poorly-supported *R. d. dilectus* group. The *R. d. dilectus* group can be further sub-divided into two groups (A and B; Figure 3); with the first subgroup (A) being well-supported (NJ: 99 %; ML: 98 %; MP: 99 %; BPP: 1.0) while the other (B) is not (NJ: 70 %). The two subgroups are separated by an average genetic distance of 3.9 %. The first subgroup (A; Figure 3) includes specimens from northern and southern Tanzania, Uganda and Malawi. The second subgroup (B) includes samples from South Africa and Zimbabwe only, with the Zimbabwe specimens forming a separate well-supported group (NJ: 96 %; ML: 87 %; MP: 95 %; BPP: 1.0). Within subgroup B, eight specimens from Hammanskraal (Gauteng Province, South Africa) and two from the University of Pretoria Experimental farm (Pretoria, Gauteng Province, South Africa) cluster with samples previously collected from South Africa, found to have a diploid number of $2n = 46$ (Rambau *et al.* 2003). The remaining 22 samples from Hammanskraal (Gauteng Province, South Africa) form a separate well-supported (NJ: 96 %; ML: 87 %; MP: 95 %; BPP: 1.0) cluster within subgroup B. Within species variation was low for *R. d. chakae* (0.68 %) but was higher for *R. d. dilectus* (2.67 %).

Discussion

Of the possible six cryptic murid species in the present study, the phylogenetic analyses revealed the presence of only three species within dataset 1 (*M. coucha, A. ineptus* and *O.*

angoniensis). The genetic distance within these species was relatively low ranging from 0.6 to 1.7 %, whereas the between species distances ranged from 3.4 % to 9.9 %. These findings agree with an evaluation of the efficacy of cytochrome *b* sequence data for distinguishing species (Bradley and Baker 2001). This study further suggests that genetic distance values smaller that 2 % are attributable to intraspecific variation; whereas values between 2 % and 11 % hold a high probability of indicating either subspecies or valid species but usually warrants additional studies (Bradley and Baker 2001). The paraphyly of the South African and Tanzanian *A. chrysophilus* samples (Figure 1) suggests that several additional cryptic species may be present within *A. chrysophilus*, highlighting the need for a comprehensive pan-African revision of this genus (Russo *et al*. 2006). Recent studies of the genetic structure of African rodent species across their entire range have revealed a large amount of genetic diversity within *M. natalensis* and possible species complex within *O. angoniensis* in East Africa (Taylor *et al.* 2011; Colangelo *et al.* 2013)

Due to small sample size and sequencing difficulty, *Saccostomus campestris* samples from the current study were not used in any phylogenetic analyses. The genus *Saccostomus* is another example of a common and widespread African rodent whose taxonomy is uncertain (Gordon and Watson 1986; Skinner and Chimimba 2005). Currently two species are recognized within the genus, *S. mearnsi* and *S. campestris*. Karyotypic and mitochondrial sequence analyses have shown that the diversity within this genus is much higher than previously thought (Gordon and Rautenbach 1980; Taylor 2000; Corti *et al.* 2004). These studies suggest that *S. mearnsi* and *S. campestris* are species complexes. Based on a large amount of karyotypic variation, several authors have suggested that *S. campestris* in southern Africa comprises of a complex of cryptic species (Taylor 2000; Corti *et al.* 2004). However, breeding experiments have shown no hybrid disadvantage between divergent chromosomal

forms (Maputla *et al*. 2011). Therefore, it is clear that there is a critical need for a taxonomic revision of this genus, as the current species delineation may be an oversimplification of the true taxonomic status of the genus (Corti *et al.* 2004).

Similarly, due to small sample size and sequencing difficulty, *Lemniscomys rosalia* samples from the current study were not used in any phylogenetic analyses. Previously, Meester *et al.* (1986) described four subspecies from the southern African sub region. Recently it was found that Tanzanian samples of *L. rosalia* (2*n* = 54) had a different diploid chromosome number compared to *L. rosalia* (2*n* = 48) from South Africa (Castiglia *et al.* 2002). Given these previously described subspecies and two different cytotypes, there may be a need for a taxonomic revision of this species in southern Africa.

Cytogenetics and molecular genetics have provided new insights into the systematics and taxonomy of murid rodents and have resulted in renewed interest in several African rodent species groups. The present study not only contributes to general small mammal studies in southern Africa but also to future epidemiological research and the refinement of the distributional data for epidemiologically important cryptic species. In the past, the reliable delimitation of the geographic ranges of these cryptic rodents has been problematic due to misidentification (Chimimba 2001; Venturi *et al.* 2004). This study also substantiates the benefits of species-specific multiplex PCR reactions in the identification of cryptic murid species. In future, this PCR approach will allow for the screening of larger samples sizes aiding especially in the identification of cryptic pest species or cryptic species known to pose a zoonotic threat (Bastos *et al.* 2005; Lecompt *et al.* 2005).

Rhabdomys taxonomy and distribution

The present study retrieved the same mitochondrial lineages corresponding to *R. pumilio, R. dilectus chakae* and *R. d. dilectus* as in previous studies; however, some of the deeper nodes were not as well-supported by all the analyses. All specimens from the current study group within *R. dilectus* and no *R. pumilio* was sampled from the current study sites. This agrees with current proposed distributional limits (Castiglia *et al* 2012; du Toit *et al*. 2012).

Within *R. dilectus* both proposed subspecies, *R. dilectus chakae* and *R. d. dilectus*, were collected from sites within the Gauteng area, which are approximately 50 km apart, *viz.* Hammanskraal and the University of Pretoria Experimental Farm (South Africa). Both *R. d. chakae* and *R. d. dilectus* samples were collected from Hammanskraal and from the University of Pretoria Experimental Farm (South Africa) during the current study. To the best of our knowledge this may represent the first record of both subspecies being sampled in the same localities. Based on niche modelling and previous trapping data, large areas of sympatry for the two subspecies were predicted for the grassland biome, especially within the Gauteng Province of South Africa (Meynard *et al*. 2012). Despite intensive sampling in previous studies, trapping yielded either only one or the other subspecies (Pillay *et al*. 2006; Ganem *et al.* 2012; Meynard *et al.* 2012). Meynard *et al*. (2012) proposed that competition could be the main driver of the species' distributional limits within this area which seems suitable for both species. Recent behavioural and ecological studies have suggested that the two subspecies may have fairly distinct and specific environmental requirements (Mackey 2011; Ganem *et al*. 2012). Although *R. d. dilectus* was more frequently sampled at the one study site (Hammanskraal, Figure 1) the current results confirms that both subspecies occur in Hammanskraal and at the University of Pretoria Experimental farm. Therefore, the present study may provide tentative evidence of a contact zone between *R. dilectus chakae* and *R. d.*

dilectus and highlights the need for more extensive sampling especially in Gauteng Province of South Africa.

The genetic divergence between *R. dilectus* and *R. pumilio* was relatively high (11.6 %) and further supports the current distinction of these two species within *Rhabdomys*. This taxonomic treatment is increasingly becoming accepted (Musser and Carleton 2005; Pillay *et al*. 2006; Rymer and Pillay 2012). It is the intraspecific variation that has proved more problematic recently (du Toit *et al*. 2012). In the present study, the genetic divergence within *R. pumilio* and *R. dilectus* is high, 8.5 % and 3.7 %, respectively. Recent studies have shown that both *R. pumilio* and *R. dilectus* are more diverse than previously thought and contain more than one distinct mitochondrial lineage (Castiglia *et al*. 2012; du Toit *et al*. 2012).

The genetic divergence between the proposed subspecies *R. d. dilectus* and *R. d. chakae* is also relatively high (5.2 %). In several mammal species, cytochrome *b* differences of 5 % have been found between morphologically distinct species and have subsequently been used to describe sister species (Baker and Bradley 2006). Although hybridization occurred under laboratory conditions (Pillay 2000b), recent studies have suggested that divergent behaviour may act as a pre-mating barrier in the wild (Pillay *et al*. 2006). Consequently, it has been proposed that *R. d. dilectus* and *R. d. chakae* be given full species ranks. The *R. d. chakae* monophyletic group showed a low within species variation (0.68 %). *Rhabdomys d. dilectus* on the other hand displayed a relatively high within species variation (2.67 %), high enough for cytochrome *b* data to suggest the presence of conspecifics (Bradley and Baker 2001). Two subgroups were identified within *R. d. dilectus*; the southern African subgroup and the eastern African subgroup (Subgroup A and B, Figure 3). The southern African subgroup (B, Figure 3), however, was not well-supported. Without additional extensive sampling

throughout the possible range of *R. d. dilectus*, a more comprehensive study of the contact zone, sequencing of additional nuclear gene regions and complete morphological investigations; the taxonomic status of these two subgroups remains unclear.

Contact zones offer an excellent opportunity to study the interaction of environmental and evolutionary mechanisms that influence speciation (Meynard *et al*. 2012). These areas also provide an opportunity to test the role of behavioural flexibility, in response to environmental changes, in species divergence (Ganem *et al.* 2012). *Rhabdomys pumilio* is frequently found in arid areas where vegetation cover is patchier and therefore there is an enhanced risk of predation; *R. dilectus* occurs in regions with higher vegetation cover and therefore, a slightly decreased vulnerability to predation (Rymer and Pillay 2012). Several behavioural studies have shown that *R. pumilio* is highly social and exhibits a high degree of social flexibility, whereas *R. dilectus* is solitary (Schradin and Pillay 2005; Rymer and Pillay 2012; Rymer *et al*. 2013). This high degree of social flexibility could have allowed *R. pumilio* to colonize different habitats (Rymer *et al*. 2013). Previous studies have suggested a South African origin for *Rhabdomys*, with *R. pumilio* being ancestral, with subsequent colonization of eastern Africa during the intense climatic oscillations of the Plio-Pleistocene era (Rambau *et al*. 2003; Castiglia *et al*. 2012; duToit *et al*. 2012). Similar phylogeographic patterns have been identified in other African rodent species such as *Saccostomus* (Corti *et al*. 2004), *Aethomys chryslophilus* (Russo *et al*. 2006) and *Micaelamys namaquensis* (Russo *et al*. 2010). Contact zones will therefore provide the unique opportunity to further evaluate the taxonomic status of newly discovered mitochondrial lineages but also to understand the pattern of muriod diversity found in Africa today.

References

Arntzen, L.,Wadee, A.A. and Isaäcson, M. 1991. Immune responses of two *Mastomys* sibling species to *Yersinia pestis*. *Infection and Immunity* **59**: 1966-1971.

Bastos, A.D.S., Chimimba, C.T., von Maltitz, E., Kirsten, F., Belmain, S. 2005. Identification of rodent species that play a role in disease transmission to humans in South Africa. *Proceedings of the South African Society for Veterinary Epidemiology and Preventive Medicine*, pp. 78-83.

Bradley, R.D. and Baker, R.J. 2001. A test of the genetic species concept: Cytochrome *b* sequences and mammals. *Journal of Mammalogy* **82**: 60-973.

Bradley, R.D. and Baker, R.J. 2006. Speciation in mammals and the genetic species concept. *Journal of Mammalogy* **87**: 643-662.

Brettschneider, H. 2010. *Bartonella* dynamics in indigenous and invasive murid rodents from South Africa: Prevalence, diversity and mathematical modelling. PhD Thesis, University of Pretoria, Pretoria, South Africa.

Bronner, G.N., Hoffman, M., Taylor, P.J., Chimimba, C.T., Best, P.B., Matthee, C.A. and Robinson, T.J. 2003. A revised systematic checklist of the extant mammals of the southern African subregion. *Durban Museum Novitates* **28**: 56-106.

Bronner, G.N. and Meester, J.A.J. 1988. *Otomys angoniensis*. *Mammalian species* **306**: 1-6.

Cantatore, P., Roberti, M., Pesole, G., Ludovico, A., Milella, F., Gadaleta, M.N. and Saccone, C. 1994. Evolutionary analysis of cytochrome *b* sequences in some perciformes: evidence for a slower rate of evolution than in mammals. *Journal of Molecular Evolution* **39**: 589-597.

Castiglia, R., Fadda, C., Corti, M., Scanzani, A., Verheyen, W. and Capanna, E. 2002. Chromosomal evolution in the African Arvicanthine rats (Murinae, Rodentia): comparative cytogenetics of *Lemniscomys* (*L. zebra, L. rosalia, L. striatus*) and *Arvicanthis dembeensis*. *Journal of Zoological Systematics and Evolutionary Research* **40**: 223-231.

Castiglia, R., Corti, M., Colangelo, P., Annesi, F., Capanna, E., Verheyen, W., Sichilima, A.M. and Makundi, R. 2003.Chromosomal and molecular characterization of *Aethomys kaiseri* from Zambia and *Aethomys chrysophilus* from Tanzania (Rodentia, Muridae). *Hereditas* **139**: 81-89.

Castiglia, R., Solano, E., Makundi, R.H., Hulselmans, J., Verheyen, E. and Colangelo, P. 2012. Rapid chromosomal evolution in the mesic four-striped grass rat *Rhabdomys dilectus* (Rodentia, Muridae) revealed by mtDNA phylogeographic analysis. *Journal of Zoological Systematics and Evolutionary Research* **50**: 165-172.

Chimimba, C.T. 1998. A taxonomic revision of synthesis of southern African *Aethomys* (Rodentia: Muridae) with a key to species. *Mammalia* **62**: 427-437.

Chimimba, C.T. 2001. Geographic variation in the Tete veld rat *Aethomys ineptus* (Rodentia: Muridae) from southern Africa. *Journal of Zoology (London)* **254**: 77-89.

Chimimba, C.T., Dippenaar, N.J. and Robinson, T.J. 1999. Morphometric and morphological delineation of southern African species of *Aethomys* (Rodentia:Muridae). *Biological Journal of the Linnean Society* **67**: 501-527.

Colangelo, P., Verheyen, E., Leirs, H., Tatard, C., Denys, C., Dobigny, G., Duplantier, J., Brouat, C., Granjon, L. and Lecompte, E. 2013. A mitochondrial phylogeographic scenario for the most widespread African rodent, *Mastomys natalensis*. *Biological Journal of the Linnean Society* **108**: 901-916.

Corti, M., Castiglia, R., Annesi, F. and Verheyen, V. 2004. Mitochondrial sequences and karyotypes reveal hidden diversity in African pouched mice (subfamily Cricetomyinae, genus *Saccostomus*). *Journal of Zoology (London)* **262**: 413-424.

Davis, D.H.S., Eisch, R.B. H., McNeill, D. and Meyer, K.F. 1968. Serological survey of plague in rodents and other small mammals in Kenya. *Transactions of the Royal Society of Tropical and Medical Hygiene* **62**: 838-861.

De Graaff, G.D. 1981. *The Rodents of Southern Africa: Notes on their Identification, Distribution, Ecology and Taxonomy*. Butterworths, Pretoria.

Ducroz, J., Granjon, L., Lombaard, M. and Volobouev, V. 1999. Comparative chromosome analysis (R- and C-bands) of two South African murid species, *Lemniscomys rosalia* and *Rhabdomys pumilio* (Rodentia, Murinae). *Cytogenetics and Cell Genetics* **87**: 69-74.

Ducroz, J., Volobouev, V. and Granjon, L. 2001. An assessment of the systematics of Arvicanthine rodents using mitochondrial DNA sequences: evolutionary and biogeographical implications. *Journal of Mammalian Evolution* **8**: 173-206.

du Toit, N., Jansen van Vuuren, B., Matthee, S. and Matthee, C.A. 2012. Biome specificity of distinct genetic lineages within the four-striped mouse *Rhabdomys pumilio* (Rodentia: Muridae) from southern Africa with implications for taxonomy. *Molecular Phylogenetics and Evolution* **65**: 75-86.

Engelbrecht, A., Taylor, P.J., Daniels, S.R. and Rambau, R.V. 2011. Cryptic speciation in the southern African vlei rat *Otomys irroratus* complex: evidence derived from mitochondrial Cyt *b* and niche modelling. *Biological Journal of the Linnean Society* **104**: 192-206.

Ganem, G., Meynard, C.N., Perrigault, M., Lancaster, J., Edwards, S., Caminade, P., Watson, J. and Pillay, N. 2012. Environmental correlates and co-occurrence of three mitochondrial

lineages of striped mice (*Rhabdomys*) in the Free State Province (South Africa). *Acta Oecologica* **42**: 30-40.

Gordon, D.H. and Rautenbach, I.L. 1980. Species complexes in medically important rodents: chromosome studies of *Aethomys*, *Tatera* and *Saccostomus* (Rodentia: Muridae, Cricetidae). *South African Journal of Science* **76**: 559-561.

Gordon, D.H. and Watson, C.R.B. 1986. Identification of cryptic species of rodents (*Mastomys*, *Aethomys*, *Saccostomus*) in the Kruger National Park. *South African Journal of Zoology* **21**: 95-99.

Green, C.A., Keogh, H., Gordon, D.H., Pinto, M. and Hartwig, E.K. 1980. The distribution, identification, and naming of the *Mastomys natalensis* species complex in southern Africa (Rodentia: Muridae). *Journal of Zoology* **192**: 17-23.

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

Hallet, A.F., McNeill, D. and Meyer, K.F. 1970. A serological survey of small mammals for plague in southern Africa. *South African Medical Journal* **44**: 831-837.

Huelsenbeck, J.P. and Ronquist, F. 2001. MrBayes: Bayesian inference of phylogeny. *Bioinformatics* **17**: 754-755.

Howald, G., Donlan, C.J., Faulkner, K.R., Ortega, S., Gellerman, H., Croll, D.A. and Tershy, B.R. 2009. Eradication of black rats *Rattus rattus* from Anacapa Island. *Oryx* **44**: 30-40.

Irwin, D.M., Kocher, T.D. and Wilson, A.C. 1991. Evolution of the Cytochrome *b* gene of mammals. *Journal of Molecular Evolution* **32**: 128-144.

Isaäcson, M., Arntzen, L. and Taylor, P. 1981. Susceptibility of members of the *Mastomys natalensis* species complex to experimental infection with *Yersinia pestis*. *Journal of Infectious Disease* **144**: 80.

Isaäcson, M., Taylor, P and Arntzen, L. 1983. Ecology of plague in Africa: Response of indigenous wild rodents to experimental plague infection. *Bulletin of the World Health Organization* **61**: 339-344.

Julius, R.S. 2013. Molecular prevalence and diversity of zoonotic bacteria of invasive *Rattus* from South Africa, with emphasis on genera *Rickettsia* and *Steptobacillus*. MSc.Thesis, University of Pretoria, Pretoria, South Africa.

Lecompte, E., Brouat, C., Duplantier, U., Galan, M., Granjon, L., Louiseau, A., Mouline, K. and Cosson, J. 2005. Molecular identification of four cryptic species of *Mastomys* (Rodentia, Murinae). *Biochemistry and Systematics Ecology* **33**: 681-689.

Lecompte, E., Aplin, K., Denys, C., Catzeflis, F., Chades, M., Chevret, P. 2008. Phylogeny and biogeography of African Murinae based on mitochondrial and nuclear gene sequences, with a new tribal classification of the subfamily. *BMC Evolutionary Biology* **8**: 199.

Mackay, M.K., 2011. The behaviour of two sub-species of the striped mouse *Rhabdomys*: the role of phylogeny and the environment. MSc Thesis, University of Witwatersrand, Johannesburg, South Africa.

Mahida, H., Campbell, G.K. and Taylor, P.J., 1999. Genetic variation in *Rhabdomys pumilio* (Sparrman, 1784) - an allozyme study. *South African Journal of Zoology* **34**: 91-101.

Maputla, N.W., Dempster, E.R., Raman, J. and Ferguson, J.W.H. 2011. Strong hybrid viability between two widely divergent chromosomal forms of the pouched mouse. *Journal of Zoology* **285**: 180-187.

Maree, S. 2002. Phylogenetic relationships and mitochondrial DNA sequence evolution in the African rodent subfamily Otomyinae (Muridae). PhD Thesis, University of Pretoria, Pretoria, South Africa.

Martin, Y., Gerlach, G., Schlötterer, C. and Meyer, A. 2000. Molecular phylogeny of European muroid rodents based on complete cytochrome *b* sequences. *Molecular Phylogenetics and Evolution* **16**: 37-47.

McKenzie, M., Chiotis, M., Pinkert, C.A. and Trounce, I.A. 2003. Functional respiratory chain analyses in murid xenomitochondrial cybrids expose coevolutionary constraints of cytochrome-b and nuclear subunits of Complex III. *Molecular Biology and Evolution* **20**: 1117-1124.

Meerburg, B.G., Singleton, G.R. and Leirs, H. 2009a. The Year of the Rat ends - time to fight hunger! *Pest Management Science* **65**: 351-352.

Meerburg, B.G., Singleton, G.R. and Kijlstra, A. 2009b. Rodent-borne diseases and their risks for public health. *Critical Reviews in Microbiology* **35**: 221-270.

Meester, J.A.J., Rautenbach, I.L., Dippenaar, N.J. and Baker, C.M. 1986. Classification of southern African mammals. *Transvaal Museum Monograph* **5**: 1-359.

Meynard, C.N., Pillay, N., Perrigault, M., Caminade, P., Ganem, G. 2012. Evidence of environmental niche differentiation in the striped mouse (*Rhabdomys* sp.): inference from its current distribution in southern Africa. *Ecology and Evolution* **5**: 1008-1023.

Michaux, J., Chevret, P.and Renaud, S. 2007. Morphological diversity of Old World rats and mice (Rodentia, Muridae) mandible in relation with phylogeny and adaptation. *Journal of Zoological Systematics and Evolutionary Research* **45**: 263-279.

Missone, X. 1974. Order Rodentia In: *The mammals of Africa: An identification Manual*, (eds) J. Meester and H.W. Setzer. Smithsonian Institution Press, Washington.

Mostert, E.M. 2009. Molecular and morphological assessment of invasive inland *Rattus* (Rodentia: Muridae) concentric in South Africa and their reservoir host potential with respect to *Helicobacter* and *Bartonella*. MSc.Thesis, University of Pretoria, Pretoria, South Africa.

Musser, G.G. and Carleton, M.D. 1993. Superfamily Muroidea. In: *Mammal Species of the World a Taxonomic and Geographic Reference*, (eds) D.E. Wilson and D.M. Reeder. Johns Hopkins University Press, Baltimore.

Musser, G.G. and Carleton, M.D. 2005. Superfamily Muroidea. In: *Mammal Species of the World a Taxonomic and Geographic Reference*, (eds) D.E. Wilson and D.M. Reeder. Johns Hopkins University Press, Baltimore.

Peppers, L.L. and Bradley, R.D. 2000. Cryptic Species in *Sigmodon hispidus*: Evidence from DNA Sequences. *Journal of Mammalogy* **81**: 332-343.

Pillay, N. 2000a. Female mate preference and reproductive isolation in populations of the striped mouse, *Rhabdomys pumilio*. *Behaviour* **137**: 1431-1441.

Pillay, N. 2000b. Reproductive isolation in three populations of the striped mouse *Rhabdomys pumilio*: interpopulation breeding studies. *Mammalia* **64**: 461-470.

Pillay,N., Eborall, J. and Ganemba, G. 2006. Divergence of mate recognition in the African striped mouse (*Rhabdomys*). *Behavioral Ecology* **17**: 757-764.

Posada, D. 2008. jModalTest: Phylogenetic model averaging. *Molecular Biology and Evolution* **25**: 1253-1256.

Rambau, R.V., Robinson, T.J. and Stanyo, R. 2003. Molecular genetics of *Rhabdomys pumilio* subspecies boundaries: mtDNA phylogeography and karyotypic analysisby fluorescence in situ hybridization. *Molecular Phylogenetics and Evolution* **28**: 564-575.

Roberts, A. 1951. Rhabdomys. In: *The Mammals of South Africa*, (eds) R. Bigalke, V. FitzSimons and D.E. Malan. Trustees of ‘‘The mammals of South Africa’’ Book Fund, Johannesburg.

Russo, I.R., Chimimba, C.T. and Bloomer, P. 2006. Mitochondrial DNA differentiation between two species of *Aethomys* (Rodentia: Muridae) from southern Africa. *Journal of Mammalogy* **87**: 545-553.

Russo, I.M., Chimimba, C.T. and Bloomer, P., 2010. Bioregion heterogeneity correlates with extensive mitochondrial DNA diversity in the Namaqua rock mouse, *Micaelamys namaquensis* (Rodentia: Muridae) from southern Africa - evidence for a species complex. *BMC Evolutionary Biology* **10**: 307.

Rymer T.L., Pillay, N. and Schradin, C. 2013. Extinction or Survival? Behavioral flexibility in response to environmental change in the African striped mouse *Rhabdomys. Sustainability* **5**: 163-186.

Rymer, T.L. and Pillay, N. 2012. The development of exploratory behaviour in the African striped mouse *Rhabdomys* reflects a gene x environment compromise. *Behavioural Genetics* **42**: 845-856.

Schradin, C., Pillay, N., 2005. Intraspecific variation in the spatial and social organization of the African striped mouse. *Journal of Mammalogy* **86**: 99-107.

Skinner, J.D. and Chimimba C.T. 2005. *The Mammals of the Southern African Subregion*. Cambridge University Press, Cambridge.

Shepherd, A.J., Leman, P.A. and Hummitzsch, D.E. 1986. Experimental plague infection in South African wild rodents. *Journal of Hygiene (Cambridge)* **96**: 171-183.

Stenseth, N.C., Leirs, H., Skonhoft, A., Davies, S.A., Pech, R.P., Andreassen, H.P., Singleton, G.R., Lima, M., Machangu, R.M., Makundi, R.H., Zhang, Z., Brown, P.B., Shi,

D. and Wan, X. 2003. Mice, rats and people: the bio-economics of agricultural rodent pests. *Frontiers in Ecology and the Environment* **1**: 367-375.

Swofford, D.L. 2000. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4.0b10. Sinauer Associates, Sunderland, MA.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution* **24**: 1596-1599.

Taylor, P.J. 2000. Patterns of chromosomal variation in southern African rodents. *Journal of Mammalogy* **81**: 317-331.

Taylor, P. J., Lavrenchenko, L.A., Carleton, M.D., Verheyen, E., Bennett, N.C., Oosthuizen, C.J. and Maree, S. 2011. Specific limits and emerging diversity patterns in East African populations of laminate-toothed rats, genus *Otomys* (Muridae: Murinae: Otomyini): Revision of the *Otomys typus* complex. *Zootaxa* **3024**: 1-66.

van Sandwyk, J.H.D. 2014. Evaluation of *Cardiovirus* reservoir potential in *Mastomys* from the Kruger National Park against a backdrop of host phylogeography and historical outbreak strain characterization. PhD Thesis, University of Pretoria, Pretoria, South Africa.

Venturi, F.P., Chimimba, C.T., van Aarde, R.J. and Fairall, N. 2004. The distribution of two medically and agriculturally important cryptic rodent species, *Mastomys natalensis* and *M. coucha* (Rodentia:Muridae) in South Africa. *African Zoology* **39**: 235-245.

Chapter 3

Molecular prevalence and diversity of bacteria associated with commensal and wild populations of indigenous rodents from South Africa

Abstract

Rodents transmit a wide variety of pathogens to humans and domestic animals including a wide variety of bacterial species. More than 50 % of emerging infectious diseases are caused by bacterial pathogens. South Africa has a large diversity of indigenous rodent species many of which have been linked to zoonotic diseases. The current study investigated the prevalence, diversity and zoonotic potential of bacteria, potentially transmitted through environmental contamination, associated with commensal and wild populations of indigenous rodent species in South Africa. Kidney samples from 55 commensal and 46 wild rodents were screened for the presence of bacterial genome using a broad-range PCR primer set targeting the 16S rRNA gene. A generalized linear mixed model was used to test for statistical differences in bacterial infection between host species, sexes and rodent populations (commensal *versus*. wild populations). An overall sequence-confirmed prevalence of 38.6 % was detected in the eight rodent species sampled (*Aethomys ineptus*; *Gerbilliscus leucogaster*; *Lemniscomys rosalia*; *Mastomys coucha*; *Mastomys natalensis*; *Otomys angoniensis*; *Rhabdomys dilectus*; *Saccostomus campestris*), with no significant differences between species, sexes or rodent populations. Analyses revealed the presence of

seven bacterial genera from two phyla namely; *Streptococcus*, *Aerococcus*, *Lactobacillus*, *Bartonella*, *Anaplasma*, *Burkholderia* and *Helicobacter*. The diverse bacterial genera detected in commensal and wild populations of indigenous rodents contain members that may be of zoonotic importance and highlights the importance of continuous disease surveillance of commensal and wild populations of indigenous rodents.

Keywords: Commensal rodents, wild rodents, 16S rRNA, PCR, bacteria prevalence, bacterial diversity, zoonoses, South Africa

Introduction

Rodent species worldwide play a significant role in the transmission of diseases to man and animals (Gratz 1997). Rodents have been implicated in the spread of numerous viruses, bacteria, helminths and protozoan pathogens (Meerburg *et al*. 2009). Many of these diseases can be transmitted by direct contact with rodents through bites and scratches or indirectly through contact with food and water contaminated with rodent faeces and urine (Meerburg *et al*. 2009). Rodents are also host to various tick, mite, flea and louse species; many of which can be transmitted to humans and other animals (Morand *et al*. 2006). These ectoparasites not only cause direct effects through their bite but can also act as vectors for several vector-borne diseases associated with rodents (Eisen *et al.* 2013).

In Africa, rodent-borne diseases are the cause of much morbidity and mortality (Gratz 1997). Plague is one of the most notorious bacterial diseases associated with rodents and is caused by the bacterium *Yersinia pestis* and is transmitted by fleas after feeding on an infected host (Hang´ombe *et al.* 2012). Originally introduced into Africa by *Rattus* species it now occurs in endemic foci across Africa (Neerinckx *et al.* 2010). Several indigenous rodents have been implicated in the spread of plague including the Natal multimammate mouse (*Mastomys natalensis*), the highveld Gerbil (*Gerbilliscus brantsii*), the four-striped grass mouse (*Rhabdomys pumilio*) and the red veld rat (*Aethomys chrysophilus*) (Gratz 1997). Almost 90 % of plague cases reported each year come from Africa and outbreaks have been reported as recently as 2007 in Tanzania (Makundi *et al*. 2008).

Bartonella, a major emerging bacterial genus, has been detected in a wide range of indigenous species from commensal and wild populations as well as invasive rodent species in Africa (Pretorius *et al*. 2004; Bastos 2007; Mostert 2009; Brettschneider 2010; Meheretu *et*

al. 2013). Prevalence rates for *Bartonella* among African rodent communities range from 8.5 % to 56 % and include known zoonotic species such as *B. elizabethae*, as well as novel strains of unknown pathogenicity (Bastos 2007; Gundi *et al*. 2012; Kamani *et al*. 2013).

Besides the above mentioned examples, African rodents are associated with many other zoonotic pathogens (Gratz 1997). Many of these indigenous rodent species occur commensally with humans, especially in informal settlements (De Graaf 1981). These informal settlements frequently lack proper sanitation, housing and infrastructure, a situation that usually leads to large commensal rodent populations (Taylor *et al*. 2008). Furthermore, changing land use patterns and human social behaviour are placing humans and domestic animals in increasing contact with wild populations of rodents that could be harbouring emerging and re-emerging infectious diseases (McMichael 2004). Given the large rodent diversity in South Africa and the increasing potential for rodent-human contact, relatively few studies have investigated the pathogen communities associated with indigenous rodent species (Taylor *et al.* 2008).

More than half of emerging infectious diseases are caused by bacterial pathogens (Jones 2008). Over the past two decades molecular methods have gained increased popularity in the study of infectious bacterial pathogens as they overcome some of the limitations associated with traditional methods such as immunochemical and culture techniques (Doern 2000). The 16S rRNA gene region is one of the most frequently used targets for molecular identification of bacteria (Srinivasan and Fredricks 2008). This is a highly conserved gene region across all bacterial lineages and the 16S rRNA gene is of sufficient length and variability to allow for phylogenetic analyses (Clarridge 2004). Consequently, a large number of 16S sequences from almost all known bacterial taxa are available on nucleotide databases

allowing for rapid comparison of bacterial samples (Clarridge 2004). Therefore many broad range bacterial studies have chosen this gene region for detection of bacterial pathogens (Nikkari *et al.* 2002). Although the kidney is not an optimal organ for detecting certain bacterial genera it has lower levels of bacterial species making it a useful organ for the identification of unknown bacteria using the broad-range molecular approach (du Bruyn *et al.* 2008). Furthermore, by screening kidney tissue, it may be possible to determine the bacterial genera that may be shed into the environment through urine or faeces.

The majority of the world's urban population growth in the next decade is set to occur in developing countries such as South Africa (Gratz 1999). Such rapid urbanisation in developing countries often leads to a large proportion of human populations living in circumstances conducive to increased rodent populations and risk of zoonotic disease transfer (Taylor et al. 2008). The current study was aimed at investigating the prevalence, diversity and zoonotic potential of the bacteria associated with the kidneys of commensal and wild populations of indigenous rodent species in South Africa.

Materials and Methods

Sample collection

Commensal rodent samples were collected at 4-6 week intervals between August 2010 and July 2011 in Hammanskraal (25°42´60´´S, 28°27´20´´E, 1111 meters above sea level) and the University of Pretoria (UP) Experimental farm (25°75´11´´S, 28°26´29´´E, 1375 m.a.s.l.), Gauteng Province, South Africa (see Figure 1 in Chapter 2) Wild rodents were sampled bi-annually over a two-year period from September 2008 to April 2010 from southern Kruger National Park (KNP; Brettschneider 2010) between the Lower Sabie and Tshokwane Rest Camps (24°48´02´´S, 31°52´45´´E to 24°89´77´´S, 39°12´33´´E, 200-400 m.a.s.l) Animals

were live-trapped using Sherman traps (H.B. Sherman Traps Inc. Florida, U.S.A.) baited with a mixture of peanut butter, oatmeal and fish oil and processed as detailed in Chapter 1. All cryptic rodent species were identified molecularly based on cytochrome *b* (cyt *b*) gene amplification and sequence analyses (Bastos *et al*. 2005) or with a species-specific multiplex protocol (See Chapter 2). A subset of 55 commensal rodent samples and 46 wild rodent samples were selected for bacterial screening.

Molecular detection, phylogenetic and statistical analyses

Total genomic DNA was extracted from kidney tissue using the High Pure PCR template preparation kit (Roche) according to the prescribed manufacturer's protocol. Broad-range, bacterial primers 16F27 (AGAGTTTGATCCTGGCTCAG) and 16R1522 (AAGGAGGTGATCCAGCCGCA) were used to amplify a region of approximately 1500 base pair (bp) of the 16S rRNA gene (Hauben *et al*. 1997). PCRs were performed in a final reaction volume of 50 µl containing 1x Buffer (Fermentas), 0.25 µM dNTP's (Fermentas), 0.4 µM of each primer (IDT), 1U of DreamTaq™ DNA polymerase and 100-200 ng DNA template. A touchdown PCR program consisting of the following thermal cycling conditions was used: Initial denaturation of 10 s at 96° C, followed by three cycles of denaturation for 12 s at 96° C, annealing for 20 s at 63° C and elongation for 1 min 40 s at 70° C. This was followed by five cycles of 12 s at 96° C, 20 s at 61.5° C and 1 min 30 s at 70° C. Finally 32 cycles of 12 s at 96° C, 20 s at 60° C and 1min 20 s at 70° C, followed by a final extension of 60 s at 70° C. Each kidney sample was screened at least twice and samples for which the results of the first two PCR reactions were contradictory were screened a third time.

Positive samples were purified using the Roche PCR Product Purification Kit, and cycle sequenced using BigDye v. 3.1 terminator cycle-sequencing kit (Perkin-Elmer, Foster

City, U.S.A.). Sequences were precipitated and run on the ABI PRISM ™ 3100 Analyser (Applied Biosystems). Nucleotide sequences were viewed and aligned in Mega5 (Tamura *et al.* 2011) prior to performing a BLAST search (www.ncbi.nm.nih.gov/blast) to identify the bacterial species with the highest sequence similarity. For phylogenetic analyses, sequences generated from this study were aligned with reference sequences downloaded from the National Centre for Biotechnology Information (GenBank; www.ncbi.nm.nih.gov/genbank) database.

Neighbour Joining (NJ) analysis was performed in MEGA5 (Tamura *et al.* 2011), Maximum Likelihood (ML) analysis was performed in PhyML (Guidon and Gascuel 2003) and Bayesian inference (BI) with MrBayes (Huelsenbeck and Ronquist 2001) using the best-fit model and parameters identified under the Akaike Information Criterion (AIC), chosen with jModelTest (Posada 2008). For the BI, four Markov Monte Carlo chains were run for 10,000,000 generations using default heating and swap settings, and were sampled every 100 generations. Trace files were viewed using Tracer version 1.5 (http://beast.bio.ed.ac.uk/) after which 20 % of the runs were discarded as burn-in. Nodal support was determined by 10,000 and 1,000 bootstrap replicates for NJ and ML analyses, respectively and from Bayesian posterior probabilities (BPP). Pairwise uncorrected p-distances, were determined in MEGA5 (Tamura *et al.* 2011). Differences in bacterial prevalence between host species, sexes and rodent populations (commensal *versus* wild populations) were analysed using Generalized Linear Models (GLM) in STATISTICA v11 (StatSoft Inc. 2012, Oklahoma, USA).

Results

Of the 101 kidney samples screened, 73.3 % ($^{74}/_{101}$) displayed positive PCR amplification. A large proportion ($^{35}/_{101}$) of samples produced mixed sequences either due to presence of more

than one bacterial species or to co-amplification of host and bacterial genomes and therefore the overall sequence-confirmed bacterial prevalence was 38.6 % ($^{39}/_{101}$). Two sequenced amplicons corresponded to host genome and were not included in the bacterial prevalence calculates. There was no significant difference in PCR-prevalence (Species: $F_{7, 91} = 1.12$, $n = 101$, $P = 0.36$; Sex: $F_{1, 91} = 0.01$, $n = 101$, $P = 0.92$; Population: $F_{1, 91} = 1.56$, $n = 101$, $P = 0.21$) or sequence-confirmed prevalence (Species: $F_{7, 91} = 1.17$, $n = 101$, $P = 0.33$; Sex: $F_{1, 91} = 0.07$, $n = 101$, $P = 0.79$; Population: $F_{1, 91} = 0.01$, $n = 101$, $P = 0.93$) between species, sexes or rodent populations (Figure 1).

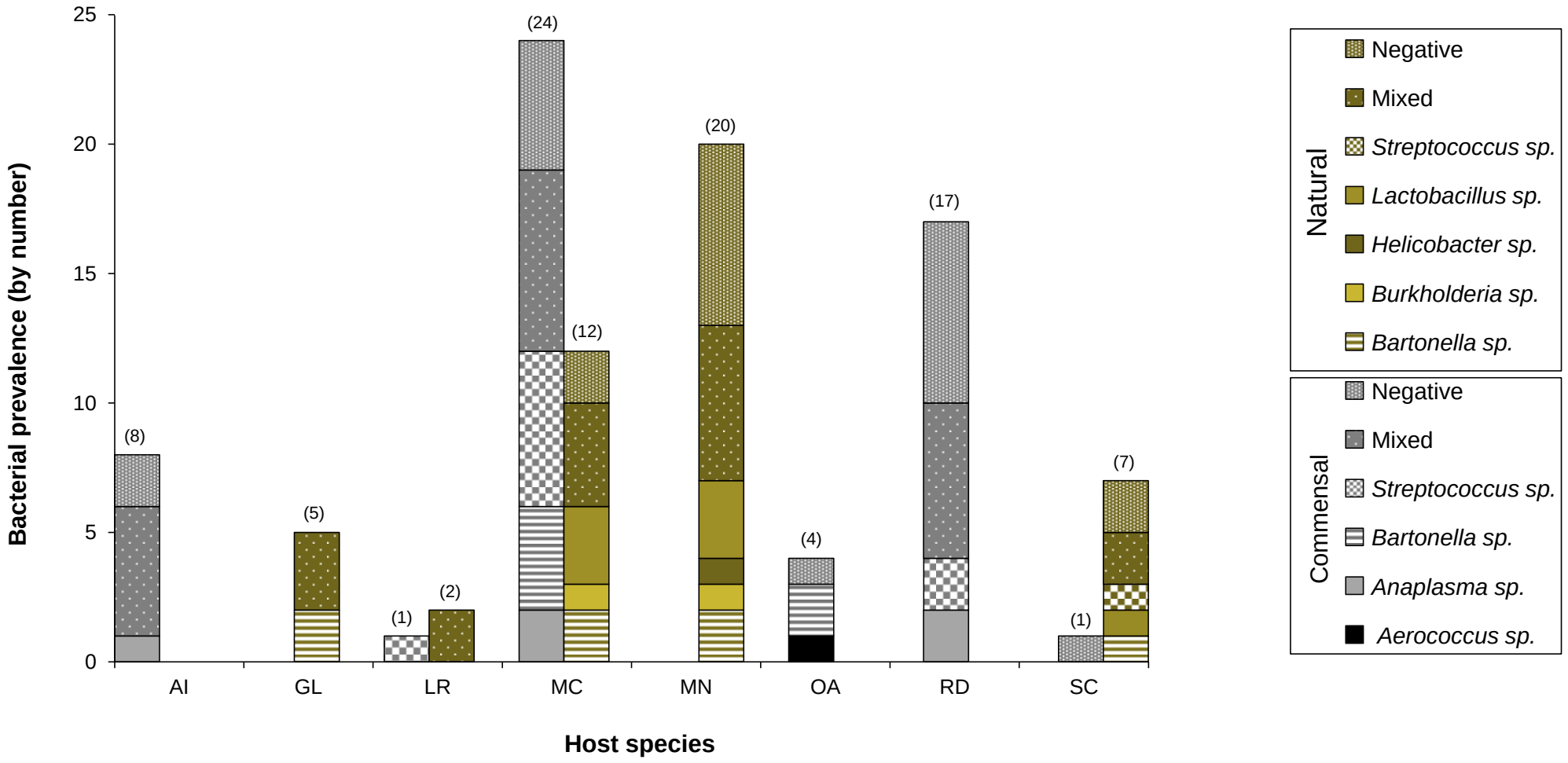

Figure 1. Bacterial prevalence in screened kidney tissue of 46 natural (green) and 55 commensal (grey) rodent species from South Africa. Sample sizes are indicated in parentheses and rodent species abbreviated as follows: AI: *Aethomys ineptus*; GL: *Gerbilliscus leucogaster*; LR: *Lemniscomys rosalia*; MC: *Mastomys coucha*; MN: *Mastomys natalensis*; OA: *Otomys angoniensis*; RD: *Rhabdomys dilectus*; SC: *Saccostomus campestris*.

Phylogenetic analyses were performed on a 77-taxon dataset, with 39 sequences generated in this study and 38 reference sequences obtained from the NCBI database. This 853 bp 16S rRNA dataset contained 408 variable sites and 362 parsimony informative sites. The average nucleotide composition was A = 0.259, C = 0.201, G = 0.306, T = 0.234, with a transition: transversion ratio R of 0.96. The TIM2+I+G (I = 0.321, G = 0.622) model of sequence evolution was chosen as the best fit model under the AIC in jModelTest. The ML, BI and NJ analyses recovered the same tree topology and support indices are summarised on relevant nodes of the NJ tree (Figure 2).

Phylogenetic analyses revealed the presence of seven bacterial genera representing two phyla namely, Proteobacteria and Firmicutes (Figure 2). The phylum Firmicutes were represented by three genera of which the genus *Streptococcus* contained the most prevalent bacterial species (*n* = 10). This genus was detected in both commensal and wild populations of rodents, although it was more prevalent in the commensal population (*n* = 9). The *Streptococcus* strains identified in the current study grouped with an uncultured strain observed in *Mus musculus* from Marion Island (de Bruyn *et al.* 2008). Within this group, commensal samples from the current study formed two well-supported clades. One clade contained samples from *Mastomys coucha* (*n* = 6) only and had between 97.23 % and 97.36 % sequence identity to the Marion Island strain (EU626397), while the other group consisted of samples from *Rhabdomys dilectus* (*n* = 2) and *Lemniscomys rosalia* (*n* = 1) and had between 95.69 % and 96.61 % sequence identity to EU626397. The single wild sample was distinct from the commensal samples.

Species of the genus *Lactobacillus* were only detected in rodents from wild population. The *Lactobacillus* strains detected formed two separate well-supported groups. The first

group contains a single sample from *Saccostomus campestris* together with *Lactobacillus murinus* and *L. animalis* (Wohlgemuth *et al.* 2010). The second group consist of samples from *M. coucha* (*n* = 3) and *M. natalensis* (*n* = 3) along with *L. frumenti*, *L. taiwanensis* and uncultured *Lactobacillus* strains from murid rodent intestinal contents (Müller *et al.* 2000; Wang *et al*. 2009; Smith *et al*. 2012).

A single commensal sample detected in *Otomys angoniensis* grouped with *Aerococcus viridans* and an *Aerococcus* strain isolated from a Damaraland mole-rat (*Cryptomys damararensis*) from South Africa (van Sandwyk 2007). The *Aerococcus* sample isolated from *O. angoniensis* shared 99.64 % and 99.76 % genetic similarity with the *A. viridans* type strain and the mole-rat strain respectively.

Within the phylum Proteobacteria, four genera were observed in the commensal and wild populations of rodents tested. The most frequently detected genus in this phylum was *Bartonella* which was detected in both commensal (*n* = 6) and wild (*n* = 7) populations of rodents (Figure 2). Genetic similarity between the *Bartonella* samples detected in the current study and valid *Bartonella* species ranged between 97.92 % and 99.84 %. The genus *Anaplasma* was only detected in commensal rodents (*n* = 5). Genetic distances between the *A. bovis* type specimen (U03775) and *Anaplasma* samples detected in commensal rodents range from 1.17 % to 1.47 %. A single sequence obtained from *M. natalensis* grouped together with *Helicobacter hepaticus* with 99.37 % similarity; this grouping however was not well supported by the analyses.

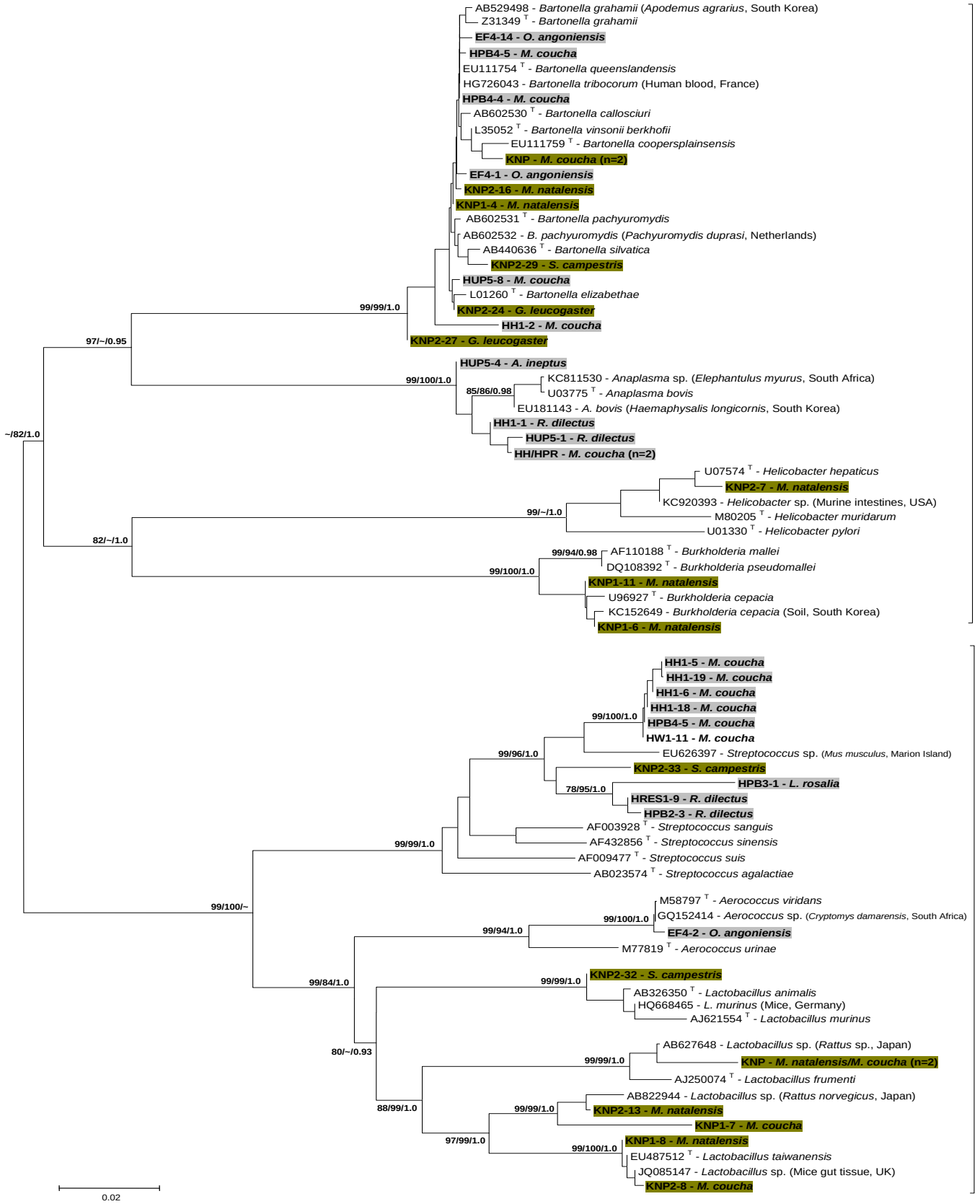

Figure 2. Neighbour-joining (NJ) tree inferred from partial 16S gene sequences of bacteria occurring in commensal and natural populations of rodents from South Africa. Bootstrap support (> 70 %) and Bayesian posterior probabilities (> 0.90) are indicated at relevant nodes (NJ/ML/BPP). Samples indicated in bold represent samples from the current study and are prefixed with KNP for natural populations (green) and with HH/HPB/HUP/HW/EF for commensal populations (grey). Samples not in bold indicate accession numbers of the sequences downloaded from Genbank, with type specimens indicated with a superscript letter T (^{T}).

Members of the genus *Burkholderia* were detected in two *M. natalensis* samples from the wild population. These samples clustered with *Bu. cepacia* with pairwise sequence dissimilarity between these samples and the *Bu. cepacia* type specimen (U96927) ranging from 0.18 % to 0.36 %. However, this grouping was not well-supported by the present analyses.

Discussion

The initial overall PCR-positive prevalence for bacteria in indigenous rodent kidneys was very high (73.3 %; $^{74}/_{101}$), however a large proportion of samples (34.7 %; $^{35}/_{101}$) produced bacterial mixtures that could not be identified. This is an acknowledged disadvantage of broad-range bacterial primers when compared to studies using species-specific primers (Clarridge 2004). However the broad range 16S rRNA PCR approach provides a good census of the bacterial community present in the kidneys of indigenous rodents. A further drawback of 16S rRNA is that this gene region is not sensitive enough to discriminate between bacterial species of certain genera (Felis and Dellaglio 2007). Other gene region such as protein-encoding genes may be better suited to distinguish between more closely related or recently diverged species (La Scola *et al.* 2003). This further emphasises the need for comprehensive single species bacterial studies in indigenous rodents. Nevertheless, bacteria from seven genera were successfully sequenced during the present study.

In the present study, bacteria belonging to two phyla were successfully identified and sequenced from the eight species of indigenous rodents. Within the phylum Firmicutes three genera belonging to the order Lactobacillales (Vos *et al.* 2009) were identified in the rodents tested. The most prevalent genus was *Streptococcus* and although members of this genus

generally form part of the normal flora of various animals, this genus also contains some important pathogens (Mas-De-Xaxars and Garcia-Gil 2009). Pathogenic streptococci can cause a range of symptoms including pneumonia, septic arthritis, endocarditis, peritonitis and severe infections such as meningitis, septicaemia and necrotizing fasciitis (Ruoff and Bisno 2010). The *Streptococcus* strains identified in the indigenous rodents in the present study may represent novel strains or species as they did not group with any described species. These strains appear to be closely related to an uncultured *Streptococcus* detected from *M. musculus* from Marion Island (du Bruyn *et al.* 2008) which is believed to be responsible for a mass mortality event of adult male sub-Antarctic fur seals on Marion Island (du Bruyn *et al.* 2008). Furthermore, a degree of host-specificity was observed in the streptococci identified from the indigenous rodents tested, however further investigation is needed to conclusively establish this specificity pattern.

Members of the genus *Aerococcus* are increasingly being identified as human pathogens (Senneby *et al*. 2013). *Aerococcus viridans* was first described in 1953 as a part of the commensal bacteria of the skin and upper respiratory tract of healthy individuals (William *et al.* 1953). Certain strains are also known to cause disease in lobsters, fish and sea turtles (Greenwood *et al*. 2005). Since its initial description as a human pathogen in 1967 it has been associated with endocarditis, urinary tract infections, arthritis, meningitis and wound infection especially in people with compromised immune systems and in nosocomial environments (Chen *et al.* 2012; Guccione *et al.* 2013). The identification of an aerococci in *O. angoniensis* and *C. damarensis* indicates the possible role of indigenous rodents as reservoirs for pathogenic members of this bacterial genus.

Lactobacillus is the biggest genus of the order Lactobacillales and contains some of the most important probiotic species and species used in fermentative food production (Müller *et al*. 2000). This bacterial genus contains members that are found in carbohydrate-rich environments such as decaying plant material, sewage and the respiratory, gastro-intestinal and genital tracts of humans and animals (Felis and Dellaglio 2007). Although lactobacilli are largely considered benign there are a small number of reports that they are associated with dental caries, septicaemia, rheumatic vascular disease, meningitis, lung abscesses and infective endocarditis in humans, particularly the elderly and immune-compromised individuals (Harty *et al.* 1994). Although the *Lactobacillus* strains associated with the indigenous rodents seem to hold minimal risk to humans, the pathogenic threat of these strains remains unknown.

The phylum Proteobacteria is divided into five classes (Gupta 2000), three of which were observed in the current study. The majority of samples belonged to the α-Proteobacteria, a class containing many plant, animal and human pathogens (Williams *et al.* 2007). To date the genus *Bartonella* contains 33 valid species with more than half having known or suspected zoonotic potential (Buffet *et al*. 2013). *Bartonella* species are mostly vector-borne and causes a wide range of diseases and symptoms (Billeter *et al*. 2008; Kaiser *et al.* 2011). Rodents worldwide have been shown to harbour a diverse assemblage of *Bartonella* species with high prevalence rates (Buffet *et al*. 2013). In South Africa recent studies have identified prevalence rates as high as 56 % and 44 % in commensal and wild rodent populations respectively (Pretorius *et al*. 2004; Bastos 2007). Bartonellae were identified in four rodent species in the present study, however these strains could not be identified to species level. La Scola *et al*. (2003) demonstrated that the 16S rRNA gene was not sensitive enough to discriminate between *Bartonella* species. Studies using phylogenetically informative gene regions such as the citrate synthase (*gltA*) and NADH

dehydrogenase gamma subunit (*nuoG*) genes (La Scola *et al.* 2003; Colborn *et al*. 2010) will allow researchers to identify known and potentially novel bartonellae circulating in indigenous rodent species.

The genus *Anaplasma* contains tick-borne pathogens of humans, dogs, cats, horses and various ruminants (Rar and Golovljova 2011). *Anaplasma bovis* causes a mild febrile illness in ruminants and is primarily transmitted by *Amblyomma variegatum* and *Rhipicephalus appendiculatus* (Rymaszewska and Grenda 2008). *Anaplasma bovis* DNA has also been detected in cottontail rabbits from the USA and raccoons from Japan suggesting that small mammals may also be involved as reservoirs of this bacteria (Goethert and Telford 2003; Sashika *et al.* 2011). Recently *A. bovis* DNA was detected in Eastern rock sengis (*Elephantulus myurus*) and an undescribed rhipicephaline tick species from South Africa, representing a possible transmission cycle (Harrison *et al.* 2011; Harrison *et al*. 2013). The detection of *A. bovis*-like strains in commensal rodents further underscores the probably role of small mammals as reservoirs of this bacterial genus.

Helicobacter is a genus belonging to the class ε-Proteobacteria (Garrity *et al.* 2005). Members of this genus infect the stomach, liver and intestines of various animals and can cause diseases such as gastric ulcers, chronic gastritis, gastroenteritis, hepatitis and adenocarcinoma of the stomach and colon (Fox *et al.* 1998). Numerous *Helicobacter* species have been detected from rodents worldwide with some rodent-associated species such as *H. heilmanii* and *H. bilis*, having known zoonotic potential (Guisti *et al*. 1998; Comunian *et al.* 2006; Whary and Fox 2006). In South Africa, a recent study of commensal *Rattus* species detected *H. rodentium* in 2.2

% of *R. rattus* and *H. muridarum* in 28.3 % of *R. norvegicus* (Mostert 2009). *Helicobacter hepaticus* is one of the most studied rodent-associated species and causes hepatitis in immunocompetent mice and can lead to liver tumours in some strains of mice (Hamada *et al*. 2009). Recently this bacterial species was also detected in bile samples of humans with cholecystitis (Hamada *et al*. 2009). Although the zoonotic potential of *H. hepaticus* remains unclear, the identification of a *Helicobacter* strain in an indigenous rodent is important as they may harbour other *Helicobacter* species that can infect humans.

The genus *Burkholderia*, class β-Proteobacteria, contains more than 60 species that can be found in a wide variety of habitats including soil, water, plant roots and legume nodules (Estrada-de los Santos *et al*. 2013). Several *Burkholderia* species are opportunistic pathogens of humans of which *Bu. pseudomallei*, *Bu. mallei*, and members of the *Bu. cepacia* complex are the most well-known. In humans, species of the *Bu. cepacia* complex mainly cause respiratory diseases in people with impaired immune systems and in nosocomial settings, however, severe infections have also been documented from immune competent individuals (Coenye and LiPuma 2003; Gautam *et al*. 2011). A study conducted in the Jukskei River catchment region (Johannesburg, South Africa) found no *Burkholderia* in the kidneys of *Rattus* species tested but detected *Bu. cepacia* in soil and water samples (Saif 2013). The detection of *Burkholderia* from the kidneys of an indigenous rodent could be due to contact with contaminated soil or water.

The bacterial genera *Bartonella* and *Anaplasma* are associated with numerous zoonotic vector-borne diseases. Nearly a third of the emerging and re-emerging infectious disease events in the last decade were caused by vector-borne diseases (Jones *et al*. 2008). Rodents are known

to host a variety of ectoparasites, several of which can be transmitted to humans and in turn act as vectors of a number of zoonotic diseases (Morand *et al*. 2006). The detection of *Anaplasma* and *Bartonella* in indigenous rodents suggests that these rodents could act as reservoirs for a range of ectoparasite-associated pathogens that are transmissible to humans and other animals. The remaining bacterial genera identified in the indigenous rodents contain species that are potentially pathogenic to humans and animals. The detection of these bacterial genera in the kidney tissues of indigenous rodents indicates the potential for these bacteria to be spread through rodent urine or faeces and therefore pose a risk for environmental contamination of food and water stores.

Many of the bacterial genera identified in this study contain pathogenic members which cause diseases which are more severe in individuals with compromised immune systems such as the very young, elderly, malnourished and individuals with chronic illnesses such as cystic fibrosis and HIV/AIDS (Harty *et al.* 1994; Coenye and LiPuma 2003; Comunian *et al.* 2006; Ismail *et al*. 2010; Chomel *et al.* 2012). South Africa has a high proportion of these individuals and this problem is frequently exacerbated by overburdened health care systems and lack of adequate knowledge of these pathogens (Richards *et al.* 2007). The presence of such diverse bacterial genera with possible pathogenic species is very important and highlights the importance of continued surveillance of indigenous rodents in commensal and natural environments.

References

Bastos, A.D.S., Chimimba, C.T.C., von Maltitz, E., Kerstin, F. and Belmain, S. 2005. Identification of rodent species that play a role in disease transmission to humans in South Africa. *The Southern African Society for Veterinary Epidemiology and Preventive Medicine*, pp 78-83.

Bastos, A.D.S. 2007. *Bartonella* incidence and diversity in endemic South African Murid rodents occurring commensally with humans. *The Southern African Society for Veterinary Epidemiology and Preventive Medicine*, pp 76-81.

Billeter, S.A., Levy, M.G., Chomel, B.B. and Breitschwerdt, E.B. 2008. Vector transmission of *Bartonella* species with emphasis on the potential for tick transmission. *Medical and Veterinary Entomology* **22**: 1-15.

Brettschneider, H. 2010. *Bartonella* dynamics in indigenous and invasive murid rodents from South Africa: Prevalence, diversity and mathematical modelling. PhD Thesis, University of Pretoria, Pretoria, South Africa.

Buffet, J., Kosoy, M. and Vayssier-Taussat, M. 2013. Natural history of *Bartonella*-infecting rodents in light of new knowledge on genomics, diversity and evolution. *Future Microbiology* **8**: 1117-1128.

Chen, L., Yu, W., Huang, S., Lin, M., Chen, T., Fung, .C. and Liu, C. 2012. Successful treatment of *Aerococcus viridans* endocarditis in a patient allergic to penicillin. *Journal of Microbiology, Immunology and Infection* **45**: 158-160.

Chomel, B.B., McMillan-Cole, A.C., Kasten, R.W., Stuckey, M.J., Sato, S., Maruyama, S., Diniz, P.P.V.P. and Breitschwerdt, E.B. 2012. Candidatus *Bartonella merieuxii*, a potential

new zoonotic *bartonella* species in canids from Iraq. *PLoS Neglected Tropical Diseases* **6**: e1843.

Clarridge, J.E. 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews* **17**: 840-862.

Coenye, T. and LiPuma, J.J. 2003 Molecular epidemiology of *Burkholderia* species. *Frontiers in Bioscience* **8**: 55-67.

Comunian, L.B., Moura, S.B., Paglia, A.P., Nicoli, J.R., Guerra, J.B., Rocha, G.A. and Queiroz, D.M.M. 2006. Detection of *Helicobacter* species in the gastrointestinal tract of wild rodents from Brazil. *Current Microbiology* **53**: 370-373.

de Bruyn, P.J.N., Bastos, A.D.S., Eadie, C., Tosh, C.A. and Bester M.N. 2008. Mass mortality of adult male sub Antarctic fur seals: are alien mice the culprits? *PLoS ONE* **3**: e3757.

De Graaff, G. 1981. *The rodents of Southern Africa*. Butterworth, Durban.

Doern, G.V. 2000. Detection of selected fastidious bacteria. *Clinical Infectious Diseases* **30**: 166-173.

Eisen, R.J., Enscore, R.E., Atiku, L.A., Zielinski-Gutierrez, E., Mpanga, J.T., Kajik, E., Andama, V., Mungujakisa, C., Tibo, E., MacMillan, K., Borchert, J.N. and Gage, K.L. 2013. Evidence that rodent control strategies ought to be improved to enhance food security and reduce the risk of rodent-borne illnesses within subsistence farming villages in the plague-endemic West Nile region, Uganda. *International Journal of Pest Management* **59**: 259-270.

Estrada-de los Santos, P., Vinuesa, P., Martınez-Aguilar, L., Hirsch, A.M. and Caballero-Mellado, J. 2013. Phylogenetic analysis of *Burkholderia* species by multilocus sequence analysis. *Current Microbiology* **67**: 51-60.

Felis, G.E. and Dellaglio, F. 2007. Taxonomy of Lactobacilli and Bifidobacteria. *Current Issues in Intestinal Microbiology* **8**: 44-61.

Fox, J.G., Dewhirst, F.E., Shen, Z., Feng, Y., Taylor, N.S., Paster, B.J., Ericson, R.L. Lau, C.N., Correa, P., Araya, J.C. and Roa, I. 1998. Hepatic *Helicobacter* species identified in bile and gallbladder tissue from Chileans with chronic cholecystitis. *Gastroenterology* **114**: 755-763.

Garrity, G.M., Bell, J.A. and Lilbur, T. 2005 Class V. Epsilonproteobacteria class. nov. In: *Bergey's Manual of Systematic Bacteriology, Volume Two: The Proteobacteria*, (eds) G.M. Garrity, D.J. Brenner, N.R. Krieg and J.R. Staley. Springer-Verlag, New York.

Gautam, V., Singhal, L. and Ray, P. 2011. *Burkholderia cepacia* complex: beyond *Pseudomonas* and *Acinetobacter*. *Indian Journal of Medical Microbiology* **29**: 4-12.

Goethert, H.K. and Telford, S.R. 2003. Enzootic transmission of *Anaplasma bovis* in Nantucket cottontail rabbits. *Journal of Clinical Microbiology* **41**: 3744-3747.

Gratz, N.G. 1997. The burden of rodent-borne diseases in Africa south of the Sahara. *Belgian Journal of Zoology* **127**: 71-84.

Gratz, N.G. 1999. Urbanization, arthropod and rodent pests and human health. In *Proceedings of the 3rd International Conference on Urban Pests*, (eds) W.H. Robinson, F. Rettich and G.W. Rambo. pp 51-59.

Greenwood, S.J., Keith, I.R., Després, B.M. and Cawthorn, R.J. 2005. Genetic characterization of the lobster pathogen *Aerococcus viridans* var. *homari* by 16S rRNA gene sequence and RAPD. *Diseases of Aquatic Organisms* **63**: 237-246.

Guccione, J., Nizza, S., Mallardo, K., Cantiello, A., Fiorito, F., Di Loria, A. and De Martino, L. 2013. Penicillin-resistant *Aerococcus viridans* bacteremia associated with bovine severe respiratory syndrome. *Open Journal of Veterinary Medicine* **3**: 131-135.

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

Guisti, A.M., Crippa, L., Bellini, O., Luini, M. and Scanziani, E. 1998. Gastric spiral bacteria in wild rats from Italy. *Journal of Wildlife Diseases* **34**: 168-172.

Gundi, V.A.K.B., Kosoy, M.Y., Makundi, R.H. and Laudisoit, A. 2012. Identification of diverse *Bartonella* genotypes among small mammals from Democratic Republic of Congo and Tanzania. *American Journal of Tropical Medicine and Hygiene* **87**: 319-326.

Gupta, R.S. 2000. The phylogeny of proteobacteria: relationships to other eubacterial phyla and eukaryotes. *FEMS Microbiology Reviews* **24**: 367-402.

Hang´ombe, B.M., Nakamura, I., Samui, K.L., Kaile, D., Mweene, A.S., Kilonzo, B.S., Sawa, H., Sugimoto, C. and Wren, B.W. 2012. Evidence of *Yersinia pestis* DNA from fleas in an endemic plague area of Zambia. *BMC Research Notes* **5**: 72-76.

Hamada, T., Yokota, K., Ayada, K., Hirai, K., Kamada, T., Haruma, K., Chayama, K. and Oguma, K. 2009. Detection of *Helicobacter hepaticus* in human bile samples of patients with biliary disease. *Helicobacter* **14**: 545-551.

Harrison, A., Bown, K.J. and Horak, I.G., 2011. Detection of *Anaplasma bovis* in an undescribed tick species collected from the eastern rock sengi *Elephantulus myurus. Journal of Parasitology* **97**: 1012-1016.

Harrison, A., Bastos, A.D.S. Medger, K. and Bennett, N.C. 2013. Eastern rock sengis as reservoir hosts of *Anaplasma bovis* in South Africa. *Ticks and Tick-borne Diseases* **4**: 503-505.

Harty, D.W.S., Oakey, H.J., Patrikakis, M., Hume, E.B.H. and Knox, K.W. 1994. Pathogenic potential of lactobacilli. *International Journal of Food Microbiology* **24**: 179-189.

Hauben, L., Vauterin, L., Swings, J. and Moore, E.R.B. 1997. Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. *International Journal of Systematic Bacteriology* **47**: 328-335.

Huelsenbeck, J.P. and Ronquist, F. 2001. MrBayes: Bayesian inference of phylogeny. *Bioinformatics* **17**: 754-755.

Ismail, N., Bloch, K.C. and McBride, J.W. 2010. Human ehrlichiosis and anaplasmosis. *Clinical Laboratory Medicine* **30**: 261-292.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L. and Daszak, P. 2008. Global trends in emerging infectious diseases. *Nature* **451**: 990-993.

Kaiser, P.O., Riess, T., O'Rourke, F., Linke, D. and Kempf, V.A.J. 2011. *Bartonella* spp.: Throwing light on uncommon human infections. *International Journal of Medical Microbiology* **301**: 7-15.

Kamani, J., Morick, D., Mumcuoglu, K.Y. and Harrus, S. 2013. Prevalence and diversity of *Bartonella* species in commensal rodents and ectoparasites from Nigeria, West Africa. *PLoS Neglected Tropical Diseases* **7**: e2246.

La Scola, B., Zeaiter, Z., Khamis, A. and Raoult, D. 2003. Gene sequence-based criteria for species definition in bacteriology: the *Bartonella* paradigm. *Trends in Microbiology* **11**: 318-321.

Makundi, R.H., Massawe, A.W., Mulungu, L.S., Katakeba, A., Mbisi, T.J. and Mgode, G. 2008. Potential mammalian reservoirs in a bubonic plague outbreak focus in Mbulu district, Northern Tanzania, in 2007. *Mammalia* **72**: 253-257.

Mas-De-Xaxars, T. and Garcia-Gil, L.J. 2009. Detection and identification of unknown streptococcal populations in clinical samples. *Microbial Ecology in Health and Disease* **21**: 233-240.

McMichael, A. J. 2004. Environmental and social influences on emerging infectious diseases: past, present and future. *Philosophical Transactions: Biological Sciences* **359**: 1049-1058.

Meerburg, B.G., Singleton, G.R. and Kijlstra, A. 2009. Rodent-borne diseases and their risks for public health. *Critical Reviews in Microbiology* **35**: 221-270.

Meheretu, Y., Leirs, H., Welegerima, K., Breno, M., Tomas, Z., Kidane, D., Girmay, K. and Goüy de Bellocq, J. 2013. *Bartonella* prevalence and genetic diversity in small mammals from Ethiopia. *Vector-Borne and Zoonotic Diseases* **13**: 164-175.

Mills, J.N. and Childs, J.E. 1998. Ecologic studies of rodent reservoirs: their relevance for human health. *Emerging Infectious Diseases* **4**: 529-537.

Morand, S. Krasnov, B.R. and Poulin, R. 2006. *Micromammals and Macroparasites: From Evolutionary Ecology to Management*. Springer-Verlag, Tokyo.

Mostert, M.E. 2009. Molecular and morphological assessment of invasive, inland *Rattus* (Rodentia: Muridae) congenerics in South Africa and their reservoir host potential with

respect to *Helicobacter* and *Bartonella*. MSc Thesis. University of Pretoria, Pretoria, South Africa.

Müller, M.R.A., Ehrmann, M.A. and Vogel, R.F. 2000. *Lactobacillus frumenti* sp. nov., a new lactic acid bacterium isolated from rye-bran fermentations with a long fermentation period. *International Journal of Systematic and Evolutionary Microbiology* 50: 2127-2133.

Neerinckx, S., Bertherat, E. and Leirs, H. 2010. Human plague occurrences in Africa: an overview from 1877 to 2008. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **104**: 97-103.

Nikkari, S., Lopez, F.A., Lepp, P.W., Cieslak, P.R., Ladd-Wilson, S., Passaro, D., Danila, R. and Rellman, D.A. 2002. Broad range bacterial detection and analysis of unexplained death and critical illness. *Emerging Infectious Diseases* **8**: 188-194.

Posada, D. 2008. jModalTest: Phylogenetic modal averaging. *Molecular Biology and Evolution* **25**: 1253-1256.

Pretorius, A., Beati, L. and Birtles, R.J. 2004. Diversity of Bartonellae associated with small mammals inhabiting Free State province, South Africa. *International Journal of Systematic and Evolutionary Microbiology* **54**: 1959-1967.

Rar, V.A. and Golovljova, I. 2011. *Anaplasma*, *Ehrlichia*, and ‘‘Candidatus *Neoehrlichia’’* bacteria: Pathogenicity, biodiversity, and molecular genetic characteristics, a review. *Infections Genetics and Evolution* **11**: 1842-1861.

Richards, R., O’leary, B. and Mutsonziwa, K. 2007. Measuring quality of life in informal settlements in South Africa. *Social Indicators Research* **81**: 375-388.

Ruoff, K.L and Bisno, A.L. 2010. Classification of Streptococci. In: *Principles and Practice of Infectious Diseases*, (eds) G.L. Mandell, J.E. Bennett and R. Dolin. Elsevier Churchill Livingstone, Philadelphia.

Rymaszewska, A. and Grenda, S. 2008. Bacteria of the genus *Anaplasma* - characteristics of *Anaplasma* and their vectors: a review. *Veterinarni Medicina* **53**: 573-584.

Saif, A. N. 2013. The detection of *Burkholderia* spp. and pathogenic *Leptospira* spp. in South Africa. MSc Thesis, University of the Witwatersrand, Johannesburg, South Africa.

Sashika, M., Abe, G., Matsumoto, K. and Inokuma, H. 2011. Molecular survey of *Anaplasma* and *Ehrlichia* infections of feral raccoons (*Procyon lotor*) in Hokkaido, Japan. *Vector Borne Zoonotic Diseases* **11**: 349-354.

Senneby, E., Nilson, B., Petersson, A. and Rasmussen, M. 2013. Matrix-assisted laser desorption ionization-time of flight mass spectrometry is a sensitive and specific method for identification of Aerococci. *Journal of Clinical Microbiology* **51**: 1303-1304.

Smith, P., Siddharth, J., Pearson, R., Holway, N., Shaxted, M., Butler, M., Clark, N., Jamontt, J., Watson, R.P., Sanmugalingam, D. and Parkinson, S.J. 2012. Host genetics and environmental factors regulate ecological succession of the mouse colon tissue-associated microbiota. *PLoS ONE* **7**: e30273.

Srinivasan, S. and Fredricks, D.N. 2008. The human vaginal bacterial biota and bacterial vaginosis. *Interdisciplinary Perspectives on Infectious Diseases*. Article ID 750479.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**: 2731-2739.

Taylor, P.J., Arntzen, L., Hayter, M., Iles, M., Frean, J and Belmain, S. 2008. Understanding and managing sanitary risks due to rodent zoonoses in an African city: beyond the Boston Model. *Integrative Zoology* **3**: 38-50.

van Sandwyk, J.H.D. 2007. Identification and classification of endogenous bacteria within mole-rats of the Family Bathyergidae. MScThesis, University of Pretoria, Pretoria, South Africa.

Vos, P., Garrity, G., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.H. and Whitman, W.B. 2009. *Bergey's Manual of Systematic Bacteriology: Volume 3: The Firmicutes*. Springer-Verlag, New York.

Wang, L., Kuo, H., Wu, Y., Tai, C and Lee, F. 2009. *Lactobacillus taiwanensis* sp. nov., isolated from silage. *International Journal of Systematic and Evolutionary Microbiology* **59**: 2064-2068.

Whary, M.T. and Fox, J.G. 2006. Detection, eradication, and research implications of *Helicobacter* infections in laboratory rodents. *Laboratory Animals* **35**: 25-36.

William, R.E.O., Hirch, A. and Cowan, S.T. 1953. *Aerococcus*, a new bacterial genus. *Journal of General Microbiology* **8**: 475-80.

Williams, K.P. Sobral, B.W. and Dickerman, A.W. 2007. A robust species tree for the Alphaproteobacteria. *Journal of Bacteriology* **189**: 4578-4586.

Wohlgemuth, S., Loh, G. and Blaut, M. 2010. Recent developments and perspectives in the investigation of probiotic effects. *International Journal of Medical Microbiology* **300:** 3-10.

Chapter 4

Bartonella prevalence and diversity in commensal and wild populations of *Mastomys coucha* (Rodentia: Muridae) from South Africa

Abstract

Bartonellae are intracellular bacteria that infect a wide variety of vertebrate hosts. Rodent species worldwide harbour a wide diversity of bartonellae often at very high prevalence rates. The aim of the present study was to compare the *Bartonella* prevalence and diversity between wild and commensal populations of the southern multimammate mouse, *Mastomys coucha* (Rodentia: Muridae) from South Africa and to test whether ectoparasite loads influence *Bartonella* prevalence. Heart samples from 58 wild and 89 commensal animals were screened for *Bartonella* genome presence using primer sets targeting the citrate synthase (*gltA*) and NADH dehydrogenase gamma subunit (*nuoG*) genes. A generalized linear mixed model was used to test for differences in *Bartonella* infection between *Mastomys* population, sex, and ectoparasite load. A prevalence of 56 % and 92 % was obtained for wild and commensal animals, respectively, representing a significant difference between the two populations. No statistically significant differences were detected between infection rate with reference to sex and ectoparasite load. Phylogenetic analyses revealed the presence of novel genotypes as well as lineages that correspond with *Bartonella elizabethae* and *B. coopersplainsensis,* which have both been reported from invasive *Rattus* species. The identification of *B. elizabethae,* a species with known

zoonotic potential implicated in causing endocarditis and neuroretinitis in humans and dogs, together with the high infection prevalence in the commensal population highlights the importance of commensal rodents in the spread of zoonotic diseases.

Keywords: Murid rodents, *Bartonella*, prevalence, ectoparasite, molecular genetics, zoonoses, southern Africa

Introduction

Members of the genus *Bartonella* are highly fastidious, Gram-negative, aerobic bacilli (Sato *et al.* 2013). Until the 1990s the genus consisted of a single species, *B. bacilliformis*, the causative agent of Carrion's disease (Brenner *et al*. 1993; Birtles *et al*. 1995). This biphasic disease, transmitted by the sandfly (*Lutzomyia verrucarum*), presents as either Oroya fever or Verruga peruana (Peruvian wart) and is endemic to the Andean region of South America (Brenner *et al*. 1991). Oroya fever is characterised by severe haemolytic anaemia and, with a death rate of 40-90 % if left untreated, has one of the highest mortality rates among human bacterial infections (Kaiser *et al.* 2011).

In the early 1990s, the discovery of *B. quintana* in HIV-positive and homeless people in North America and Europe sparked a renewed interest in this bacterial genus (Spach *et al*. 1993; Ohl and Spach 2000). *Bartonella quintana* is transmitted by the human body louse and causes Trench Fever, a disease that first received attention during World War I and II when it affected millions of soldiers (Ohl and Spach 2000). The renewed scientific interest in this bacterial genus after the re-discovery of this almost forgotten disease, together with improved culturing and molecular detection methods has led to a rapid increase in the number of described *Bartonella* species in the last two decades (Ko *et al*. 2013).

To date, 33 species and 3 subspecies have been formally described and many novel variants remain to be formally described (Mediannikov *et al*. 2013a; Mediannikov *et al*. 2013b; Buffet *et al*. 2013). Almost half of the formally described and candidate *Bartonella* species have known or suspected zoonotic potential (Table 1). Bartonellae are facultative intracellular bacteria

Table 1. Worldwide *Bartonella* species and subspecies, primary reservoirs, incidental hosts, vectors, associated diseases and reference of original descriptions based on Chomel and Kasten (2010), and Pulliainen and Dehio (2012). *Bartonella* species with confirmed or suspected zoonotic potential are indicated in bold. Unconfirmed suspected vectors are indicated with a question mark (?).

Species	Host			Vector	Reference
	Primary reservoir	Incidental	Clinical diagnosis		
Bartonella acomydis	Golden Spiny Mouse (*Acomys russatus*)	Unknown	Unknown	Unknown	Sato *et al.* 2013
Bartonella alsatica †	European Rabbit (*Oryctolagus cuniculus*)	Human	Endocarditis, Lymphadenopathy	Rabbit Flea? (*Spilopsyllus cuniculi*)	Heller *et al.* 1999
Bartonella australis §	Kangaroo (*Macropus giganteus*)	Unknown	Unknown	Unknown	Fournier *et al.* 2007
Bartonella bacilliformis †	Humans	None	Carrion's disease (Oroya fever, Verruga peruana)	Sandfly (*Lutzomyia verrucarum*)	Strong *et al.* 1913
Bartonella birtlesii	Wild rodents (*Apodemus* spp.)	Mouse (*Mus nusculus*)	Unknown	Tick (*Ixodes ricinus*)	Bermond *et al*. 2000
Bartonella bovis	Ruminants (Cow, Moose, Roe deer)	Cat , Dog	Endocarditis	Hippoboscid flies?	Bermond *et al.* 2002
Bartonella callosciuri	Plantain Squirrel (*Callosqurus notatus*)	Unknown	Unknown	Unknown	Sato *et al*. 2013
Bartonella capreoli	Cervids (Roe deer, Elk)	Unknown	Unknown	Hippoboscid flies?	Bermond *et al*. 2002
Bartonella chomelii	Cow	Unknown	Unknown	Hippoboscid flies?	Maillard *et al*. 2004
Bartonella clarridgeiae †	Cat	Human, Dog	Cat Scratch Disease (CSD), Lymphadenopathy	Cat Flea (*Ctenocephalide felis*)	Lawson and Collins 1996
Bartonella coopersplainsensis	Rodents (*Rattus* spp., *Uromys* spp., *Melomys* spp.)	Unknown	Unknown	Unknown	Gundi *et al.* 2009
Bartonella doshiae	Field Vole (*Microtus agrestis*)	Unknown	Unknown	Rodent Fleas? Mites?	Birtles *et al.* 1995
Bartonella elizabethae †	Rodents	Human, Dog	Endocarditis, Neuroretinitis	Rodent Fleas (*Xenopsylla cheopis*?)	Brenner *et al.* 1993

Bartonella florenciae	Shrew (*Crocidura russula*)	Unknown	Unknown	Unknown	Mediannikov *et al.* 2013b
***Bartonella grahamii* †**	Rodents	Human, Water Deer	Neuroretinitis	Rodent Flea (*Ctenophtalmus nobilis nobilis*)	Birtles *et al.* 1995
***Bartonella henselae* †**	Felids	Human, Dog, Horse, Marine mammals	CSD, Endocarditis, Neuroretinitis, Bacillary angiomatosis, Bacillary peliosis, Fever, Epitheloid hemangioendothelioma	Cat Flea	Brenner *et al.* 1993
Bartonella jaculi	Greater Egyptian Jerboa (*Jaculus orientalus*)	Unknown	Unknown	Unknown	Sato *et al.* 2013
Bartonella japonica	Small Japanese Field Mouse (*Apodemus argenteus*)	Unknown	Unknown	Unknown	Inoue *et al.* 2010
***Bartonella koehlerae* †**	Cat	Human, Dog	Endocarditis, Epitheloid hemangioendothelioma	Cat Flea	Droz *et al.* 1999
Bartonella pachyuromydis	Fat-tail Gerbil (*Pachyuromys duprasi*)	Unknown	Unknown	Unknown	Sato *et al.* 2013
Bartonella peromysci	Mice (*Peromyscus* spp.)	Unknown	Unknown	Unknown	Birtles *et al*. 1995
Bartonella phoceensis §	Rodents	Unknown	Unknown	Lice? (*Hoplopleura pacifica*?)	Gundi *et al.* 2004
Bartonella queenslandensis	Rodents (*Rattus* spp., *Uromys* spp., *Melomys* spp.)	Unknown	Unknown	Unknown	Gundi *et al.* 2009
***Bartonella quintana* †**	Humans	Macaque (*Macaca* spp.), Cat, Dog	Trench fever, Endocarditis, Neuroretinitis, Bacillary angiomatosis, Lymphadenopathy	Human body louse (*Pediculus humanis humanis*)	Brenner *et al.* 1993
Bartonella rattaustraliani	Rodents (*Rattus* spp., *Uromys* spp., *Melomys* spp.)	Unknown	Unknown	Unknown	Gundi *et al.* 2009
Bartonella rattimassiliensis §	Rodents	Unknown	Unknown	Lice? (*H. pacifica*? *Polyplax spinulosa*?) Ticks?	Gundi *et al.* 2004
***Bartonella rochalimae* †**	Canids, Raccoon	Human, Rodents (*R. norvegicus*),	Bacteraemia with fever	Fleas? (*Pulex irritans, P. simulans*)	Eremeeva *et al.* 2007
Bartonella schoenbuchensis	Cervids (Moose, Roe deer)	Unknown	Unknown	Deer Keds? (*Lipoptena* spp.)	Dehio *et al.* 2001
Bartonella senegalensis	Rodents?	Unknown	Unknown	Soft tick? (*Ornithodoros sonrai*)	Mediannikov *et al.* 2013a
Bartonella silvatica	Large Japanese Field Mouse (*A. speciosus*)	Unknown	Unknown	Unknown	Inoue *et al*. 2010

Bartonella talpae	Rodents, Moles	Unknown	Unknown	Unknown	Birtles *et al.* 1995
***Baronella tamiae* § †**	Rodents?	Human	Bacteraemia with fever	Ticks? Mites?	Kosoy *et al.* 2008
Bartonella taylorii	Woodmouse? (*Apodemus* spp.)	Unknown	Unknown	Rodent Flea (*Ctenophtalmus nobilis nobilis*)	Birtles *et al.* 1995
Bartonella tribocorum	Rodents	Unknown	Unknown	Rodent Fleas? Lice?	Heller *et al.* 1998
***Bartonella vinsonii arupensis* †**	White-footed Mouse (*Peromyscus leucopus*)	Human, Dog	Endocarditis, Bacetraemia with fever	Flea? Ticks? (*Ixodes* spp.)	Welch *et al.* 1999
***Bartonella vinsonii berkhoffii* †**	Dog, Coyote (*Canis latrans*), Grey Fox (*Urocyon cinereoargenteus*)	White-tailed deer (*Odocoileus virginianus*) Human, Cat	Endocarditis, Epitheloid hemangioendothelioma	Fleas? Ticks?	Kordick *et al.* 1996
Bartonella vinsonii vinsonii	Meadow vole (*Microtus pennsylvaicus*)	Unknown	Unknown	Rodent Fleas? Vole ear mite? (*Trombicula miroti*)	Kordick *et al.* 1996
***Bartonella washoensis* § †**	Ground Squirrels (*Spermophilus beecheyii*), Rodents	Human, Dog	Endocarditis, Myocarditis	Rodent Fleas?	Regnery *et al.* 1992

† *Bartonella* species with confirmed or suspected zoonotic potential

§ *Bartonella* species that do not appear on the "List of Prokaryotic names with Standing in Nomenclature" to date

that infect the erythrocytes and endothelial cells of various vertebrate hosts (Birtles 2005). In the primary reservoir host, infections often lead to chronic intra-erythrocytic bacteraemia but usually either remain asymptomatic or cause low morbidity (Chomel *et al*. 2009). In incidental hosts, however, *Bartonella* causes a broad spectrum of symptoms ranging from acute febrile illnesses, lymphadenopathy and septicaemia to vascuproliferative disorders and diverse manifestations involving cardiovascular, neurological, ocular and musculoskeletal systems (Kaiser *et al.* 2011; Breitschwerdt *et al.* 2012; Maritsi *et al.* 2013; Mascarelli *et al*. 2013; Minadakis *et al.* 2013). The presentation of symptoms caused by *Bartonella* infections also depend on the immune status of an individual (Jacomo *et al.* 2002). *Bartonella henselae* infections in humans, the predominant cause of Cat Scratch Disease, typically cause self-limiting infections in immune-competent individuals but result in severe systemic diseases in immune-compromised individuals (Chomel *et al.* 2012).

Bartonella is primarily transmitted by heamatophagous arthropods but transmission through animal scratches, bites and direct contact with infected blood has also been reported (Heller *et al.* 1998; Dehio *et al.* 2001). Various *Bartonella* species have been isolated from a wide range of arthropods including fleas, lice, ticks, mites and biting flies and even from non-heamatophagous arthropods (Billeter *et al.* 2008; Mascarelli *et al*. 2013). An increasing number of studies have focussed on identifying potential vectors for *Bartonella* species; however, thus far only five ectoparasites have been confirmed as vectors (Billeter *et al.* 2008; Tsai *et al.* 2011). The sandfly is the confirmed vector for *B. bacilliformis*, the human body louse for *B. quintana*, the rodent flea *Ctenophtalmus nobilis* transmits *B. grahamii* and *B. taylorii*, the cat flea *Ctenocephalides felis* transmits *B. henselae* and recently it has been demonstrated that the tick

species *Ixodes ricinus* is a competent vector for *Bartonella birtlesii* (Bouhsira *et al.* 2013). *Bartonella* species are distributed worldwide and have been isolated from a wide diversity of mammalian orders including carnivores, ruminants, ungulates, cetaceans, bats, lagomorphs, insectivores and rodents (Birtles 2005; Harms and Dehio 2012) (Table 1).

Bartonella in rodents and their ectoparasites

Many zoonotic viral, bacterial and protozoal diseases are known to be spread by rodents (Meerburg *et al* 2009). Numerous rodent species all over the world have been shown to harbour a wide diversity of bartonellae often at very high prevalence rates (Paziewska *et al.* 2012; Table 2). *Bartonella* infections in rodents do not seem to have a direct effect on rodent mortality and the proportion of infected individuals appears to decrease with age (Birtles *et al.* 2001). Long-term studies of several species have shown that there is a significant decrease in *Bartonella* prevalence in older animals (Fichet-Calvet 2000; Kosoy *et al.* 2004a; Bai *et al.* 2008; Kosoy 2010). These studies also noted that the level of bacteraemia was higher in juveniles (Kosoy *et al.* 2004a; Jardine *et al.* 2006). Several authors have suggested that acquired immunity explains these age-related changes in *Bartonella* prevalence but there is no conclusive evidence to support this hypothesis (Fichet-Calvet 2000; Kosoy *et al.* 2004a; Kosoy *et al.* 2004b).

Table 2. Worldwide *Bartonella* species and prevalence in rodents (adapted from Brettschneider 2010, see also Inoue *et al*. 2008, Saisongkorth *et al*. 2009, Gundi *et al.* 2012, Meheretu *et al.* 2013, Karagöz *et al.* 2013).

Rodent species	Country Sampled	Prevalence	Bartonella species
Acomys cahirinus	Israel	25%	*Bartonella* spp.
Apodemus agrarius	Korea, Russia	6.7% - 73%	*B. elizabethae, B. grahamii, B.taylorii*
Apodemus argenteus	Japan	54%	*B. grahamii, B. japonica*
Apodemus chevrieri	China	62%	*B. elizabethae, B. grahamii*
Apodemus draco	China	33%	*B. elizabethae, B. grahamii*
Apodemus flavicollis	Sweden	42%	*B. grahamii*
Apodemus latronum	China	71%	*B. elizabethae, B. grahamii*
Apodemus peninsulae	Russia	60%	*B. grahamii, B.taylorii*
Apodemus spp.	Germany	82%	*B. birtlesii*
Apodemus speciosus	Japan	60%	*B. grahamii, B. silvatica, B.taylorii*
Apodemus sylvaticus	UK	24%	*B. doshiae, B. grahamii, B.taylorii*
Arvicanthis neumanni	Democratic Republic of Congo (DRC)	40%	*B. elizabethae*
Arvicanthis dembeensis	Ethiopia	37%	*B. elizabethae*
Bandocota indica	Thailand	8%	*Bartonella* spp.
Clethrionomys grapperi	Canada	66%	*B. elizabethae, B. grahamii*
Clethrionomys glareolus	Canada, Poland, Sweden, UK	15% - 64%	*B. doshiae, B. grahamii, B.taylorii*
Clethrionomys rufocanus	Japan, Russia	23% - 60%	*B. grahamii, B.taylorii*
Eothenomys spp.	China, Korea	11% - 18%	*B. elizabethae, B. grahamii*
Gerbilliscus leocogaster	South Africa	63 - 96%	*B. elizabethae, B. grahamii, B. tribocorum*
Lemniscomys rosalia	South Africa	10%	*Bartonella* spp.
Lophuromys rita	DRC	25%	*Bartonella* spp.
Lophuromys spp.	Tanzania	50%	*B. elizabethae*
Mastomys awashensis	Ethiopia	37%	*B. elizabethae*
Mastomys natalensis	South Africa	60%	*B. elizabethae, B. grahamii, B. tribocorum*
Mastomys spp.	South Africa	58%	*B. elizabethae*
Micaelamys namaquensis	South Africa	57%	*B. elizabethae, B. grahamii, B. tribocorum*
Microtus agrestis	Sweden	33%	*B. grahamii*
Microtus arvalis	Poland	32%	*B. grahamii*
Microtus fortis	Russia	83%	*B. grahamii, B.taylorii*
Microtus oeconomus	Poland	11%	*B. grahamii*
Microtus socialis	Turkey	57%	*B. grahamii, B.taylorii*
Mus minutoides	DRC	66%	*Bartonella* spp.
Mus musculus	Sweden	5%	*B. grahamii*
Otomys irroratus	South Africa	50%	*B. elizabethae, B. grahamii, B. tribocorum*
Praomys delectorum	Tanzania	66%	*B. tribocorum*
Peromyscus leucopus	USA	76%	*B. grahamii, B. vinsonii, arupensis*
Peromyscus maniculatus	Canada	33%	*B. vinsonii arupensis*
Psammomys obesus	Tunisia	90%	*Bartonella* spp.
Rattus berdmorei	Thailand	25%	*B. coopersplainsensis, B. queenslandensis*
Rattus exulans	Thailand	1.4%	*Bartonella* spp.
Rattus losea	Thailand	18% - 66%	*B. elizabethae, B. grahamii*
Rattus norvegicus	China, France, Nigeria, Portugal, South Africa, Taiwan, Thailand, USA	19% - 52%	*B. elizabethae, B. grahamii, B. tribocorum, B. phoceensis, B. rattimassiliensis*
Rattus rattus	DRC, Ethiopia, Israel, Japan, Nigeria, Portugal, South Africa, Thailand, USA	4% - 24%	*B. elizabethae, B. grahamii, B. rattimassiliensis, B. vinsonii spp.*
Rattus savilei	Thailand	6%	*B. queenslandensis, B. rochalimae*
Rattus surifer	Thailand	24%	*B. coopersplainsensis, B. phoceensis, B. queenslandensis*
Rattus tanezumi	China, South Africa	7% - 41%	*B. elizabethae, B. grahamii*
Rhabdomys spp.	South Africa	44%	*B. elizabethae, B. grahamii, B. tribocorum*
Saccostomus campestris	South Africa	50%	*B. elizabethae, B. grahamii, B. tribocorum*
Stenocephalemys albipes	Ethiopia	64%	*B. elizabethae*

Most longitudinal studies of *Bartonella* in rodents have been conducted in the northern hemisphere and have indicated that transmission mostly occurs during summer and autumn (Fichet-Calvet 2000; Kosoy *et al.* 2004a; Jardine *et al.* 2006; Bai *et al.* 2008). Conversely, recent studies from Ethiopia and South Africa found no significant difference in prevalence rates between the wet and the dry seasons (Brettschneider 2010; Brettschneider *et al.* 2012a; Meheretu *et al.* 2013). Differences in *Bartonella* infections between sexes are not consistent. Some studies show no significant differences between sexes (Gundi *et al.* 2004; Jardine *et al.* 2006; Bai *et al.* 2008; Brettschneider 2010; Brettschneider *et al.* 2012a), while others show higher infection rates in females (Brettschneider 2010; Meheretu *et al.* 2013) and one study found that bacteraemic male cotton rats had significantly higher colony forming units than bacteraemic female rats (Kosoy *et al.* 2004a).

Although *Bartonella* infections do not appear to cause any outward harm in rodents, there may be indirect effects on population fitness (Kosoy 2010). *Bartonella* has been isolated from embryos and neonates of naturally and experimentally infected rodents (Kosoy *et al.* 1998; Boulouis *et al.* 2001). The *in-utero* transmission in experimentally infected mice led to reduced number of embryos, increased foetal resorption, increased foetal death rates and low birth weights (Boulouis *et al.* 2001). Studies that investigate the influence of *Bartonella* infections on parameters such as reproductive output or survival of rodent populations are limited and represent a major gap in the understanding of the disease dynamics of this bacterial genus (Jardine *et al.* 2006).

Reports of host-specificity of rodent-associated *Bartonella* are variable. Certain species, such as *B. doshiae, B. elizabethae, B. grahamii*, and *B. taylorii* occur in a wide range of rodent hosts worldwide and show little host-specificity (Kosoy 2010; Meheretu *et al*. 2013). However, experimental infections of cotton rats (*Sigmodon hispidus*) and white-footed mice (*Peromyscus leucopus*) with *Bartonella* led to bacteraemia only when the inoculated strain was isolated from naturally infected individuals from either the same species or close taxonomic relatives (Kosoy *et al.* 2000). Several recently described rodent-associated bartonellae exhibit very narrow host ranges, although this may be due to the limited number of species screened (Inoue *et al.* 2010; Sato *et al.* 2013). Co-infection in rodents with different *Bartonella* species but not different strains of the same species has been documented (Kosoy *et al.* 1999; Birtles *et al*. 2001). These observations indicate that cross-protection between *Bartonella* species does not occur but that immunity to one strain provides immunity against related strains of the same species (Kosoy *et al.* 1999; Birtles *et al*. 2001).

It is estimated that vector-borne diseases, inclusive of Bartonellosis, are responsible for almost 30 % of the emerging infectious diseases reported in the last decade (Jones *et al.* 2008). The arthropod vectors and transmission dynamics for most *Bartonella* species in rodents are either unknown or largely understudied (Reeves *et al.* 2006; Chomel *et al.* 2009). *Bartonella* species have been detected in fleas collected from a wide diversity of rodents (Morway *et al*. 2008; Tsai *et al.* 2011). Fleas do not seem to be negatively affected by *Bartonella* infection and this may suggest a long history of co-evolution (Morick *et al*. 2013a). *Bartonella* DNA has been isolated from the reproductive tissue of fleas collected from small mammals suggesting that vertical transmission may occur (Brinkerhoff *et al*. 2010). A recent study also demonstrated

potential vertical non-transovarial transmission of *Bartonella* to flea larvae with *Bartonella*-positive flea faeces (Morick *et al.* 2013b). Although fleas have been proposed as predominant vectors among rodents, *Bartonella* has also been detected from rodent ticks, mites and lice demonstrating their vector potential (Tsai *et al.* 2011). The above-mentioned studies only broadly summarise the current knowledge on *Bartonella* infections in rodents and highlight the substantial gaps that still exist in the understanding of the dynamics of this bacterial genus, particularly in the southern hemisphere and African rodent species.

Rodent-associated Bartonella in Africa

Bartonellae have been reported from an increasingly wide range of rodent species from Africa. *Bartonella* infection prevalence and dynamics in African rodent species was investigated for the first time in the fat sand rat (*Psammomys obesus*) from Tunisia (Fichet-Calvet *et al.* 2000). Later *Bartonella* infections were detected in a number of murid rodent species from South Africa (Pretorius *et al*. 2004; Bastos 2007). Increasing interest in this bacterial genus has led to a number of reports on *Bartonella* prevalence from several African countries. Rodents trapped in domestic and peri-domestic areas in Ethiopia and Nigeria showed *Bartonella* prevalence rates of 34 % and 26 %, respectively (Kamani *et al*. 2013; Meheretu *et al*. 2013). In Tanzania and the Democratic Republic of Congo (DRC), rodents from peri-domestic and natural environments had *Bartonella* infection rates ranging from 8.5 % to 38.5 % (Gundi *et al*. 2012). The bartonellae isolated in these countries displayed a degree of host specificity and several genotypes detected in these rodents corresponded with formally recognised species, *B. elizabethae, B. grahamii, B. tribocorum* and *B. queenslandensis* (Gundi *et al*. 2012; Kamani *et al*. 2013; Meheretu *et al*. 2013). A number of distinct possibly novel genotypes were also detected, re-emphasising the

diversity of rodent-associated bartonellae. In addition, 28 % of ectoparasites collected from Nigerian rodents tested positive for *Bartonella* DNA, however significant differences between ectoparasite load and *Bartonella* infection rates were not determined (Kamani *et al*. 2013). Although ectoparasites collected from rodents in Ethiopia were not tested for *Bartonella*, Meheretu *et al*. (2013) did not find an increased probability of *Bartonella* infection with increased ectoparasite load.

More specifically, in South Africa few studies have investigated *Bartonella* prevalence and diversity in indigenous rodents. The first study to assess *Bartonella* prevalence and diversity in wild rodent populations from South Africa found an overall prevalence of 44 % in eight rodent species (Pretorius *et al*. 2004). Phylogenetic analysis recovered genotypes that corresponded with *B. elizabethae* and *B. grahamii*, as well as genotypes exclusively isolated from South African rodents at that time (Pretorius *et al*. 2004). A study by Bastos (2007) concentrated on *Bartonella* infections in commensal rodent populations. In the eight indigenous rodent species tested that included the southern multimammate mouse, *Mastomys coucha*, an overall prevalence of 56 % was found and phylogenetic analyses identified genotypes that corresponded with genotypes detected in the previous South African study and several that appeared to be novel (Bastos 2007). In a longitudinal study investigating *Bartonella* dynamics in the Namaqua rock mouse, *Micaelamys namaquensis*, an overall prevalence of 44 % was found with no significant difference between infection rates and sex or season (Brettschneider *et al.* 2012a). Interestingly, *Bartonella* prevalence in invasive commensal species of the genus *Rattus* in South Africa ranged from 5 % to 27 % (Mostert 2009; Brettschneider 2010).

It is evident that South African rodents harbour a diverse range of known and potentially novel *Bartonella* strains. From these studies it also appears that commensal rodents in South Africa may have a higher incidence of *Bartonella* infection than wild populations, however, to date no study focussing on a single rodent species has investigated this phenomenon. Therefore, the aim of this study was to compare the *Bartonella* prevalence and diversity between wild and commensal populations of *M. coucha* from South Africa and to test whether ectoparasite loads affect *Bartonella* infection prevalence.

Members of the genus *Mastomys* have long been recognised as a pest species (Leirs *et al.* 1996). They are capable of very high reproductive rates and are, therefore, prone to population eruptions (Lima *et al.* 2003). Not only are they regarded as a major agricultural pest but they have also been associated with the spread of zoonotic diseases such as plague, leptospirosis and lassa fever (Gratz 1997). High *Bartonella* prevalence rates have been detected in *Mastomys* species from Africa, ranging from 37 % in *M. awashensis* in Ethiopia (Meheretu *et al.* 2013) to 60 % in *M. natalensis* in South Africa (Pretorius *et al.* 2004). The southern multimammate mouse is widely distributed throughout southern Africa and frequently occurs commensally with humans (Skinner and Chimimba 2005). *Bartonella* has been detected in *M. coucha* from South Africa (Bastos 2007) but not in animals from the DRC (Gundi *et al.* 2012). This study represents the first detailed investigation contrasting *Bartonella* infections in wild and commensal populations of a single rodent host species, *M. coucha*, in Africa.

Materials and methods

Sample collection

Commensal *M. coucha* were sampled at 4-6 week intervals between August 2010 and July 2011 in Hammanskraal (25°42´60´´S; 28°27´20´´E; 1111 meters above sea level) and the University of Pretoria (UP) Experimental farm (25°75´11´´S; 28°26´29´´E; 1375 m.a.s.l.), Gauteng Province, South Africa (see Figure 1 in Chapter 2). Wild *M. coucha* populations were sampled bi-annually over a two-year period from September 2008 to April 2010 from southern Kruger National Park (KNP; Brettschneider 2010) between the Lower Sabie and Tshokwane Rest Camps (24°48´02´´S, 31°52´45´´E to 24°89´77´´S, 39°12´33´´E, 200-400 m.a.s.l). Animals were live-trapped using Sherman live traps (H.B. Sherman Traps Inc. Florida, U.S.A.) baited with a mixture of peanut butter, oatmeal and fish oil and processed as detailed in Chapter 1. All *M. coucha*, which is morphologically indistinguishable from its sibling species, the Natal multimammate mouse (*M. natalensis*) were positively-identified using cytochrome *b* (cyt *b*) amplification and sequence analyses (Bastos *et al*. 2005) or with a *Mastomys*-specific multiplex PCR protocol (see Chapter 2).

Molecular detection and phylogenetic analysis

Total genomic DNA was extracted from heart tissue using the High Pure PCR template preparation kit (Roche) according to the prescribed manufacturer's protocol. All samples were screened for *Bartonella* genome presence using two *Bartonella*-specific primer sets: (i) Bart-EF and BhCs1137n amplifying a 722 bp region of the citrate synthase gene (*gltA*) (Bastos *in prep*.; Norman *et al.* 1995); (ii) NuoGF and NuoGR that amplify a 346 bp amplicon of the NADH dehydrogenase gamma subunit (*nuoG*) gene (Colborn *et al.* 2010). In a previous study

(Brettschneider 2010), wild population samples were screened with a third *Bartonella*-specific primer set; (iii) Bart-EF and Bart-ER which targets a 513 bp region of the citrate synthase gene (*gltA*). PCRs were performed in a final reaction volume of 50 µl containing 1x Buffer (Biotools), 0.25 µM dNTP's (Fermentas), forward and reverse primer at concentrations indicated in Table 3, 1U of BiotoolsTaq™ DNA polymerase and 100-200 ng DNA template. A touchdown PCR program was used for all three primer sets and all PCR reactions for a specific primer set were run on the same thermocycler (ABI 2720, Applied Biosystems, Table 3).

Table 3. Final primer concentrations and reaction conditions for *Bartonella*-specific primer sets.

Final concentration of Forward and Reverse primer		Initial denaturation	Step 1 (3 cycles)	Step 2 (5 cycles)	Step 3 (35 cycles)	Final extension
(i) BartEF and BhCs1137n	0.5 µM	96°C, 10s	96°C, 12s; 51°C, 30s; 70°C, 60s	96°C, 12s; 49.5°C, 25s; 70°C, 55s	96°C, 12s; 48°C, 25s; 70°C, 50s	70°C, 60s
(ii) NuoGF and NuoGR	0.4µM	96°C, 10s	96°C, 12s; 53°C, 30s; 70°C, 60s	96°C, 12s; 51.5°C, 25s; 70°C, 55s	96°C, 12s; 50°C, 30s; 70°C, 50s	70°C, 60s
(iii) BartEF and BartER	0.8µM	96°C, 10s	96°C, 12s; 53°C, 30s; 70°C, 60s	96°C, 12s; 51.5°C, 25s; 70°C, 55s	96°C, 12s; 50°C, 30s; 70°C, 50s	70°C, 60s

Positive samples were purified using the Roche PCR Product Purification Kit, and cycle sequenced using BigDye v 3.1 terminator cycle-sequencing kit (Perkim-Elmer, Foster City, U.S.A.). Sequences were precipitated and run on the ABI PRISM ™ 3100 Analyser (Applied Biosystems). Nucleotide sequences were viewed and aligned in MEGA5 (Tamura *et al.* 2011) prior to performing a BLAST search (www.ncbi.nm.nih.gov/blast) to identify the *Bartonella* reference species with the highest sequence similarity. For phylogenetic analyses, sequences generated from this study were aligned with reference sequences downloaded from the National Centre for Biotechnology Information (GenBank; www.ncbi.nm.gov/genbank) database and from previous studies performed on murid rodents from South Africa (Pretorius *et al.* 2004; Bastos 2007; Brettschneider *et al. in prep*.).

Neighbour-Joining (NJ) analyses were performed in MEGA5 (Tamura *et al.* 2011), Maximum Likelihood (ML) analyses in PhyML (Guidon and Gascuel 2003) and Bayesian Inference (BI) with MrBayes (Huelsenbeck and Ronquist 2001) using the best-fit model and parameters identified under the Akaike Information Criterion (AIC), chosen with jModelTest (Posada 2008). For the BI, four Markov Monte Carlo chains were run for 15,000,000 generations using default heating and swap settings, and were sampled every 100 generations. Trace files were viewed using Tracer version 1.5 (http://beast.bio.ed.ac.uk/) after which 20 % of the runs were discarded as burn-in. Nodal support was determined by 10,000 and 1,000 bootstrap replications for NJ and ML analyses, respectively and from Bayesian posterior probabilities (BPP). Genetic distances (uncorrected p-distance) were determined in MEGA5 (Tamura *et al.* 2011).

Statistical analyses

Ectoparasite loads were calculated by dividing the total number of a specific ectoparasites collected from a population of *M. coucha*, by the number of rodents sampled. Tick, mite, flea, louse and total ectoparasite indices were calculated for each *M. coucha* population and are summarised in Table 5. For statistical analyses, final *Bartonella* prevalence was based on the recovery of at least one unambiguous sequence with either *gltA* or *nuoG* or both. Differences between final *Bartonella* prevalence and *M. coucha* population, sex and ectoparasite load were tested using Generalized Linear Models in STATISTICA v11 (StatSoft Inc., Oklahoma, USA, 2012).

Results

Bartonella prevalence and ectoparasite load

Bartonella prevalence for the commensal and wild *M. coucha* populations is summarised in Table 4. A small proportion of ambiguous sequences (based on multiple peaks observed in sequence chromatographs) were recovered for both gene regions from the commensal population but not from the wild population samples (Table 4). Samples yielding at least one unambiguous sequence for one of the two gene regions tested were considered *Bartonella*-positive and included in the statistical analyses. Sequencing of positive samples in cases where there was a conflicting result between the two gene regions, confirmed *Bartonella* in all cases and these sequences were therefore included in that respective gene region's phylogenetic dataset. The data showed a statistically significant difference in the final *Bartonella* prevalence between the commensal (92.9 %) and wild (56.9 %) populations of *M. coucha* but not between sexes (Population: $F_{1, 139} = 28.49$; $n = 142$; $P < 0.001$; Sex: $F_{1, 139} = 0.10$; $n = 142$; $P = 0.75$).

Table 4. *Bartonella* prevalence (%) based on PCR screening and sequencing of *gltA* (722 bp) and *nuoG* (328 bp) partial gene fragments for commensal and wild *Mastomys coucha* populations from South Africa.

Commensal population			
	nuoG	***gltA***	**Final**
Prevalence % (No. positive/tested)	88.1 (74/84)	77.4 (65/84)	92.9 (78/84)
Male	85 (57/67)	76 (51/67)	91 (61/67)
Female	100 (17/17)	82 (14/17)	100 (17/17)
Ambiguous sequences †	2.3 (2/84)	3.5 (3/84)	
Conflicts ‡	15.4 (13/84)	4.7 (4/84)	
Natural population			
	nuoG	***gltA***	**Final**
Prevalence % (No. positive/tested)	46.6 (27/58)	46.6 (27/58)	56.9 (33/58)
Male	45.8 (11/24)	58.3 (14/24)	58.3 (14/24)
Female	47 (16/34)	38.2 (13/34)	55.9 (19/34)
Ambiguous sequences †	0	0	
Conflicts ‡	10.3 (6/58)	10.3 (6/58)	

† Samples where multiple peaks were observed in sequence chromatographs

‡ Samples that were positive with one gene region but negative with other gene region

Ectoparasite load was higher in all cases for commensal populations. Female commensal *M. coucha* showed higher ectoparasite loads for all ectoparasites counted except lice. There was no clear trend between male and female *M. coucha* from the natural population (Table 5). There was a statistically significant difference in total ectoparasite load between natural and commensal populations but not for individual ectoparasite groups (Total: $F_{1, 137} = 6.92$; $n = 142$; $P < 0.05$; Ticks: $F_{1, 137} = 2.51$; $n = 142$; $P = 0.11$; Mites: $F_{1, 137} = 0.77$; $n = 142$; $P = 0.38$; Fleas: $F_{1, 137} = 0.12$; $n = 142$; $P = 0.73$). Statistically significant differences were obtained for ectoparasite load

between sexes for mites and total ectoparasite load but not for ticks, fleas or lice (Total: $F_{1, 137}$ = 5.13; n = 142; P < 0.05; Mites: $F_{1, 137}$ = 6.43; n = 142; P < 0.001; Ticks: $F_{1, 137}$ = 3.65; n = 142; P = 0.06; Fleas: $F_{1, 137}$ = 0.11; n = 142; P = 0.74). There was no statistically significant difference between *M. coucha* infection status and ectoparasites indices (Total: $F_{1, 137}$ = 2.96; n = 142; P = 0.08; Ticks: $F_{1, 137}$ = 2.05; n = 142; P = 0.15; Mites: $F_{1,137}$ = 0.73; n = 142; P = 0.39; Fleas: $F_{1, 137}$ = 0.69; n = 142; P = 0.41).

Table 5. Summary of ectoparasite loads for wild and commensal populations of *Mastomys coucha* from South Africa.

	Total	Ticks	Mites	Fleas	Lice
Commensal	18.54	3.10	12.42	1.06	1.96
Male	15.85	2.78	9.84	0.99	2.25
Female	29.12	4.35	22.59	1.35	0.82
Natural	2.81	0.52	2.14	0.14	0.02
Male	2.79	1.13	1.5	0.13	0.04
Female	2.82	0.09	2.59	0.15	0.00

Phylogenetic analyses

Phylogenetic analyses were performed on the *gltA* and *nuoG* datasets separately. Phylogenetic analyses of the *gltA* dataset were performed on a 98-taxon dataset, with 53 *Bartonella*-positive *M. coucha* sequences generated from this study and 45 *Bartonella* reference sequences obtained from the NCBI database and from a previous study by Bastos (2007). This 444 bp *gltA* dataset contained 152 variable sites and 121 parsimony informative sites. The TIM1+I+G (G=0.234) model of sequence evolution was chosen as the best fit model under the AIC in jModelTest (Posada 2008). The ML, BI and NJ analyses recovered the same tree topology and support indices are summarised on relevant nodes of the NJ tree (Figure 1).

Based on a 0.05-0.14 mean genetic distance (uncorrected p-distance) between lineages, five distinct *gltA* lineages were recovered (Birtles and Raoult 1996; La Scola 2003). From this phylogeny two of these lineages appear to be specific to the genus *Mastomys*. The first of these lineages (Lineage II; Figure 1) contains *Bartonella* isolated from the commensal population from this study and samples isolated from wild populations of *Mastomys* collected from the Free State Province in the central part of South Africa and commensal populations of *Mastomys* collected from Limpopo Province in the northern part of South Africa (Pretorius *et al*. 2004; Bastos 2007). The second species-specific lineage (Lineage V; Figure 1) contains *Bartonella* isolated from commensal and natural populations from this study. They group together with samples isolated from commensal *M. coucha* identified previously from Limpopo Province (Bastos 2007).

The remaining three lineages contain *Bartonella* isolated from various murid rodent species. Of these, one lineage appears to be specific to South African rodents (Lineage I; Figure 1) and contains both commensal and wild *M. coucha* samples and coincides with a bacterial lineage described previously by Pretorius *et al.* (2004) and Bastos (2007). Although the South African-specific lineage does not have reliable support, together with Lineage II and *Bartonella* isolated from wild populations of *Rhabdomys* sp., it falls within a well-supported, previously-described cluster (Pretorius *et al*. 2004). Another lineage corresponds with the formally recognised *B. elizabethae* (Lineage III; Figure 1). The largest proportion of samples (38.2 %, $^{34}/_{89}$) grouped within this lineage. Reference strains from *Rattus* species group with both wild and commensal samples indicating possible transmission of *Bartonella* between invasive and indigenous murid species.

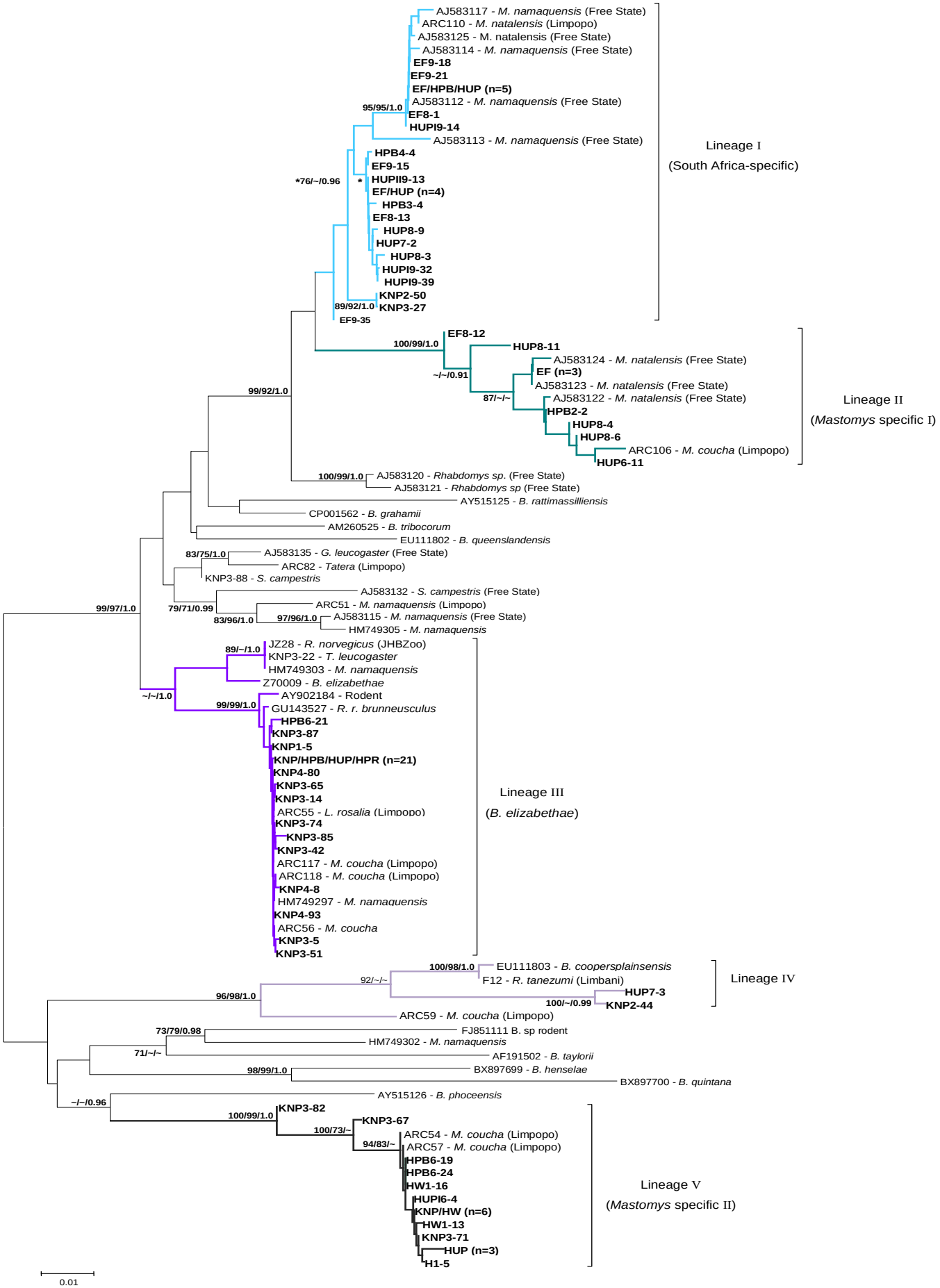

Figure 1. Neighbour Joining (NJ) tree inferred from partial *gltA* gene sequences of *Bartonella* occurring in commensal and wild populations of *Mastomys coucha* from South Africa. Bootstrap support (> 70 %) and Bayesian posterior probabilities (> 0.90) are indicated at relevant nodes (NJ/ML/BPP). Samples indicated in bold represent *Bartonella* isolated from *M. coucha* from the current study and are prefixed with KNP (Kruger National Park, South Africa) for wild populations and with HW/HPB/HUP/EF for commensal populations. Samples not in bold indicate accession numbers of the sequences downloaded from Genbank and samples prefixed with ARC indicate sequences obtained from a previous study (Bastos 2007).

The remaining lineage contains samples isolated from both wild and commensal populations of *M. coucha* (Lineage IV; Figure 1). The *gltA* reference sequence that shared the highest sequence similarity (94.6 %) with this lineage was that of *B. coopersplainsensis*, a species that has only recently been found to be present in South Africa (Mostert 2009; Brettschneider 2010). This lineage is also sister to a novel bacterial lineage previously recovered from *M. coucha* by Bastos (2007).

Phylogenetic analyses of the *nuoG* dataset were performed on a 93-taxon dataset, with 69 *Bartonella* sequences generated from this study and 24 *Bartonella* reference sequences obtained from the NCBI database and from a previous study (Brettschneider *et al.* unpublished). This *nuoG* dataset, 328 nucleotides in length, contained 99 variable sites and 75 parsimony informative sites. The TVM+I+G (G=0.479) model of sequence evolution was chosen as the best fit model under the AIC in jModelTest. The NJ and ML analyses recovered the same tree topology. The tree topology recovered by BI analysis was similar, however it differed in the placement of Lineages C and Lineage F; with Lineage C being basal instead of Lineage F. Apart from this difference, the tree topologies were similar therefore support indices are summarised on relevant nodes of the NJ tree (Figure 2).

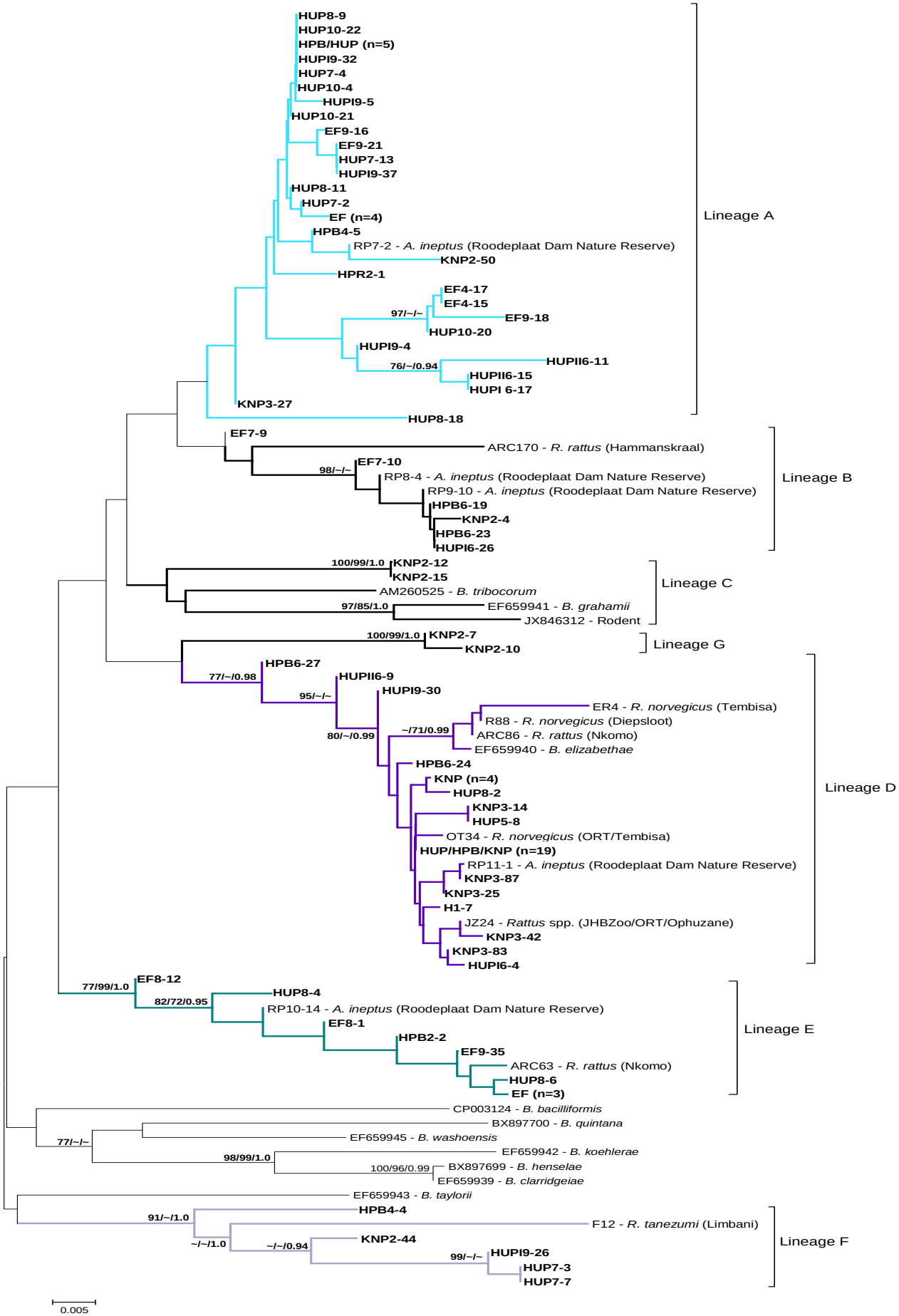

Figure 2. Neighbour Joining (NJ) tree inferred from partial *nuoG* gene sequences of *Bartonella* occurring in commensal and wild populations of *Mastomys coucha* from South Africa. Bootstrap support (> 70 %) and Bayesian posterior probabilities (> 0.90) are indicated at relevant nodes (NJ/ML/BPP). Samples indicated in bold represent *Bartonella* isolated from *Mastomys coucha* from the current study and are prefixed with KNP (Kruger National Park, South Africa) for wild populations and with HW/HPB/HUP/EF for commensal populations. Samples not in bold indicate accession numbers of the sequences downloaded from Genbank or samples obtained from a previous study (Brettschneider *et al*. *in prep*.). Clade colours correspond to concordant *gltA* lineages in Figure 1.

Based on a 0.043-0.106 mean genetic distance (uncorrected p-distance) between lineages, seven distinct *nuoG* lineages were recovered (Birtles and Raoult 1996; La Scola 2003). Four of these lineages (Lineages A, D, E and F) are concordant with lineages recovered in the *gltA* phylogeny, whilst the remaining three (Lineages B, C and G) contained samples that were negative for the *gltA* gene region (Figure 2). As in the *gltA* phylogeny, the largest proportion of samples (36 %; $^{36}/_{99}$) grouped with the formally recognised *B. elizabethae* (Lineage D). Sister to this lineage is Lineage G containing two samples from the natural *M. coucha* population that were negative for the *gltA* gene region (Figure 1). Lineage A corresponds with the South African-specific lineage identified in the *gltA* phylogeny in Figure 1 and contains samples isolated from commensal and natural South African rodents. Lineage E contains samples from the *gltA Mastomys*-specific Lineage I in Figure 1. In the *nuoG* phylogeny however, these samples grouped with *Bartonella* isolated from the Tete veld rat, *Aethomys ineptus* and the house rat, *Rattus rattus*, indicating that this lineage may not represent a species-specific bacterial genotype (Figure 2). Lineage F, consisting of five samples from this study and one sample isolated from the Asian house rat, *R. tanezumi*, corresponds to Lineage IV of the *gltA* phylogeny in Figure 1. Within the two lineages that contain samples that were negative with *gltA*, Lineage B contains *Bartonella* sequences isolated from murid rodent species from Hammanskraal and

Roodeplaat Nature Reserve all in Gauteng Province, South Africa; whilst in Lineage C *Bartonella* from wild *M. coucha* populations grouped with *B. tribocorum* and *B. grahamii*.

Discussion

This study represents the first comparison of *Bartonella* infection between a commensal and wild population of an African murid rodent species. The *Bartonella* prevalence of 56 % detected in the wild *M. coucha* population is within the infection range previously detected in other rodents from South Africa and other countries worldwide (Bastos 2007; Gundi *et al.* 2012). In contrast, the commensal population had an infection prevalence which was almost double that of the wild population. Similar high prevalence rates have been detected in the fat sand rat, *Psammomys obesus* from Tunisia (90 %) and the bushveld gerbil, *Gerbilliscus leucogaster* from South Africa (96 %); however these prevalence rates were from wild populations (Fichet-Calvet 2000; Brettschneider 2010). Data focussing on differences in disease prevalence between commensal and wild populations of single rodent species are limited. In a study conducted in Spain on the prevalence of *Rickketsia typhi* in five species of rodents, there was no significant difference in the seroprevalence among rural and urban populations, although in that case single species differences were not investigated (Lledó *et al.* 2003).

It has been suggested that an increase in ectoparasites can lead to an increase in the prevalence of vector-borne diseases (Brettschneider *et al*. 2012b). The commensal samples assessed in the present study were significantly more infested with ectoparasites. However, similar to previous studies, the data in the present study indicate no significant difference between ectoparasite loads and *Bartonella* infection. In free-ranging white-tailed deer,

Odocoileus virginianus, from the U.S.A. there was no difference in tick load between deer infected and not infected by *Bartonella* (Chitwood *et al.* 2013). In an attempt to explain differences in *Bartonella* infection rates between two species of *Rattus*, Brettschneider *et al.* (2012b) found that differences in ectoparasite loads did not fully account for observed differences in *Bartonella* prevalence between *R. rattus* and *R. norvegicus*. Similarly, in Ethiopian rodents there was no increased probability of *Bartonella* infection in rodents with increasing ectoparasite infection (Meheretu *et al*. 2013). The present study did not investigate the difference between *Bartonella* prevalence and different ectoparasite species. It is possible that there could be a difference between certain *Bartonella* strains and specific ectoparasite species, however, this was not explored in this study and requires further investigation.

Recently, *Bartonella* DNA was detected in ectoparasites sampled from indigenous South African rodents but no differences between the presence of ectoparasites and the infection status of the host was detected (Brettschneider 2010). Although the presence of *Bartonella* DNA in these ectoparasites does not necessarily indicate vector competence, it demonstrates that these ectoparasites do come into contact with the bacteria, which is the first step in identifying potential vectors of *Bartonella* in these rodent genera (Reeves *et al*. 2007). It is suggested that future studies should be targeted towards investigating the differences between specific ectoparasite species and *Bartonella* prevalence in commensal and wild populations of rodents in general and *M. coucha* in particular.

A diverse assemblage of *Bartonella* lineages are present in *M. coucha* from South Africa. The largest proportion of samples grouped with vector-borne zoonotic *B. elizabethae*. In both the

nuoG and *gltA* phylogenies the *B. elizabethae*-associated lineage (Lineage III, Figure 1; Lineage D, Figure 2) contained samples isolated from commensal populations of indigenous (*Aethomys ineptus, Lemniscomys rosalia, Mastomys* spp.) and invasive (*Rattus rattus* and *R. norvegicus*) species. As suggested in previous studies (Bastos 2007; Brettschneider 2010), this could indicate possible spill-over of *Bartonella* from invasive species to indigenous species in a commensal environment. However, samples from wild populations of indigenous rodent species (*Micaelamys namaquensis, Gerbilliscus leucogaster* and *M. coucha*) also grouped in this lineage. *Bartonella elizabethae* has been isolated from several native rodent species from other parts of Africa suggesting that this bacterial species may not be specific to *Rattus* species (Gundi *et al*. 2012; Meheretu *et al*. 2013).

Two samples from Lineage IV (Figure 1) in the present study together with one *R. tanezumi* isolate grouped with *B. coopersplainsensis*. This bacterial species was first detected in *R. norvegicus* from Australia (Gundi *et al.* 2009). Recently, *B. coopersplainsensis* was detected in South Africa in two *R. tanezumi* sampled from a rural settlement in the northern part of Limpopo Province (Mostert 2009; Brettschneider 2010). This *B. coopersplainsensis* lineage was also recovered in the *nuoG* phylogeny (Lineage F).

A degree of host-specificity was observed, with some lineages containing *Bartonella* obtained from specific rodent genera only. Lineage II (Figure 1) includes *M. natalensis* and *M. coucha* isolates from previous studies conducted in South Africa (Pretorius *et al.* 2004; Bastos 2007) together with *M. coucha* isolates from the present study and appears to be *Mastomys*-specific. Although this host rodent-specific group was recovered in the *nuoG* phylogeny

(Lineage E, Figure 2) the species-specific pattern was not consistently observed as the *nuoG* lineage also included isolates from *A. ineptus* and *R. rattus*. This could possibly indicate a recent spill-over of *Bartonella* strains between *Rattus* species and indigenous species in a commensal setting.

Another lineage that appears to be specific to *M. coucha* was observed in the *gltA* phylogeny (Lineage V, Figure 1); however, this lineage was not recovered in the *nuoG* phylogeny. Interestingly, a large proportion of isolates from this lineage were negative for *Bartonella* in the *nuoG* PCR (38 %, $^{7}/_{18}$), with the remaining samples grouping within Lineages A, B, D and F. Non-concordance between phylogenies based on different gene regions have previously been observed in *Bartonella* studies (Renesto *et al*. 2000; Colborne *et al*. 2010). One possible explanation that has been proposed is that this is partly the result of genetic rearrangements and lateral gene transfer which frequently occurs in *Bartonella* (Lindroos *et al*. 2006; Berglund *et al*. 2009). This poses a particular problem when using single gene-phylogenies (Pitulle *et al*. 2002). Therefore, more robust phylogenies based on multiple gene regions are needed in future to allow better insights into the evolutionary relationships within *Bartonella* (Renesto *et al*. 2000).

Apart from *B. elizabethae* and *B. coopersplainsensis*, samples isolated from rodents in South Africa show little association with other known *Bartonella*. This seems to support the hypothesis that there is a phylogenetic division between *Bartonella* associated with hosts native to the Old World and those found in hosts native to the New World (Ellis *et al*. 1999). Approximately 37 % of the world's murid species are found in Africa, and given this large murid

rodent diversity further studies in a broader range of rodent species need to be conducted to fully understand the evolutionary history and pathogenicity of *Bartonella* in Africa.

The high infection prevalence in the commensal *M. coucha* and the presence of *B. elizabethae* highlights the importance of commensal rodents in the potential spread of zoonotic diseases in South Africa, especially in informal settlements. Up to 73 % of people in sub-Saharan Africa live in informal settlements (Wekesa *et al*. 2011). These informal settlements are characterised by a lack of basic infrastructure and housing, poor sanitation, inadequate health care systems and a large proportion of immune-compromised individuals (Richards *et al*. 2007; Wekesa *et al*. 2011). Recently, a quarter of HIV-positive patients tested in an informal settlement in South Africa were positive for *Bartonella*, however, due to difficulties in diagnosis and over-burdened healthcare systems these infections remain largely under-diagnosed and untreated (Trataris *et al*. 2012). Increased knowledge of *Bartonella* in rodents will not only contribute to a better understanding of *Bartonella* dynamics but also assist with the planning of health, environment and conservation management practices that will mitigate the effect of vector-borne diseases.

References

Bastos, A.D.S., Chimimba, C.T.C., von Maltitz, E., Kerstin, F. and Belmain, S. 2005. Identification of rodent species that play a role in disease transmission to humans in South Africa. *The Southern African Society for Veterinary Epidemiology and Preventive Medicine*, pp 78-83.

Bastos, A.D.S., 2007. *Bartonella* incidence and diversity in endemic South African Murid rodents occurring commensally with humans. *The Southern African Society for Veterinary Epidemiology and Preventive Medicine*, pp 76-81.

Bai, Y., Kosoy, M., Martin, A., Ray, C., Sheff, K., Chalcraft, L. and Collinge, S.K. 2008. Characterization of *Bartonella* strains isolated from black-tailed prairie dogs (*Cynomys ludovicianus*). *Vector-borne and Zoonotic Diseases* **8**: 1-5.

Berglund, E.C., Frank, A.C., Calteau, A., Vinnere Pettersson, O., Granberg, F., Eriksson, A.S., Naslund, K., Holmberg, M., Lindroos, H. and Andersson, S.G. 2009. Run-off replication of host-adaptability genes is associated with gene transfer agents in the genome of mouse-infecting *Bartonella grahamii*. *PLoS Genetics* **5**: e1000546.

Bermond, D., Heller, R., Barrat, F., Delacour, G., Dehio, C., Alliot, A., Monteil, H., Chomel, B., Boulouis, H.J. and Piémont, Y. 2000. *Bartonella birtlesii* sp. nov., isolated from small mammals (*Apodemus* spp.). *International Journal Systematic and Evolutionary Microbiology* **50**: 1973-1979.

Bermond, D., Boulouis, H., Heller, R., Van Laere, G., Monteil, H., Chomel, B.B., Sander, A., Dehio, C. and Piemont, Y. 2002. *Bartonella bovis* sp. nov. and *Bartonella capreoli* sp. nov.,

isolated from European ruminants. *International Journal of Systematic Microbiology* **52**: 383-390.

Billeter, S.A., Levy, M.G., Chomel, B.B. and Breitschwerdt, E.B. 2008. Vector transmission of *Bartonella* species with emphasis on the potential for tick transmission. *Medical and Veterinary Entomology* **22**: 1-15.

Birtles, R.J. 2005. Bartonellae as elegant hemotropic parasites. *Annals of the New York Academy of Sciences* **1063**: 270-279.

Birtles, R.J. and Raoult, D. 1996. Comparison of partial Citrate Synthase gene (*gltA*) sequences for phylogenetic analysis of *Bartonella* species. *International Journal of Systematic Bacteriology* **46**: 891-897.

Birtles, R.J., Harrison, T.G., Saunders, N.A. and Molyneux, D.H. 1995. Proposals to unify the genera *Grahamella* and *Bartonella*, with descriptions of *Bartonella talpae* comb. nov., *Bartonella peromysci* comb. nov., and three new species, *Bartonella grahamii* sp. nov., *Bartonella taylorii* sp. nov., and *Bartonella doshiae* sp. nov. *International Journal of Systematic Bacteriology* **45**: 1-8.

Birtles, R.J., Hazel, S.M., Bennet, M., Brown, K., Raoult, D. and Begon, M. 2001. Longitudinal monitoring of the dynamics of infections due to *Bartonella* species in UK woodland rodents. *Epidemiology Infectious Diseases* **126**: 323-329.

Bouhsira, E., Ferrandeza, Y., Liua, M., Franc, M., Boulouisa, H. and Biville, F. 2013. *Ctenocephalides felis* an in vitro potential vector for five *Bartonella* species. *Comparative Immunology, Microbiology and Infectious Diseases* **36**: 105-111.

Boulouis, H. J., Barrat, F., Bermond, D., Bernex, F., Thibault, D., Heller, R., Fontaine, J.C., Piedmont, Y. and Chomel, B.B. 2001. Kinetics of *Bartonella birtlesii* infection in experimentally infected mice and pathogenic effect on reproductive functions. *Infection and Immunity* **69**: 5313-5317.

Breitschwerdt, E.B., Sontakke, S. and Hopkins, S. 2012. Neurological manifestations of Bartonellosis in immunocompetent patients: a composite of reports from 2005-2012. *Journal of Neuroparasitology* **3**: 1-15.

Brenner, D.J., O'Connor, S.P., Hollis, D.G., Weaver, R.E. and Steigerwalt, A.G. 1991. Molecular characterization and proposal of a neotype strain for *Bartonella bacilliformis*. *Journal of Clinical Microbiology* **29**: 1299-1302.

Brenner, D.J., O'Connor, S.P., Winkler, H.H. and Steigerwalt, A.G. 1993. Proposals to unify the genera *Bartonella* and *Rochalimaea*, with descriptions of *Bartonella quintana* comb. nov., *Bartonella vinsonii* comb. nov., *Bartonella henselae* comb. nov., and *Bartonella elizabethae* comb. nov., and to remove the family Bartonellaceae from the order Rickettsiales. *International Journal of Systematic and Evolutionary Microbiology* **43**: 777-786.

Brettschneider, H. 2010. *Bartonella* dynamics in indigenous and invasive murid rodents from South Africa: Prevalence, diversity and mathematical modelling. PhD Thesis, University of Pretoria, Pretoria, South Africa.

Brettschneider, H., Bennett, N.C., Chimimba, C.T. and Bastos, A.D.S. 2012a. Bartonellae of the Namaqua rock mouse, *Micaelamys namaquensis* (Rodentia: Muridae) from South Africa. *Veterinary Microbiology* **157**: 132-136.

Brettschneider, H., Anguelov, R., Chimimba, C.T. and Bastos, A.D.S. 2012b. A mathematical epidemiological model of gram-negative *Bartonella* bacteria: does differential ectoparasite load fully explain the differences in infection prevalence of *Rattus rattus* and *Rattus norvegicus*? *Journal of Biological Dynamics* **6**: 763-781.

Brinkerhoff, R.J., Kabeya, H., Inoue, K., Bai, Y. and Maruyama, S. 2010. Detection of multiple *Bartonella* species in digestive and reproductive tissues of fleas collected from sympatric mammals. *ISME Journal* **4**: 955-958.

Buffet, J., Kosoy, M. and Vayssier-Taussat, M. 2013. Natural history of *Bartonella*-infecting rodents in light of new knowledge on genomics, diversity and evolution. *Future Microbiology* **8**: 1117-1128.

Chitwood, M.C., Maggi, R.G., Kennedy-Stoskopf, S., Toliver, M. and DePerno, C.S. 2013. *Bartonella vinsonii* subsp. *berkhoffii* in Free-Ranging White-Tailed Deer (*Odocoileus virginianus*). *Journal of Wildlife Diseases* **49**: 468-470.

Chomel, B.B., Boulouis, H., Breitschwerdt, E.B., Kasten, R.W., Vayssier-Taussat, M., Birtles, R.J., Koehler, J.E. and Dehio, C. 2009. Ecological fitness and strategies of adaptation of *Bartonella* species to their hosts and vectors. *Veterinary Research* **40**: 29-51.

Chomel, B.B. and Kasten, R.W. 2010. Bartonellosis, an increasingly recognized zoonosis. *Journal of Applied Microbiology* **109**: 743-750.

Chomel, B.B., McMillan-Cole, A.C., Kasten, R.W., Stuckey, M.J., Sato, S., Maruyama, S., Diniz, P.P.V.P. and Breitschwerdt, E.B. 2012. Candidatus *Bartonella merieuxii*, a potential new zoonotic *bartonella* species in canids from Iraq. *PLoS Neglected Tropical Diseases* **6**: e1843.

Colborn, J.M., Kosoy, M.Y., Motin, V.L., Telepnev, M.V., Valbuena, G., Myint, K.S., Fofanov, Y., Putonti, C., Feng, C. and Peruski, A.2010. Improved detection of *Bartonella* DNA in mammalian hosts and arthropod vectors using the NADH dehydrogenase gamma subunit (*nuoG*) by real-time PCR. *Journal of Clinical Microbiology* **48**: 4630-4633.

Dehio, C., Lanz, C., Pohl, R., Behrens, P., Bermond, D., Piémont, Y., Pelz, K. and Sander, A. 2001. *Bartonella schoenbuchii* sp. nov., isolated from the blood of wild roe deer. *International Journal of Systematic and Evolutionary Microbiology* **51**: 1557-1565.

Droz, S., Chi, B., Horn, E., Steigerwalt, A.G., Whitney, A.M. and Brenner, D.J. 1999. *Bartonella koehlerae* sp. nov., isolated from cats. *Journal Clinical Microbiology* **37**: 1117-1122.

Ellis, B.A., Regnery, R.L., Bacellar, F., Rood, M., Glass, G.G., Marston, E., Ksiazek, T.G., Jones, D. and Childs, J.E. 1999. Rats from the genus *Rattus* are reservoir hosts for pathogenic *Bartonella* species: an old world origin for a new world disease? *Journal of Infectious Diseases* **180**: 220-224.

Eremeeva, M.E., Gerns, H.L., Lydy, S.L., Goo, J.S., Ryan, E.T., Mathew, S.S., Ferraro, M.J., Holden, J.M., Nicholson, W.L., Dasch, G.A. and Koehler, J.E. 2007. Bacteremia, fever, and splenomegaly caused by a newly recognized *Bartonella* species. *The New England Journal of Medicine* **356**: 2381-2387.

Fichet-Calvet, E., Jomaa, I., Ismael, B. and Ashford, R.W. 2000. Patterns of infection of haemoparasites in the fat sand rat, *Psammomys obesus*, in Tunisia, and effect on the host. *Annals of Tropical Medicine and Parasitology* **94**: 55-68.

Fournier, P.E., Taylor, C., Rolain, J.M. Barrassi, L., Smith, G. and Raoult, D. 2007. *Bartonella australis* sp. nov. from Kangaroos, Australia. *Emerging Infectious Diseases* **13**: 1961-1962.

Gratz, N.G. 1997. The burden of rodent-borne diseases in Africa south of the Sahara. *Belgian Journal of Zoology* **127**: 71-84.

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

Gundi, V.A.K.B., Davoust, B., Khamis, A. Boni, M., Raoult, D. and La Scola, B. 2004. Isolation of *Bartonella rattimassiliensis* sp. nov. and *Bartonella phoceensis* sp. nov. from European *Rattus norvegicus*. *Journal of Clinical Microbiology* **42**: 3816-3818.

Gundi, V.A.K.B., Taylor, C., Raoult, D. and La Scola, B. 2009. *Bartonella rattaustraliani* sp. nov., *Bartonella queenslandensis* sp. nov. and *Bartonella coopersplainsensis* sp. nov., identified in Australian rats. *International Journal of Systematic and Evolutionary Microbiology* **59**: 2956-2961.

Gundi, V.A.K.B., Kosoy, M.Y., Makundi, R.H. and Laudisoit, A. 2012. Identification of diverse *Bartonella* genotypes among small mammals from Democratic Republic of Congo and Tanzania. *American Journal of Tropical Medicine and Hygiene* **87**: 319-326.

Harms, A. and Dehio, C. 2012. Intruders below the radar: molecular pathogenesis of *Bartonella* spp. *Clinical Microbiology Reviews* **25**: 42-78.

Heller, R., Riegel, P., Hansmann, Y., Delacour, G., Bermond, D., Dehio, C., Lamarque, F., Monteil, H., Chomel, B. and Piémont, Y. 1998. *Bartonella tribocorum* sp. nov., a new *Bartonella* species isolated from the blood of wild rats. *International Journal of Systematic Bacteriology* **48**: 1333-1339.

Heller, R., Kubina, M., Mariet, P., Riegel, P., Delacour, G., Dehio, C., Lamarque, F., Kasten, R., Boulouis, H.J., Monteil, H., Chomel, B. and Piémont, Y. 1999. *Bartonella alsatica* sp. nov., a

new *Bartonella* species isolated from the blood of wild rabbits. *International Journal of Systematic Bacteriology* **49**: 283-288.

Huelsenbeck, J.P. and Ronquist, F. 2001. MrBayes: Bayesian inference of phylogeny. *Bioinformatics* **17**: 754-755.

Inoue, K., Maruyama, S., Kabeya, H., Yamada, N., Ohashi, N., Sato, Y., Yukawa, M., Masuzawa, T., Kawamori, F., Kadosaka, T., Takada, N., Fujita, H. and Kawabata, H. 2008. Prevalence and genetic diversity of *Bartonella* species isolated from wild rodents in Japan. *Applied Environmental Microbiology* **74**: 5086-5092.

Inoue, K., Kabeya, H., Shiratori, H., Ueda, K., Kosoy, M.Y., Chomel, B.B., Boulouis, H.J. and Maruyama, S. 2010. *Bartonella japonica* sp. nov. and *Bartonella silvatica* sp. nov., isolated from *Apodemus* mice in Japan. *International Journal of Systematic and Evolutionary Microbiology* **60**: 759-763.

Jacomo V., Kelly P.J. and Raoult D. 2002. Natural history of *Bartonella* infections (an exception to Koch's postulate). *Clinical and Diagnostic Laboratory Immunology* **9**: 8-18.

Jardine, C., Waldner, C., Wobeser, G. and Leighton, F. 2006. Demographic features of *Bartonella* infections in Richardson's ground squirrels (*Spermophilus richardsonii*). *Journal of Wildlife Diseases* **42**: 739-749.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L. and Daszak, P. 2008. Global trends in emerging infectious diseases. *Nature* **451**: 990-993.

Kaiser, P.O., Riess, T., O'Rourke, F., Linke, D., Kempf, V.A.J., 2011. *Bartonella* spp.: Throwing light on uncommon human infections. *International Journal of Medical Microbiology* **301**: 7-15.

Kamani, J., Morick, D., Mumcuoglu, K.Y. and Harrus, S. 2013. Prevalence and diversity of *Bartonella* species in commensal rodents and ectoparasites from Nigeria, West Africa. *PLoS Neglected Tropical Diseases* **7**: e2246.

Karagöz, A., Çelebi, B., Şimşek, H., Taner, M., Kiliç, S., Durmaz, R. and Ertek, M. 2013. Detection of *Bartonella* spp. in field mice (*Microtus socialis*) by culture and PCR. *Veterinary Journal of Ankara University* **60**: 235-239.

Ko, S., Kim, S., Kang, J., Won, S., Lee, H., Shin, N., Choi, K., Youn, H. and Chae, J. 2013. Molecular detection of *Bartonella grahamii* and *B. schoenbuchensis*-related species in Korean Water Deer (*Hydropotes inermis argyropus*). *Vector-Borne and Zoonotic Diseases* **13**: 415-418.

Kordick, D.L., Swaminathan, L.B., Greene, C.E., Wilson, K.H., Whitney, A.M., O'Connor, S., Hollis, D.G., Matar, G.M., Steigerwalt, A.G., Malcolm, G.B., Hayes, P.S., Widfield, T.F., Breitschwerdt, E.B. and Brenner, D.J. 1996. *Bartonella vinsonii* subsp. *berkhofii* isolated from Dogs; *Bartonella vinsonii* subsp. *vinsonii* and emended description of *Bartonella vinsonii*. *International Journal of Systematic Bacteriology* **46**: 704-709.

Kosoy, M.Y., Regnery, R.L., Kosaya, O.I., Jones, D.C., Marston, E.L. and Childs, J.E. 1998. Isolation of *Bartonella* spp. from embryos and neonates of naturally infected rodents. *Journal of Wildlife Diseases* **34**: 305-309.

Kosoy, M.Y., Regnery, R.L. Kosaya, O.I. and Childs, J.E. 1999. Experimental infection of cotton rats with three naturally occurring *Bartonella* species. *Journal of Wildlife Diseases* **35**: 275-284.

Kosoy, M.Y., Saito, E.K., Green, D., Marston, E.L., Jones, D.C. and Childs, J.E. 2000. Experimental evidence of host specificity of *Bartonella* infection in rodents. *Comparative Immunology, Microbiology and Infectious Diseases* **23**: 2211-2238.

Kosoy, M., Mandel, E., Green, D., Marston, E. and Childs, J. 2004a. Prospective studies of *Bartonella* of rodents. Part I. Demographic and temporal patterns in population dynamics. *Vector-borne and Zoonotic Diseases* **4**: 285-295.

Kosoy, M., Mandel, E., Green, D., Marston, E., Jones, D. and Childs, J. 2004b. Prospective studies of *Bartonella* of rodents. Part II. Diverse infections in a single rodent community. *Vector-borne and Zoonotic Diseases* **4**: 296-305.

Kosoy, M., Morway, C. Sheff, K.W., Bai, Y., Colborn, J., Chalcraft, I., Dowell, S.F., Peruski, L.F., Maloney, S.A., Baggett, H., Sutthirattana, H., Sidhirat, A., Maruyama, S., Kabeya, H., Chomel, B.B., Kasten, R., Popov, V., Robinson, J., Kruglov, A. and Petersen, I.R. 2008. *Bartonella tamiae* sp. nov., a newly recognized pathogen isolated from three human patients from Thailand. *Journal of Clinical Biology* **46**: 772-775.

Kosoy, M.Y. 2010. Ecological associations between bacteria of the genus *Bartonella* and mammals. *Biology Bulletin* **37**: 716-724.

La Scola, B., Zeaiter, Z., Khamis, A. and Raoult, D. 2003. Gene-sequence-based criteria for species definition in bacteriology: the *Bartonella* paradigm. *Trends in Microbiology* **11**: 318-321.

Lawson, P.A. and Collins, M.D. 1996. Description of *Bartonella clarridgeiae* sp. nov.isolated from the cat of a patient with *Bartonella henselae* septicemia. *Medical Microbiology Letters* **5**: 64-73.

Leirs, H., Verhagen, R., Verheyen, W., Mwanjabe, P. and Mbise, T. 1996. Forecasting rodent outbreaks in Africa: an ecological basis for *Mastomys* control in Tanzania. *Journal of Applied Ecology* **33**: 937-943.

Lima, M., Stenseth, N.C., Leirs, H. and Jaksic, F.M. 2003. Population dynamics of small mammals in semi-arid regions: a comparative study of demographic variability in two rodent species. *Proceedings: Biological Sciences* **270**: 1997-2007.

Lindroos, H., Vinnere, O., Mira, A., Repsilber, D., Naslund, K. and Andersson, S.G. 2006. Genome rearrangements, deletions, and amplifications in the natural population of *Bartonella henselae*. *Journal of Bacteriology* **188**: 7426-7439.

Lledó, L., Gegúndez, I., Ruiz, E., Rodríguez, L., Bacellar, F. and Saz, J.V. 2003. *Rickettsia typhi* infection in wild rodents from central Spain. *Annals of Tropical Medicine and Parasitology* **97**: 411-414.

Maillard, R., Riegel, P., Barrat, F., Bouillin, C., Thibault, D., Gandoin, C., Halos, L., Demanche, C., Alliot, A., Guillot, J. and Piemont, Y. 2004. *Bartonella chomelii* sp.nov., isolated from French domestic cattle (*Bos taurus*). *International Journal of Systematic and Evolutionary Microbiology* **54**: 215-220.

Maritsi, D.N., Zarganis, D., Metaxa, Z., Papaioannou, G. and Vartzelis, G. 2013. *Bartonella henselae* infection: an uncommon mimicker of autoimmune disease. *Case Reports in Pediatrics* **2013** Article ID 726826.

Mascarelli, P.E., Maggi, R.G., Hopkins, S., Mozayeni, B.R., Trull, C.L., Bradley, J.M., Hegarty, B.C. and Breitschwerdt, E.B. 2013. *Bartonella henselae* infection in a family experiencing

neurological and neurocognitive abnormalities after woodlouse hunter spider bites. *Parasites and Vectors* **6**: 98-107.

Mediannikov, O., El Karkouri, K., Diatta, G., Robert, C., Fournier, P.E. and Raoult, D. 2013a. Non-contiguous finished genome sequence and description of *Bartonella senegalensis* sp. nov. *Standards in Genomic Sciences* **8**: 2.

Mediannikov, O., El Karkouri, K., Robert, C., Fournier, P. E., and Raoult, D. 2013b. Non-contiguous finished genome sequence and description of *Bartonella florenciae* sp. nov. *Standards in Genomic Sciences* **9**: 185-196.

Meerburg, B.G., Singleton, G.R. and Kijlstra, A. 2009. Rodent-borne diseases and their risks for public health. *Critical Reviews in Microbiology* **35**: 221-270.

Meheretu, Y., Leirs, H., Welegerima, K., Breno, M., Tomas, Z., Kidane, D., Girmay, K. and Goüy de Bellocq, J. 2013. *Bartonella* prevalence and genetic diversity in small mammals from Ethiopia. *Vector-Borne and Zoonotic Diseases* **13**: 164-175.

Minadakis, G., Koutentakis, P., Koukoulakis, M., Chochlakis, D., Tselentis, Y, and Psaroulaki, A. 2013. Sudden loss of vision due to retinitis following *Bartonella henselae* infection. *Archives of Hellenic Medicine* **30**: 85-87.

Morick, D., Krasnov, B.R., Khokhlova, I.S., Gutiérrez, R., Fielden, L.J. Gottlie, Y. and Harrus, S. 2013a. Effects of *Bartonella* spp. on flea feeding and reproductive performance. *Applied and Environmental Microbiology* **79**: 3438-3443.

Morick, D., Krasnov, B.R., Khokhlova, I.S., Gutierrez, R. Gottlieb, Y. and Harrus, S. 2013b. Vertical nontransovarial transmission of *Bartonella* in fleas. *Molecular Ecology* **22**: 4747-4752.

Morway, C., Kosoy, M., Eiseni, R., Montenierii, J., Sheff, K., Reynolds, P.J. and Powers, N. 2008. A longitudinal study of *Bartonella* infection in populations of woodrats and their fleas. *Journal of Vector Ecology* **33**: 353-364.

Mostert, E.M. 2009. Molecular and morphological assessment of invasive inland *Rattus* (Rodentia: Muridae) concentric in South Africa and their reservoir host potential with respect to *Helicobacter* and *Bartonella*. MSc.Thesis, University of Pretoria, Pretoria, South Africa.

Norman, A.F., Regnery, R., Jameson, P., Greene, C. and Krause, D.C. 1995. Differentiation of *Bartonella*-like isolates at the species level by PCR-restriction fragment length polymorphisms in the citrate synthase gene. *Journal of Clinical Microbiology* **33**: 1797-1803.

Ohl, M.E. and Spach, D.H. 2000. *Bartonella quintana* and urban trench fever. *Clinical Infectuous Diseases* **31**: 131-135.

Paziewska, A., Siński, E. and Harris, P.D. 2012. Recombination, diversity and allele sharing of infectivity proteins between *Bartonella* species from rodents. *Microbial Ecology* **64**: 525-536.

Pitulle, C., Strehse, C., Brown, J.W. and Breitschwerdt, E.B. 2002. Investigation of the phylogenetic relationships within the genus *Bartonella* based on comparative sequence analysis of the *rnpB* gene, 16S rDNA and 23S rDNA. *International Journal of Systematic and Evolutionary Microbiology* **52**: 2075-2080.

Posada, D. 2008. jModalTest: Phylogenetic modal averaging. *Molecular Biology and Evolution* **25**: 1253-1256.

Pretorius, A., Beati, L. and Birtles, R.J. 2004. Diversity of bartonellae associated with small mammals inhabiting Free State province, South Africa. *International Journal of Systematic and Evolutionary Microbiology* **54**: 1959-1967.

Pulliainen, A.T. and Dehio, C. 2012. Persistence of *Bartonella* spp. stealth pathogens: from subclinical infections to vasoproliferative tumor formation. *FEMS Microbiology Reviews* **36**: 563-599.

Reeves, W.K., Szumlas, D.E., Moriarity, J.R., Loftis, A.D., Abbassy, M.M., Helmy, I.M. and Dasch, G.A. 2006. Louse-borne bacterial pathogens in lice (Phthiraptera) of rodents and cattle from Egypt. *The Journal of Parasitology* **92**: 313-318.

Reeves, W.K., Rogers, T.E., Durden, L.A. and Dasch, G.A. 2007. Association of *Bartonella* with the fleas (Siphonaptera) of rodents and bats using molecular techniques. *Journal of Vector Ecology* **32**: 118-122.

Regnery, R.L., Olson, J.G., Perkins, B.A. and Bibb, W. 1992. Serological response to "*Rochalimaea henselae*" antigen in suspected cat-scratch disease. *Lancet* **339**: 1443-1445.

Renesto, R., Gouvernet, J., Drancourt, M., Roux, V. and Raoult, D. 2000. Determination of the rpoB gene sequences of *Bartonella henselae* and *Bartonella quintana* for phylogenetic analysis. *Research in Microbiology* **151**: 831-836.

Richards, R., O'leary, B. and Mutsonziwa, K. 2007. Measuring quality of life in informal settlements in South Africa. *Social Indicators Research* **81**: 375-388.

Saisongkorh, W., Wootta W., Sawanpanyalert, P., Raoult, D. and Rolain, J. 2009. ''Candidatus *Bartonella thailandensis''*: A new genotype of *Bartonella* identified from rodents. *Veterinary Microbiology* **139**: 197-201.

Sato, S., Kabeya, H., Fujinaga, Y., Inoue, K., Une, Y., Yoshikawa, Y. and Maruyama, S. 2013. *Bartonella jaculi* sp. nov., *Bartonella callosciuri* sp. nov., *Bartonella pachyuromydis* sp. nov.

and *Bartonella acomydis* sp. nov., isolated from wild Rodentia. *International Journal of Systematic and Evolutionary Microbiology* **63**: 1734-1740.

Skinner, J.D. and Chimimba, C.T. 2005. *The Mammals of the Southern African Subregion*. Cambridge University Press, Cape Town.

Spach, D.H., Callis, K.P., Paauw D.S., Houze,Y. B., Schoenknecht, F.D., Welch, D.F., Rosen, H. and Brenner, D.J. 1993. Endocarditis caused by *Rochalimaea quintana* in a patient infected with human immunodeficiency virus. *Journal of Clinical Microbiology* **31**: 692-4.

Strong, R.P., Tyzzer, E.E., Brues, C.T., Sellards, A.W. and Gastiaberg, K.C. 1913. Verruga peruviana, Oroya fever and Uta. *Journal of the American Medical Association* **61**: 1713-1716.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**: 2731-2739.

Trataris, A.N., Rossouw, J., Arntzen, L., Karstaedt, A. and Frean, J. 2012. *Bartonella* spp. in human and animal populations in Gauteng, South Africa, from 2007 to 2009. *Onderstepoort Journal of Veterinary Research* **79**: 18-25.

Tsai, Y.L., Chang, C.C., Chuang, S.T. and Chomel, B.B. 2011. *Bartonella* species and their ectoparasites: selective host adaptation or strain selection between the vector and the mammalian host? *Comparative Immunology, Microbiology and Infectious Diseases* **34**: 299-314.

Wekesa, B.W., Steyn, G.S. and Otieno, F.A.O. 2011. A review of physical and socio-economic characteristics and intervention approaches of informal settlements. *Habitat International* **35**: 238-245.

Welch, D.F., Carroll, K.C., Hofmeister, E.K., Persing, D.H., Robison, D.A., Steigerwalt, A.G. and Brenner, D.J. 1999. Isolation of a new subspecies, *Bartonella vinsonii* subsp. *arupensis*, from a cattle rancher: identity with isolates found in conjunction with *Borrelia burgdorferi* and *Babesia microti* among naturally infected mice. *Journal of Clinical Microbiology* **37**: 2598-2601.

Chapter 5

Prevalence and diversity of tick-borne Anaplasmataceae in indigenous, commensal rodents from Gauteng Province, South Africa

Abstract

Tick-borne members of the family Anaplasmataceae include the genera *Anaplasma* and *Ehrlichia* and the proposed genus *Neoehrlichia*. *Anaplasma* and *Ehrlichia* species have been known to veterinary medicine for a long time but the emergence of human pathogens in the last 20 years has renewed interest in these bacterial genera. Despite this, the prevalence and diversity of zoonotic *Anaplasma* and *Ehrlichia* in Africa is understudied. The aim of the present study was to investigate the prevalence and diversity of tick-borne Anaplasmataceae in indigenous, commensal rodents in South Africa and to assess whether tick load influences infection rates. A total of 175 commensal rodent kidney samples were screened for Anaplasmataceae DNA using a primer set that targets the 16S rRNA gene. A generalized linear mixed model was used to test for differences between bacterial infection with reference to rodent species, sex and tick load. Two genera of the Anaplasmataceae were identified in 60 of 175 commensal rodents, corresponding to an overall prevalence of 34.3 %. Both bacterial prevalence and tick load showed significant differences between rodent species but not between sexes. A significant

difference was observed between bacterial prevalence and tick load. *Anaplasma bovis*-like bacteria were detected in five of the rodent species (*Aethomys ineptus, Lemniscomys rosalia, Mastomys coucha, Otomys angoniensis* and *Rhabdomys dilectus*) sampled, whereas *Ehrlichia ewingii* was only detected in *Mastomys coucha*. This study represents the first report of both *A. bovis* and *E. ewingii* in South African rodents.

Keywords: *Anaplasma*, *Ehrlichia*, rodents, epidemiology, zoonotic diseases, South Africa

Introduction

The family Anaplasmataceae comprise of small, gram-negative, obligate intracellular bacteria (Dumler *et al.* 2005). After a major taxonomic revision, based on molecular, antigenic and biological data, this family includes four genera, namely, *Anaplasma, Ehrlichia, Neorickettsia* and *Wolbachia* (Dumler *et al.* 2001). Apart from *Wolbachia*, members of all genera in this family replicate in the hemapoietic cells of vertebrate hosts and epithelial cells of specific invertebrate vectors (Table 1; Dumler *et al.* 2001; Rar and Golovljova 2011). The genera *Anaplasma* and *Ehrlichia* include pathogens of veterinary and medical importance that are transmitted by ixodid ticks (Rar *et al*. 2010). *Neorickettsia* include bacteria transmitted by helminthes, causing severe disease in canids, horses and humans (Table 1; Vaughan *et al.* 2012). *Wolbachia* is an endosymbiont found in the germinal and epithelial cells of various insect species, filarial nematodes and a number of other arthropods (Lo *et al.* 2007). The family also includes one candidate genus, *Neoehrlichia*, found in ticks and rodents from Eurasia and raccoons from the United States of America (Yabsley *et al.* 2008). The veterinary significance of *Anaplasma* and *Ehrlichia* species have been known since the early 20th century, however, the discovery of zoonotic species fuelled renewed interest in the Anaplasmataceae (Doudier *et al.* 2010). The diseases caused by these bacteria are therefore considered as emerging diseases since the majority have only been described in the last two decades (Sashika *et al*. 2011).

Table 1. Anaplasmataceae of veterinary and medical importance in the world (Rymaszewska and Grenda 2008; Ismail *et al.* 2010; Esemu *et al.* 2011; Rar and Golovljova 2011).

Species	Infected host cells	Main Hosts	Primary Vectors	Diseases	Geographic distribution
Anaplasma					
A. bovis	Monocytes	Cattle, Wild ruminants, Rodents, Cotton-tailed rabbits	*Amblyomma variegatum*, *Rhipicephalus appendiculatus*, *Rhipicephalus* spp., *Hyalomma* spp. *Haemaphysalis* spp.	Bovine anaplasmosis	Africa, Asia, North America, South America
A. centrale	Erythrocytes	Cattle, Wild ruminants	*Rhipicephalus simus*	Bovine anaplasmosis (vaccine strain)	
A. marginale	Erythrocytes	Cattle, Wild ruminants	*Rhipicephalus (Boophilus) microplus*, *Rhipicephalus* spp., *Dermacentor* spp.	Bovine anaplasmosis	Worldwide in tropical and subtropical regions
A. ovis	Erythrocytes	Sheep, Goats, Wild ruminants	*Rhipicephalus bursa* *Rhipicephalus* spp., *Dermacentor* spp.	Ovine anaplasmosis	
A. phagocytophilum	Granulocytes (neutrophils, basophils, eosinophils)	Humans, Horses, Ruminants, Dogs, Cats, Rodents, Cervids	*Ixodes* spp.	Human granulocytic anaplasmosis (HGA), Equine anaplasmosis, Tick-borne fever of ruminants, Anaplasmosis of cats and dogs	Asia, Europe, North America, South America, North Africa
A. platys	Platelets	Dogs	*Rhipicephalus sanguineus*	Canine cyclic thrombocytopenia	Africa, Asia, Europe, North America

Ehrlichia					
E. canis	Monocytes / macrophages	Dogs, Wild canids	*Rhipicephalus sanguneus* *Dermacentor variabilis*	Canine monocytic ehrlichiosis (CME)	Worldwide
E. chaffeensis	Monocytes / macrophages	Humans, Dogs, Cervids (White-tailed deer)	*Amblyomma americanum*	Human monocytic ehrlichiosis (HME), Canine ehrlichiosis	Africa, Asia, North America, South America
E. ewingii	Granulocytes (neutrophils)	Humans, Dogs, Cervids (White-tailed deer)	*Amblyomma americanum*	Human ewingii ehrlichiosis (HEE), Canine granulocytic ehrlichiosis	Africa, Asia, North America
E. muris	Monocytes / macrophages	Murine rodents	*Haemaphysalis flava*, *Ixodes* spp.	Murine splenomegaly	Eurasia, Japan
E. ruminantium	Endothelial cells, Granulocytes (neutrophils), Macrophages	Cattle, Sheep, Goats, Wild ruminants	*Amblyomma* spp.	Heartwater	Africa, Caribbean
Neorickettsia					
N. helminthoeca	Monocytes / macrophages	Canids	Digenean fluke (*Nanophyetus salmincola*)	Salmon poisoning disease	U.S.A
N. risticii	Monocytes / macrophages, Mast cells, Enterocytes	Horses	Lecithodendriid digeneans	Equine monocytic ehrlichiosis (Potomac horse fever)	North America, South America, Europe, India
N. sennetsu	Monocytes / macrophages	Humans, Dogs, Rodents	Digenean fluke	Sennetsu fever	Japan, Malaysia
Candidatus *Neohrlichia*					
Candidatus *N. lotoris*	Unknown	Raccoons (*Procyon lotor*)	Unknown	Unknown	U.S.A
Candidatus *N. mikurensis*	Endothelial cells, Granulocytes	Rodents, Dogs	*Ixodes* ssp.	Febrile infection in Humans	Eurasia

To date, all described *Anaplasma* and *Ehrlichia* species are biologically transmitted by ticks of the family Ixodidae (Rar and Golovljova 2011). These bacteria are transmitted transtadially between ticks and with the exception of one unverified case, transovarial transmission appears inefficient in ticks and does not seem to play a major role in the spread of these diseases (Bezuidenhout and Jacobsz 1986; Long *et al*. 2003; Doudier *et al*. 2010). In addition to biological transmission, certain species such as *A. marginale* can also be transmitted mechanically by biting flies or via blood-contaminated fomites or transplacentally (Da Silva *et al.* 2013).

The diseases caused by the tick-borne Anaplasmataceae generally present as non-specific febrile illnesses with the most common symptoms including fever, headache, myalgia, and malaise (Dumler *et al.* 2007; Ismail *et al.* 2010). Due to the non-pathognomonic symptoms of anaplasmosis and ehrlichiosis, diagnosis frequently relies on laboratory tests such as blood smear examination, *in vitro* isolation, serological tests and detection of bacterial DNA by PCR (Massung *et al*. 1998; Schouls *et al.* 1999). Currently, the genus *Anaplasma* includes six species and the genus *Ehrlichia* has five species, of which three species (*A. phagocytophilum, E. chaffeensis, and E. ewingii*) are considered major emerging human pathogens (Rar and Golovljova 2011).

Human Monocytic Ehrlichiosis (HME) is caused by *Ehrlichia chaffeensis* and can range from a mild febrile illness to more severe multisystemic infections (Rar and Golovljova 2011). HME is potentially the most life-threatening zoonotic ehrlichial species with between 42 % and 60 % of cases requiring hospitalisation and a fatality rate of up to 3 % (Ismail *et al*. 2010; Rar

and Golovljova 2011). The majority of confirmed HME cases have been reported in the U.S.A. where the main vector is the lone star tick (*Amblyomma americanum*) and the primary reservoir is the white-tailed deer (*Odocoileus virginianus*) (Yabsley 2010). *Anaplasma phagocytophilum* is the causative agent of Human Granulocytic Anaplasmosis (HGA) as well as equine anaplasmosis and severe febrile diseases in dogs, cats and ruminants (Rar and Golovljova 2011). Various rodents and cervids serve as reservoir hosts and the infection is primarily transmitted by ticks of the genus *Ixodes* (Rymaszewska and Grenda 2008). HGA is generally less severe than HME, with severe complications frequently associated with opportunistic infections (Dumler *et al.* 2007). *Ehrlichia ewingii* was originally described as a canine pathogen causing a less severe form of canine ehrlichiosis than *E. canis* (Anderson *et al*. 1992). Subsequently, *E. ewingii* was detected in patients with suspected ehrlichiosis (Buller *et al.* 1999). Human Ewingii Ehrlichiosis (HEE) seems to cause milder infections than HME or HGA with the majority of cases being detected in immunocompromised patients (Ismail *et al*. 2010).

The remaining species of this family are known for their importance as veterinary pathogens; however, several isolated reports indicate that some of these species are associated with human diseases. Together with *A. marginale* and *A. centrale*, *A. ovis* is an intra-erythrocytic member of this family that causes febrile diseases of wild and domestic ruminants worldwide (Aubry and Geale 2011). Recently a variant of *A. ovis* was found to be associated with a case of human anaplasmosis in Cyprus (Chochlakis *et al.* 2010). *Ehrlichia canis* is the most frequently detected Anaplasmataceae species in dogs and causes the potentially fatal disease canine monocytic ehrlichiosis (Rar and Golovljova 2011). Since its discovery *E. canis* was considered as strictly a canine pathogen until 1996 when *E. canis* DNA was detected in humans in

Venezuela (Perez *et al.* 1996). Subsequently, the same *E. canis* strain was detected in patients with human ehrlichiosis in Venezuela (Perez *et al.* 2006). The first ehrlichial species to be described, *Ehrlichia ruminantium*, causes Heartwater an economically important disease of ruminants in Africa and some of the islands in the Caribbean (Allsopp 2010). Recently, *E. ruminantium* was detected in four fatal cases of human disease in South Africa suggesting that infection of this *Ehrlichia* species can be fatal to humans (Louw *et al.* 2005).

Tick-borne Anaplasmataceae in Africa

Not much is known about the prevalence and diversity of zoonotic Anaplasmataceae in Africa (Esemu *et al.* 2011). The first evidence of human ehrlichiosis in Africa came from a well-documented HME case acquired in Mali, subsequently diagnosed in the U.S.A. (Uhaa *et al.* 1992). A serological survey of eight countries found evidence of *E. chaffeensis* infection, but concluded that ehrlichioses do not occur often in Africa (Brouqui *et al.* 1994). In 1999, a possible case of human ehrlichiosis was reported from South Africa (Pretorius *et al*. 1999). Recently, *E. chaffeensis* DNA was detected in patients presenting with non-specific febrile illness in Cameroon (Ndip *et al.* 2009). Potentially, zoonotic strains of *E. ruminantium* have also been described from South Africa (Allsopp and Allsopp 2001). Furthermore, *A. phagocytophilum*, the agent of HGA, was detected in sub-Saharan Africa in 2013 for the first time. Although this bacterial species was detected in sheep it suggests that this known zoonotic pathogen is circulating in animals in Africa (Djiba *et al.* 2013).

Several Anaplasmataceae that are able to infect dogs have also been detected throughout Africa (Inokuma *et al.* 2005). *Ehrlichia canis* was initially described in Algeria in 1935 and is

the most widespread *Ehrlichia* species detected in dogs in Africa (Rar and Golovljova 2011). It has been detected serologically in a number of African countries including South Africa, Tunisia, Senegal, Egypt, Chad, Zimbabwe and Cameroon (Ndip *et al.* 2005). Serological evidence also exists for the presence of *E. chaffeensis* in dogs in South Africa (Pretorius and Kelly 1998). Recently, the DNA of *A. platys* and *E. ewingii* was detected in dogs from the Democratic Republic of Congo (DRC) and Cameroon, respectively (Sanogo *et al*. 2003; Ndip *et al*. 2005). Furthermore, a number of new genotypes closely related to *Anaplasma phagocytophilum* and *Ehrlichia ruminantium* have been detected in dogs from South Africa (Allsopp and Allsopp 2001; Inokuma *et al*. 2005). However, the pathogenicity of these variants and their possible vectors remain unstudied.

In contrast, members of this family that infect livestock have a long history in Africa. Heartwater, initially called *Rickettsia ruminantium*, was first described in 1925 in South Africa (Cowdry 1925a; b). *Ehrlichia ruminantium* is widespread throughout sub-Saharan Africa, where the distribution coincides with the tick (*Amblyomma* spp) vector (Esemu *et al*. 2011; Table 1). It is estimated that 76 % of cattle in Africa are at high risk of exposure to *E. ruminantium* and mortality rates can be as high as 95 % (Allsopp 2010). The economic losses due to Heartwater are difficult to quantify for the entire continent but certain African countries have estimated losses exceeding US$21 million per annum (Kivaria 2006). With no reliable vaccine available, *E. ruminantium* represents a major limitation to livestock production in Africa (Bekker *et al.* 2001).

Anaplasma marginale, the cause of bovine anaplasmosis in tropical and subtropical areas worldwide, was also first described in South Africa (Kocan *et al.* 2010). A large proportion of cattle farming in Africa occur in areas where bovine anaplasmosis occurs endemically (De Waal 2000). Furthermore, in southern Africa, *Anaplasma marginale* is one of the most commonly reported causes of cattle mortality especially in low input farming systems where there is limited access to vaccines and veterinary care (Marufu *et al.* 2012).

Another ruminant anaplasmosis, *A. bovis*, was recently detected in eastern rock sengis (*Elephantulus myurus*) and an undescribed Rhipicephaline tick in South Africa (Harrison *et al.* 2011; Harrison *et al*. 2013). *Anaplasma bovis* is a monocytotropic bacterium that usually causes a mild febrile illness in ruminants, however, more severe infections have been observed in cattle (Goethert and Telford 2003; Sashika *et al.* 2011). Infections of *A. bovis* have been reported in cattle, goats, deer and buffalo from Africa, Asia and South America (Rar and Golovljova 2011). *Anaplasma bovis* DNA has also been detected in several ixodid tick species from Africa, South America, Korea, Japan, the Russian Far East and Canada (Rar and Golovljova 2011). Ruminants are generally considered the main reservoirs but the detection of *A. bovis* DNA in cottontail rabbits from the U.S.A. and raccoons from Japan suggests that small mammals may also be involved as reservoirs of this infection (Goethert and Telford 2003; Sashika *et al.* 2011).

It is clear that several tick-borne Anaplasmataceae are present on the African continent. It also appears that novel strains and novel reservoirs for certain *Anaplasma* and *Ehrlichia* species may be present in Africa (Allsopp and Allsopp 2001; Inokuma *et al*. 2005; Harrison *et al*. 2013). To date, no studies have investigated Anaplasmataceae in rodents in Africa. Therefore, the

objective of the present study was to investigate the prevalence and diversity of tick-borne members of this family in indigenous commensal rodents in South Africa and to test whether tick load influences bacterial infection prevalence.

Ticks transmit a wider variety of pathogens than any other heamatophagous arthropod vector and are considered one of the most important vectors of human and animal diseases (Jongejan & Uilenberg 2004). It has been estimated that vector-borne diseases are responsible for almost 30 % of the emerging infectious diseases reported in the last decade and that tick-borne diseases are responsible for more than 100,000 cases of these diseases worldwide (de la Fuente *et al.* 2008; Jones *et al.* 2008). Globally, rodents are hosts to a large variety of tick species (Morand *et al.* 2006). Similarly, in South Africa, rodents are the preferred host of several tick species of veterinary and medical importance (Petney *et al.* 2004). However, due to insufficient information the true prevalence and diversity of tick-borne diseases in sub-Saharan Africa remains difficult to estimate (Hotez and Kamath 2009). The present study represents one of the first studies to investigate the tick-borne Anaplasmataceae in rodents in sub-Saharan Africa.

Materials and Methods

Sample collection

Commensal rodent samples were collected at 4-6 week intervals between August 2010 and July 2011 in Hammanskraal (25°42′60′′S, 28°27′20′′E, 1111 meters above sea level) and the University of Pretoria (UP) Experimental farm (25°75′11′′S, 28°26′29′′E, 1375 m.a.s.l.), Gauteng Province, South Africa (see Figure 1 in Chapter 2). Animals were live-trapped and processed as detailed in Chapter 1. All cryptic murid species were identified molecularly by

means of cytochrome *b* gene amplification and sequence analyses (Bastos *et al*. 2005) or with a species-specific multiplex protocol (see Chapter 2). Total genomic DNA was extracted from rodent kidney tissue using the High Pure PCR template preparation kit (Roche) according to the prescribed manufacturer's protocol. A total of 175 commensal rodents representing six species (*Aethomys ineptus*, *Lemniscomys rosalia*, *Mastomys coucha*, *Otomys angoniensis*, *Rhabdomys dilectus*, and *Saccostomus campestris*) belonging to two families (Muridae and Nesomyidae) (Musser and Carlton 2005) (see Chapter 2) were used to screen for tick-borne Anaplasmaceae in the present study.

Primer assessment

Four primer sets were evaluated for their specificity and sensitivity for detecting members of the family Anaplasmataceae (Table 2). Each primer set was tested using four samples that were previously screened with a universal bacterial 16S rRNA primer (see Chapter 3) and a negative control was included with each run. Of the samples used to test the primers, two samples were confirmed by sequencing of 16S rRNA to be positive for *Anaplasma*, one sample was positive for *Bartonella* and the remaining sample was negative for bacterial genome presence.

For optimisation of annealing temperatures (Ta) all PCR reactions were performed in a final reaction volume of 50 µl containing 1x Buffer (Fermentas), 0.25 µM dNTP's (Fermentas), 0.4 µM of the forward and reverse primer (IDT), 1U of DreamTaq™ DNA polymerase and 100-200 ng DNA template and run on the same thermal cycler (Veriti™, Applied Biosystems). A touchdown PCR program was used with an initial denaturation of 10 s at 96° C, followed by three cycles of denaturation for 12 s at 96° C, annealing for 30 s at the temperatures listed in

Table 2 and elongation for 50 s at 70° C. This was followed by the second step consisting of five cycles of denaturation for 12 s at 96° C, annealing for 25 s at the temperatures listed in Table 2 and elongation for 45 s at 70° C. The last step comprised 32 cycles of denaturation for 12 s at 96° C, annealing for 20 s at the temperatures listed in Table 2 and elongation for 40 s at 70° C, followed by a final extension of 60 s at 70° C.

Additional optimisation of primer concentration, DNA polymerase concentration and template concentration was also conducted; however the specificity and sensitivity of the primers tested did not improve significantly. The primer targeting the major surface protein4 gene (de la Fuente *et al*. 2007) was the least sensitive and was unable to detect *Anaplasma* from any of the known positive samples. Primer set EhrF and EhrR (Schouls *et al*. 1999; Bekker *et al*. 2002) was unable to detect *Anaplasma* from the two known positive samples. Primer set Ana-ge3a and Ana-ge2 (Massung *et al*. 1998) amplified host-genome. The Anaplasmataceae-specific primer set targeting the 345 bp region of the 16S rRNA gene displayed a high degree of sensitivity and specificity and was selected to screen the remaining kidney samples.

Table 2. Forward and reverse sequences of Anaplasmataceae primers tested, target gene regions, expected amplicon size, annealing temperatures used during temperature optimisation touchdown PCR and the associated references.

	Target gene	Primer sequence (5' - 3')	Product size	Annealing temperatures (°C)			Reference
				Step 1	Step 2	Step 3	
(i)	*Anaplasma* spp. Major surface protein 4	Ana-msp4F: CTCCTATGAATTACAGAGAATTG Ana-msp4R: TCCTTAGCTGAACAGGAATCTT	860bp	60 59 58 57 55	58 57 56 55 53	56 55 54 53 51	de la Fuente *et al*. 2007, modified
(ii)	*Anaplasma* spp. 16S rRNA	Ana-ge3a: CACATGCAAGTCGAACGGATTATTC Ana-ge2: GGCAGTATTAAAAGCAGCTCCAGG	555bp	63 62 60 58 57	61 60 58 56 56	59 58 56 54 55	Massung *et al*. 1998, modified
(iii)	*Anaplasma* spp. & *Ehrlichia* spp. 16S rRNA	EhrF: GGAATTCAGAGTTGGATCMTGGYTCAG EhrR: CGGGATCCCGAGTTTGCCGGGACTTYTTCT	492-498bp	60.5 59 57.5	59 57 56	57.5 55 54.5	Schouls *et al*. 1999; Bekker *et al*.2002
(iv)	Anaplasmataceae 16S rRNA	EHR16SD: GGTACCYACAGAAGAAGTCC EHR16SR: TAGCACTCATCGTTTACAGC	345bp	61 59 57 55 53	59 57 55 53 51	57 55 53 51 49	Parola *et al*. 2000

Molecular detection and phylogenetic analysis

After primer selection each sample was screened at least twice with the Anaplasmataceae-specific primer set EHR16SD and EHR16SR (Parola *et al*. 2000) using similar reaction conditions as described above using the following thermal cycling conditions: Initial denaturation of 10 s at 96° C, followed by three cycles of denaturation for 12 s at 96° C, annealing for 30 s at 61° C and elongation for 50 s at 70° C. This was followed by five cycles of 12 s at 96° C, 25 s at 59° C and 45 s at 70° C. Finally, 32 cycles of 12 s at 96° C, 20 s at 57° C and 40 s at 70° C, followed by a final extension of 60 s at 70° C. Samples for which the results of the first two PCR reactions were contradictory were screened a third time, with those producing two positive results being scored as positive.

Samples that tested positive were purified using the Roche PCR Product Purification Kit, and cycle sequenced using BigDye v. 3.1 terminator cycle-sequencing kit (Perkin-Elmer, Foster City, U.S.A.). Sequences were precipitated and run on the ABI PRISM ™ 3100 Analyser (Applied Biosystems). Nucleotide sequences were viewed and aligned in MEGA5 (Tamura *et al.* 2011) prior to performing a BLAST search (www.ncbi.nm.nih.gov/blast) to identify the Anaplasmataceae reference species with the highest sequence similarity. For phylogenetic analyses, sequences generated from this study were aligned with reference sequences downloaded from the National Centre for Biotechnology Information database (GenBank; www.ncbi.nm.nih.gov/genbank).

Neighbour joining (NJ) analysis was performed in MEGA5 (Tamura *et al.* 2011), Maximum Likelihood (ML) analysis was performed in PhyML (Guidon and Gascuel 2003) and

Bayesian inference (BI) was performed with MrBayes (Huelsenbeck and Ronquist 2001) using the best-fit model and parameters identified under the Akaike Information Criterion (AIC), chosen with jModelTest (Posada 2008). For the BI, four Markov Monte Carlo chains were run for 10,000,000 generations using default heating and swap settings, and were sampled every 100 generations. Trace files were viewed using Tracer version 1.5 (http://beast.bio.ed.ac.uk/) after which 10 % of the runs were discarded as burn-in. Nodal support was determined by 10,000 and 1,000 bootstrap replicates for NJ and ML analyses, respectively and from Bayesian posterior probabilities (BPP). Pairwise uncorrected p-distances were determined in MEGA5 (Tamura *et al.* 2011).

Statistical analyses

Tick load was calculated by dividing the total number of ticks collected from a species of rodent, by the number of rodents sampled. Tick load was determined for each species of rodent captured in this study and is summarized in Table 3. Differences between Anaplasmataceae prevalence and rodent species, sex, sampling locality, and tick load were tested using Generalized Linear Models in Statistica v11 (StatSoft Inc., Oklahoma, USA, 2012).

Results

Anaplasmataceae prevalence and tick load

The overall Anaplasmataceae prevalence for all rodent species (*Aethomys ineptus*, *Lemniscomys rosalia*, *Mastomys coucha*, *Otomys angoniensis*, *Rhabdomys dilectus*, and *Saccostomus campestris*) sampled was 34.3 % (Table 3), with no significant difference between males and females ($F_{1, 166} = 0.25$; $n = 175$; $P = 0.61$). Although tick-borne Anaplasmataceae bacteria were

detected from all sampling localities in Hammanskraal, no Anaplasmataceae bacteria were detected from the University of Pretoria experimental farm. Significant differences in overall Anaplasmataceae prevalence were observed between the six rodent species sampled ($F_{5,\ 166}$ = 3.01; n = 175; $P < 0.05$). The highest overall bacterial prevalence was observed in *A. ineptus* (51.7 %), while *O. angoniensis* showed the lowest infection rate (4.5 %). No Anaplasmataceae bacteria were detected from the *S. campestris* sampled.

Overall the tick load showed no statistically significant difference between males and females ($F_{1,\ 166}$ = 0.65; n = 175; P = 0.42); however the tick load differed significantly between the six rodent species sampled ($F_{5,\ 166}$ = 112.14; n = 175; $P < 0.001$). There was also a significant difference in the overall tick load between sampling localities in Hammanskraal (4.9) and the UP Experimental farm (0.2) ($F_{1,\ 166}$ = 33.39, n = 175, $P < 0.001$). Furthermore a significant difference was found between rodent infection status and tick load ($F_{1,\ 166}$ = 3.84; n = 175; $P < 0.05$).

Table 3. Summary of overall tick load and Anaplasmataceae prevalence based on PCR screening and sequencing of 16S rRNA partial gene fragments for six rodent species sampled from Hammanskraal and University of Pretoria Experimental farm, Pretoria, Gauteng Province, South Africa. Numbers in parentheses indicate number of positive rodents of a species and the total number of that rodent species sampled.

	Aethomys ineptus	*Lemniscomys rosalia*	*Mastomys coucha*	*Otomys angoniensis*	*Rhabdomys dilectus*	*Saccostomus campestris*	Overall
Prevalence % (No. positive/tested)	51.7 (15/29)	50 (1/2)	39.3 (33/84)	4.5 (1/22)	27.77 (10/36)	0 (0/2)	34.3 (60/175)
Female	50 (6/12)	NA	35.3 (6/17)	12.5 (1/8)	18.18 (2/11)	NA	31.3 (15/45)
Male	52.9 (9/17)	50 (1/2)	40.3 (27/67)	0	32 (8/25)	0 (0/2)	35.4 (45/127)
Tick load †	10.0	44.0	3.1	0.9	1.2	8.0	4.1
Female	6.3	NA	4.4	2.0	2.8	NA	4.08
Male	12.6	44.0	2.8	0.3	0.4	8.0	4.1

† Tick load: total number of ticks collected from a species of rodent divided by the number of that rodent species sampled

Anaplasma was detected in five of the rodent species that tested positive for Anaplasmataceae bacteria, whereas *Ehrlichia* was only detected from *M. coucha* (Table 4). Overall *Anaplasma* prevalence was 39.2 %, with no significant difference in bacterial prevalence between rodent species, sex or tick load (Species: $F_{5, 145} = 1.99$; $n = 153$; $P = 0.08$; Sex: $F_{1, 145} = 0.16$; $n = 153$; $P = 0.69$; Tick infestation: $F_{1, 145} = 2.35$; $n = 145$; $P = 0.13$). Similar statistical results were obtained for *Ehrlichia* prevalence, with an overall *Ehrlichia* prevalence of 5.3 % (Species: $F_{5, 145} = 1.81$; $n = 153$; $P = 0.11$; Sex: $F_{1, 145} = 0.10$; $n = 153$; $P = 0.75$; Tick infestation: $F_{1, 145} = 0.03$; $n = 145$; $P = 0.85$).

Phylogenetic analyses

Phylogenetic analyses were performed on a 96-taxon dataset, with 60 sequences generated from this study and 36 Anaplasmataceae reference sequences obtained from the NCBI database. This 305 bp 16S rRNA dataset contained 98 variable sites and 72 parsimony informative sites. The average nucleotide composition was A = 0.273, C = 0.192, G = 0.323, T = 0.213, and the transition: transversion ratio (R) was 3.08. The TIM3+I+G (I = 0.302, G = 0.653) model of sequence evolution was chosen as the best fit model under the AIC in JModelTest. The ML, BI and NJ analyses recovered the same tree topology and support indices are summarised on relevant nodes of the NJ tree (Figure 1).

Table 4. Summary of *Anaplasma* and *Ehrlichia* prevalence and tick load based on PCR screening and sequencing of 16S rRNA partial gene fragments for six rodent species sampled from Hammanskraal only (Gauteng Province, South Africa). Numbers in parentheses indicate number of positive rodents of a species and total number of that rodent species sampled.

	Anaplasma							*Ehrlichia*	
	Aethomys ineptus	*Lemniscomys rosalia*	*Mastomys coucha*	*Otomys angoniensis*	*Rhabdomys dilectus*	*Saccostomus campestris*	Overall	*Mastomys coucha*	Overall
Prevalence	51.7 (15/29)	50 (1/2)	44.6 (33/74)	7.1 (1/14)	31.3 (10/32)	0 (0/2)	39.2 (60/153)	10.8 (8/74)	5.2 (8/153)
Female	50 (6/12)	NA	40 (6/15)	25 (1/4)	22.2 (2/9)	NA	37.5 (15/40)	13.3 (2/15)	5.0 (2/40)
Male	52.9 (9/17)	50 (1/2)	45.8 (27/59)	0	34.8 (8/23)	0 (0/2)	39.8 (45/113)	10.2 (6/59)	3.3 (6/113)
Tick load †	10.0	44.0	3.9	1.4	1.3	8.0	4.8	3.1	4.8
Female	6.3	NA	5.5	4.0	3.4	NA	5.0	3.0	5.0
Male	12.6	44.0	3.5	0.3	0.4	8.0	4.7	3.2	4.7

† Tick load: total number of ticks collected from a species of rodent divided by the number of that rodent species sampled

Phylogenetic analysis of the partial 16S rRNA sequences obtained from commensal rodents sampled during the current study revealed the presence of two lineages; however, this pattern was not well-supported by all analyses (Figure 1). The majority of samples (86.7 %; $^{52}/_{60}$) grouped in Lineage I (Figure 1). Genetic distances between the *A. bovis* type specimen (U03775) and members of Lineage I range from 0.9 % to 2.3 %. A well-known pathogen of ruminants, *A. bovis* is widespread in Africa and Asia (Rar and Golovljova 2011). Within Lineage I, a large proportion of samples (43.3 %; $^{26}/_{60}$) were identical based on the partial 16S rRNA fragment sequenced. These samples were obtained from various sampling localities in Hammanskraal and various rodent species (*L. rosalia, M. coucha and R. dilectus*) and are 99.7 % similar to an *Anaplasma* variant detected in a tick (*Amblyomma javanense*) removed from a pangolin in Thailand (Parola *et al*. 2003).

Certain variants within Lineage I were detected more often from particular rodent host species indicating a potential for bacterial host specificity. Six ($^{6}/_{10}$) of the samples from *R. dilectus* grouped together and all ($^{15}/_{15}$) the sequences obtained from *A. ineptus* grouped together, separate from the other species. This host-species-specific pattern was, however, only supported by the Neighbour joining analysis (Figure 1). The single sequence obtained from *O. angoniensis* grouped together with an uncultured *Anaplasma* sample from a goat from China with 99.2 % similarity. Interestingly, the *A. bovis* strain previously detected in the eastern rock sengi (*E. myurus*) and an undescribed Rhipicephaline tick species sampled from Limpopo Province, South Africa, did not group together with sequences isolated from the current study.

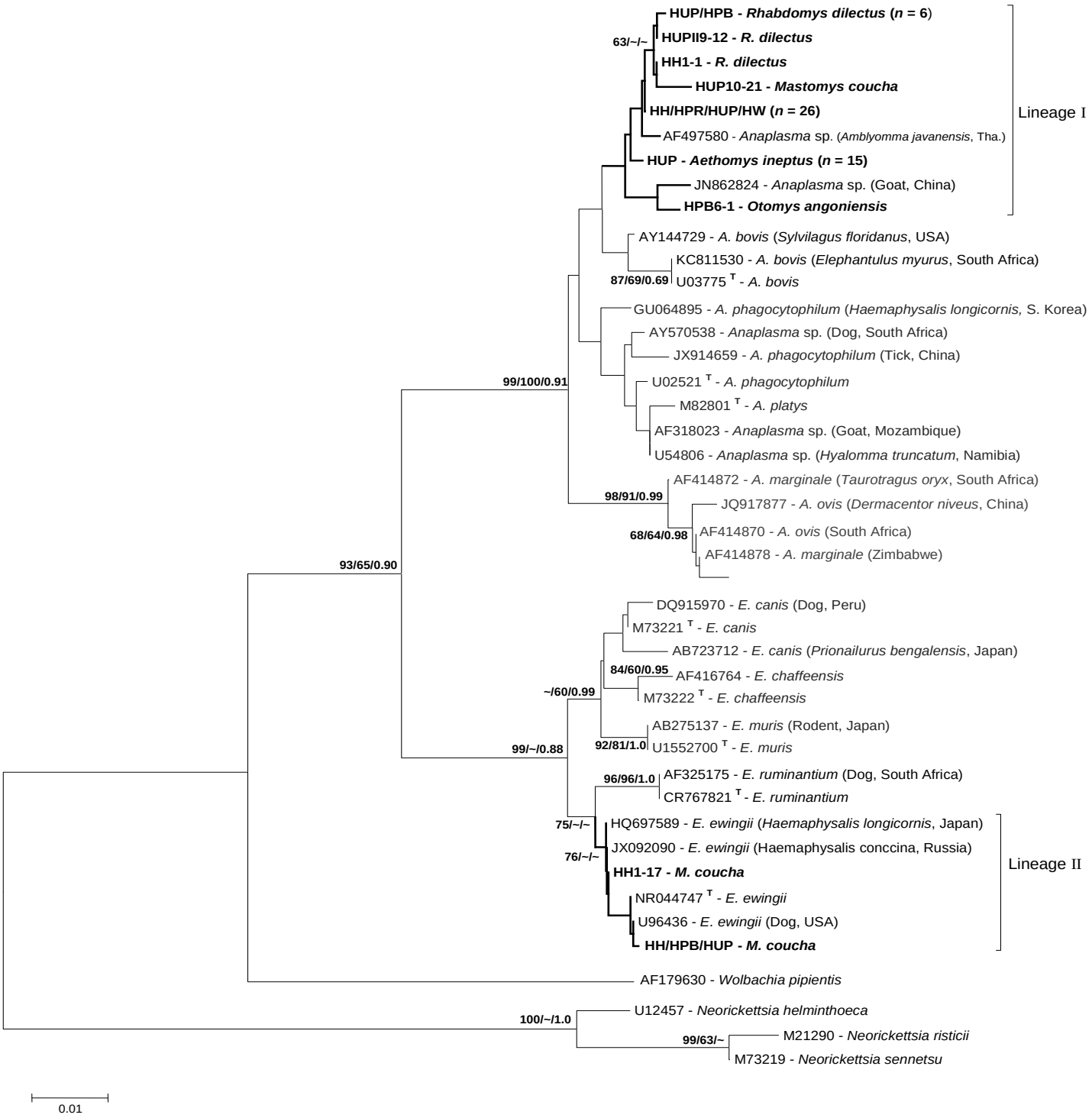

Figure 1. Neighbour joining (NJ) tree inferred from partial 16S rRNA gene sequences of Anaplasmataceae bacteria occurring in commensal rodents sampled from Hammanskraal and from the University of Pretoria experimental farm, Gauteng Province, South Africa. Bootstrap support (> 60 %) and Bayesian posterior probabilities (> 0.90) are indicated at relevant nodes (NJ/ML/BPP). Samples indicated in bold represent sequences generated from the current study and are prefixed with HUP/HPB/HH/HPR/HW to indicate specific sampling localities in Hammanskraal. Samples not in bold indicate accession numbers of the sequences downloaded from GenBank, with type specimens indicated with a superscript letter T (^{T}).

Lineage II consists of eight *Ehrlichia* sequences from the current study that corresponds with the described species *Ehrlichia ewingii*, a pathogenic agent to humans and dogs (Matsumoto *et al*. 2011). Genetic distances between the *E. ewingii* type specimen (NR044747) and members of Lineage II range from only 0 % to 0.3 %. The ehrlichiae were only obtained from *M. coucha* suggesting potential host species specificity. This lineage also contains *E. ewingii* variants from *Haemaphysalis* ticks from Russia and Japan (Matsumoto *et al*. 2011; Rar *et al*. 2013).

Discussion

The present study represents one of the first studies to investigate the prevalence and diversity of tick-borne Anaplasmataceae in rodents in Africa in general and South Africa in particular. The overall bacterial prevalence rate observed in the present study (34.3 %) is slightly higher than the prevalence rate observed in elephant shrews (*E. myurus*) from Limpopo Province, South Africa (28.6 %; Harrison *et al.* 2013). For both bacterial prevalence and tick load there was no significant difference between sexes but there was a significant difference between the six rodent species tested. Similar results were found in *E. myurus*, where neither tick load nor infection rate were influenced significantly by sex (Luterman *et al.* 2012; Harrison *et al.* 2013). There was however, a significant difference found between rodent infection status and tick load. It has been suggested that an increase in ectoparasites could lead to an increase in vector-borne disease prevalence (Brettschneider *et al*. 2012). This has been demonstrated for *A. bovis* infection in feral raccoons in Japan, where the rate of infection in animals with ticks was significantly higher (Sashika *et al.* 2011).

No Anaplasmataceae were detected in rodent samples from the University of Pretoria Experimental farm (Pretoria). Various species of livestock are housed on the experimental farm and they are frequently treated with acaricides. Since *Anaplasma* and *Ehrlichia* are transmitted by ticks, it is possible that a lack of tick-control in the resource-poor Hammanskraal could explain the observed difference in infection prevalence. Similar results were observed for *E. canis* in the Bloemfontein area, Free State Province, South Africa. Dogs from less-wealthy suburbs, with a lack of effective tick-control programs, had significantly higher antibodies to *E. canis* than those from more wealthy areas (Pretorius and Kelly 1998). However, the effect of acarides on the prevalence of *Anaplasma* and *Ehrlichia* in cattle does not necessarily follow this pattern (De Waal 2000).

The largest proportion of samples grouped in a lineage that was closely related to *A. bovis* (Figure 1). Interestingly, the sequence from *E. myurus* hosts did not group with *Anaplasma* sequences detected in the rodents sampled in this study, but rather grouped more closely with the *A. bovis* type specimen in a well-supported group. This suggests that different *A. bovis* variants could be circulating in rodents and elephant shrews. A degree of host specificity was observed with certain variants within Lineage I being more frequently associated with particular rodent species. However, the species-specific pattern was not well-supported by all analyses. Sequencing of additional gene regions and increased sample sizes are needed to conclusively demonstrate possible species specificity of these bacteria. Harrison and *et al*. (2013) also detected *A. bovis* from an undescribed tick species that was almost exclusively found on *E. myurus* and these authors proposed a putative transmission cycle of *A. bovis*.

In Africa, *Amblyomma variegatum* and *Rhipicephalus appendiculatus* are considered to be vectors for *A. bovis* (Rymaszewska and Grenda 2008). The present study did not screen ticks removed from rodents for Anaplasmataceae and therefore potential vectors of the identified bacteria are not known. *Anaplasma bovis* has also been detected in several other tick species in Asia, most notably tick species from the genus *Haemaphysalis* (Rar *et al*. 2013). In southern Africa, the immature stages of two *Haemaphysalis* species (*H. elliptica* and *H. leachi*) are frequently found on rodents (Apanaskevich *et al.* 2007). It is possible that these tick species play a role in transmitting *A. bovis* in South Africa and future studies should include screening of these and other tick species.

The veterinary and medical importance of the *A. bovis*-like bacteria detected in small mammals in South Africa remains unclear. *Anaplasma bovis* infections in cattle are usually self-limiting or include mild symptoms; however, other studies have reported mortality rates as high as 50 % (Rymaszewska and Grenda 2008). To date, there are no case reports of *A. bovis* infection in humans, although experimental infection of macaques led to a flu-like illness, suggesting that human infection may cause illness (Goethert and Telford 2003). It is therefore critical to isolate and fully describe these bacterial strains.

The remaining PCR-positive samples were recovered from *M. coucha* and grouped with the zoonotic *E. ewingii*. This is the first time that *E. ewingii* DNA has been detected in rodents from South Africa. *Ehrlichia ewingii* is the etiological agent of Human Ewingii Ehrlichiosis (HEE). *Ehrlichia ewingii* DNA has been detected in *Rhipicephalus sanguineus* ticks and dogs from Cameroon and in *H. longicornis* ticks

and voles from South Korea (Ndip *et al*. 2005; Kim *et al.* 2006). However, confirmed human cases of *E. ewingii* infection have only been reported in the U.S.A. (Ndip *et al.* 2005). The majority of confirmed HEE cases have been reported from immune-compromised patients (Rar and Golovljova 2011). In patients with underlying immune deficiencies ehrlichioses can be potentially life-threatening (Paddock *et al.* 2001). South Africa has a large number of immune-compromised individuals, especially in informal settlements where this problem is exacerbated by a lack of adequate health care (Richards *et al.* 2007). Therefore, the identification of this ehrlichial species in rodents from an informal settlement in South Africa is significant and further studies are needed to describe the epidemiology of *E. ewingii* in the country.

Worldwide, the incidence of vector-borne diseases, especially tick-borne diseases, is increasing (Dantas-Torres *et al*. 2012). Several factors have played a role in the increase of tick-borne pathogens including improved diagnostic techniques, surveillance programs, expansion of susceptible populations and climate and habitat change (Doudier *et al*. 2010). Since most studies in Africa have focused on *A. marginale* and *E. ruminantium*, very little is known about the prevalence, diversity and geographic distribution of zoonotic Anaplasmataceae (Djiba *et al*. 2013). This study not only contributes to the limited information available regarding the diversity of Anaplasmataceae in Africa but also emphasizes the importance of on-going surveillance for novel reservoirs and vectors of tick-borne pathogens. Increased knowledge of the Anaplasmataceae in rodents in South Africa will not only contribute to a better understanding of transmission dynamics but will also assist with the

planning of health, environment and conservation management practices that will mitigate the effect of this and other vector-borne diseases.

References

Allsopp, B.A. 2010. Natural history of *Ehrlichia ruminantium*. *Veterinary Parasitology* **167**: 123-135.

Allsopp, M.T. and Allsopp, B.A. 2001. Novel *Ehrlichia* genotype detected in dogs in South Africa. *Journal of Clinical Microbiology* **39**: 4204-4207.

Anderson, B.E., Greene, C.E., Jones, D.C. and Dawson, J.E., 1992. *Ehrlichia ewingii*. sp. nov., the etiologic agent of canine granulocytic ehrlichiosis. *International Journal of Systematic Bacteriology* **42**: 299-302.

Apanaskevich, D.A., Horak, I.G. and Camicas, J.L. 2007. Redescription of *Haemaphysalis* (*Rhipistoma*) *elliptica* (Koch, 1844), an old taxon of the *Haemaphysalis* (*Rhipistoma*) *leachi* group from East and southern Africa, and of *Haemaphysalis* (*Rhipistoma*) *leachi* (Audouin, 1826) (Ixodida, Ixodidae). *Onderstepoort Journal of Veterinary Research* **74**: 181-208.

Aubry, P. and Geale, D.W. 2011. A review of bovine anaplasmosis. *Transboundary and Emerging Diseases* **58**: 1-30.

Bastos, A.D.S., Chimimba, C.T.C., von Maltitz, E., Kerstin, F. and Belmain, S. 2005. Identification of rodent species that play a role in disease transmission to humans in South Africa. *The Southern African Society for Veterinary Epidemiology and Preventive Medicine*, pp 78-83.

Bekker, C.P., Vink, D., Pereira, C.M., Wapenaar W., Langa, A. and Jongejan, F. 2001. Heartwater (*Cowdria ruminantium* infection) as a cause of post restocking mortality of goats in Mozambique. *Clinical and Diagnostic Laboratory Immunology* **8**: 843-846.

Bekker, P.J., de Vos, S., Taoufik, A., Sparango, O.A.E. and Jongejan, F. 2002. Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization. *Veterinary Microbiology* **89**: 223-238.

Bezuidenhout, J.D. and Jacobsz, C.J. 1986. Proof of transovarial transmission of *Cowdria ruminantium* by *Amblyomma herbraeum*. *Onderstepoort Journal of Veterinary Research* **53**: 31-34.

Brettschneider, H., Anguelov, R., Chimimba, C.T. and Bastos, A.D.S. 2012. A mathematical epidemiological model of gram-negative *Bartonella* bacteria: does differential ectoparasite load fully explain the differences in infection prevalence of *Rattus rattus* and *Rattus norvegicus*? *Journal of Biological Dynamics* **6**: 763-781.

Brouqui, P., Le Cam, C., Kelly, P.J., Laurens, R., Tounkara, A., Sawadogo, S., Velo, M., Gondao, L., Faugere, B., Delmont, J., Bourgeades, A. and Raoult, D. 1994. Serologic evidence for human ehrlichiosis in Africa. *European Journal of Epidemiology* **10**: 695-698.

Buller, R.S., Arens, M., Hmiel, S.P., Paddock, C.D., Sumner, J.W., Rikhisa, Y., Unver, A., Gaudreault-Keener, M., Manian, F.A., Liddell, A.M., Schmulewitz, N. and Storch, G.A. 1999. *Ehrlichia ewingii*, a newly recognized agent of human ehrlichiosis. *New England Journal of Medicine* **15**: 148-155.

Chochlakis, D., Ioannou, I., Tselentis, Y. and Psaroulaki, A. 2010. Human anaplasmosis and *Anaplasma ovis* variant. *Emerging Infectious Diseases* **16**: 1031-1032.

Cowdry, E.V. 1925a. Studies on the etiology of heartwater. 1. Observation of a rickettsia, *Rickettsia ruminantium* (n. sp.), in the tissues of infected animals. *Journal of Experimental Medicine* **42**: 231-252.

Cowdry, E.V. 1925b. Studies on the etiology of heartwater. 2. *Rickettsia ruminantium* (n. sp.) in the tissues of ticks transmitting the disease. *Journal of Experimental Medicine* **42**: 253-274.

Dantas-Torres, F., Chomel, B.B. and Otranto, D. 2012. Ticks and tick-borne diseases: a One Health perspective. *Trends in Parasitology* **28**: 437-446.

Da Silva, A.S., Lopes, L.S., Diaz, J.D.S., Tonin, A.A., Stefani, L.M. and Araújo, D.N. 2013. Lice outbreak in buffaloes: Evidence of *Anaplasma marginale* transmission by sucking lice *Haematopinus tuberculatus*. *The Journal of Parasitology* **99**: 546-547.

de la Fuente, J., Atkinson, M.W., Naranjo, V., Fernandez de Mera, I.G., Mangold, A.J., Keating, K.A. and Kocan, K.M. 2007. Sequence analysis of the msp4 gene of *Anaplasma ovis* strains. *Veterinary Microbiology* **119**: 375-381.

de la Fuente, J., Estrada-Pena, A., Venzal, J.M., Kocan, K.M. and Sonenshine, D.E. 2008. Overview: Ticks as vectors of pathogens that cause disease in humans and animals. *Frontiers in Bioscience* **13**: 6938-6946.

De Waal, D.T. 2000. Anaplasmosis control and diagnosis in South Africa. *Annals of the New York Academy of Sciences* **916**: 474-483.

Djiba, M.L., Mediannikov, O., Mbengue, M., Thiongane, Y., Molez, J., Seck, M.T., Fenollar, F., Raoult, D. and Ndiaye, M. 2013. Survey of Anaplasmataceae bacteria in sheep from Senegal. *Tropical Animal Health and Production* **45**: 1557-1561.

Doudier, B., Olano, J., Parola, P. and Brouqui, P. 2010. Factors contributing to emergence of *Ehrlichia* and *Anaplasma* spp. as human pathogens. *Veterinary Parasitology* **167**: 149-154.

Dumler, J.S., Barbet, A.F., Bekker, C.P.J., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y. and Rurangirwa, F.R. 2001. Reorganisation of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. *International Journal of Systematic and Evolutionary Microbiology* **51**: 2145-2165.

Dumler, J.S., Rikihisa, Y. and Dasch, G.A. 2005. Family II. Anaplasmataceae Philip 1957, 980^{AL}. In: *Bergey's Manual of Systematic Bacteriology, Volume Two: The Proteobacteria*, (eds) G.M. Garrity, D.J. Brenner, N.R. Krieg and J.R. Staley, Springer-Verlag, New York.

Dumler, J.S., Madigan, J.E., Pusterla, N. and Bakken, J.S. 2007. Ehrlichioses in humans: epidemiology, clinical presentation, diagnosis, and treatment. *Clinical Infectuous Diseases* **4**: S45-51.

Esemu, S.N., Ndip, L.M. and Ndip, R.N. 2011. *Ehrlichia* species, probable emerging human pathogens in sub-Saharan Africa: environmental exacerbation. *Reviews on Environmental Health* **26**: 269-279.

Goethert, H.K. and Telford, S.R. 2003. Enzootic transmission of *Anaplasma bovis* in Nantucket cottontail rabbits. *Journal of Clinical Microbiology* **41**: 3744-3747.

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

Harrison, A., Bown, K.J. and Horak, I.G., 2011. Detection of *Anaplasma bovis* in an undescribed tick species collected from the eastern rock sengi *Elephantulus myurus. Journal of Parasitology* **97**: 1012-1016.

Harrison, A., Bastos, A.D.S. Medger, K. and Bennett, N.C. 2013. Eastern rock sengis as reservoir hosts of *Anaplasma bovis* in South Africa. *Ticks and Tick-borne Diseases* **4**: 503-505.

Hotez, P.J. and Kamath, A. 2009. Neglected tropical diseases in sub-Saharan Africa: Review of their prevalence, distribution, and disease burden. *PLoS Neglected Tropical Diseases* **3**: e412.

Huelsenbeck, J.P. and Ronquist, F. 2001. MrBayes: Bayesian inference of phylogeny. *Bioinformatics* **17**: 754-755.

Inokuma, H., Oyamada, M., Kelly, P.J., Jacobson, L.A., Fournier, P., Itamoto, K., Okuda, M. and Brouqui, P. 2005. Molecular Detection of a New *Anaplasma* Species Closely Related to *Anaplasma phagocytophilum* in Canine Blood from South Africa. *Journal of Clinical Microbiology* **43**: 2934-2937.

Ismail, N., Bloch, K.C. and McBride, J.W. 2010. Human ehrlichiosis and anaplasmosis. *Clinical Laboratory Medicine* **30**: 261-292.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L. and Daszak, P. 2008. Global trends in emerging infectious diseases. *Nature* **451**: 990-993.

Jongejan, F. and Uilenberg, G. 2004. The global importance of ticks. *Parasitology* **129**: 3-14.

Kim, C.M., Yi, Y.H., Yu, D.H., Lee, M.J., Cho, M.R., Desai, A.R., Shringi, S., Klein, T.A., Kim, H.C., Song, J.W., Baek, L.J., Chong, S.T., O'Guinn, M.L., Lee, J.S., Lee, I.Y., Park, J.H., Foley, J. and Chae, J.S., 2006. Tick-borne rickettsial pathogens in ticks and small mammals in Korea. *Applied Environmental Microbiology* **72**: 5766-5776.

Kivaria, F.M. 2006. Estimated direct economic costs associated with tick-borne diseases on cattle in Tanzania. *Tropical Animal Health and Production* **38**: 291-299.

Kocan, K.M., de la Fuente, J., Blouin, E.F., Coetzee, J.F. and Ewing, E.A. 2010. The natural history of *Anaplasma marginale*. *Veterinary Parasitology* **167**: 95-107.

Lo, N., Paraskevopoulos, C., Bourtzis, K., O'Neill, S.L., Werren, J.H., Bordenstein, S.R. and Bandi, C. 2007. Taxonomic status of the intracellular bacterium *Wolbachia pipientis*. *International Journal of Systematic and Evolutionary Microbiology* **57**: 654-657.

Long, S.W, Zhang, X., Zhang, J., Ruble, R.B., Teel, P. and Yu, X. 2003. Evaluation of transovarial transmission and transmissibility of *Ehrlichia chaffeensis* (Rickettsiales: Anaplasmataceae) in *Amblyomma americanum* (Acari: Ixodidae). *Journal of Medical Entomology* **40**: 1000-1014.

Louw, M., Allsopp, M.T. and Meyer, E.C. 2005. *Ehrlichia ruminantium*, an emerging human pathogen - a further report. *South African Medical Journal* **95**: 948.

Lutermann, H., Medger, K. and Horak, I.G. 2012. Abiotic and biotic determinants of tick burdens in the eastern rock sengi (*Elephantulus myurus*). *Medical and Veterinary Entomology* **26**: 255-262.

Marufu, M.C., Chimonyo, M., Mtshali, M.S. and Dzama, K.G. 2012. Molecular prevalence of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in Nguni and local Crossbred cattle under the low input production system in South Africa. *The Southern African Society for Veterinary Epidemiology and Preventive Medicine (SASVEPM)*, pp 57-67.

Massung, R. F., Slater, K., Owens, J. H., Nicholson, W. L., Mather, T. N., Solberg, V. B. and Olson, J. G. 1998. Nested PCR assay for the detection of granulocytic ehrlichiae. *Journal of Clinical Microbiology* **36**: 1090-1095.

Matsumoto, K., Takeuchi, T., Yokoyama, N., Katagiri, Y., Ooshiro, M., Zakimi, S., Kawamori, F., Ohashi, N. and Inokuma, H. 2011. Detection of the new *Ehrlichia* species closely related to *Ehrlichia ewingii* from *Haemaphysalis longicornis* in Yonaguni Island, Okinawa, Japan. *Journal of Veterinary Medical Science* **73**: 1485-1488.

Morand, S. Krasnov, B.R. and Poulin, R. 2006. *Micromammals and Macroparasites: From Evolutionary Ecology to Management*. Springer-Verlag, Tokyo.

Musser, G.G. and Carleton, M.D. 2005. Superfamily Muroidea. In: *Mammal Species of the World*, (eds) D.E. Wilson and D.M. Reeder.,3rd edn. The Johns Hopkins University Press, Baltimore.

Ndip, L.M., Ndip, R.N., Esemu, S.N., Dickmu, V.L., Fokam, E.B., Walker, D.H. and McBride, J.W. 2005. Ehrlichial infection in Cameroonian canines by *Ehrlichia canis* and *Ehrlichia ewingii*. *Veterinary Microbiology* **111**: 59-66.

Ndip, L.M., Labruna, M., Ndip, R.N., Walker, D.H. and McBride, J.W. 2009. Molecular and clinical evidence of *Ehrlichia chaffeensis* infection in Cameroonian

patients with undifferentiated febrile illness. *Annals of Tropical Medicine and Parasitology* **103**: 719-725.

Paddock, C.D., Folk, S.M., Shore, G.M., Machado, L.J., Huycke, M.M., Slater, L.N., Liddell, A.M., Buller, R.S., Storch, G.A., Monson, T.P., Rimland, D., Sumner, J.W., Singleton, J., Bloch, K.C., Tang, Y.W., Standaert, S.M. and Childs, J.E., 2001. Infections with *Ehrlichia chaffeensis* and *Ehrlichia ewingii* in persons coinfected with human immunodeficiency virus. *Clinical Infectious Diseases* **33**: 1586-1594.

Parola, P., Roux, V., Camicas, J.L., Baradji, I., Brouqui, P. and Raoult, D. 2000. Detection of ehrlichiae in African ticks by PCR. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **94**: 707-708.

Parola, P., Cornet, J.P., Sanogo, Y.O., Miller, R.S., Thien, H.V., Gonzalez, J.P., Raoult, D., Telford S.R. and Wongsrichanalai, C. 2003. Detection of *Ehrlichia* spp., *Anaplasma* spp., *Rickettsia* spp., and other Eubacteria in ticks from the Thai-Myanmar border and Vietnam. *Journal Clinical Microbiology* **41**: 1600-1608.

Perez, M., Rikihisa, Y. and Wen, B. 1996. *Ehrlichia canis*-like agent isolated from a man in Venezuela: antigenic and genetic characterization. *Journal of Clinical Microbiology* **34**: 2133-2139.

Perez, M., Bodor, M., Zhang, C., Xiong, Q. and Rikihisa, Y. 2006. Human infection with *Ehrlichia canis* accompanied by clinical signs in Venezuela. *Annals of the New York Academy of Science* **1078**: 110-117.

Petney T.N., Horak, I.G., Howell, D.J. and Meyer, S. 2004. Striped mice, *Rhabdomys pumilio*, and other murid rodents as hosts for immature ixodid ticks. *Onderstepoort Journal of Veterinary Research* **71**: 313-318.

Posada, D. 2008. jModelTest: Phylogenetic modal averaging. *Molecular Biology and Evolution* **25**: 1253-1256.

Pretorius, A.M. and Kelly, P.J. 1998. Serological survey for antibodies reactive with *Ehrlichia canis* and *E. chaffeensis* in dogs from the Bloemfontein area, South Africa. *Journal of South African Veterinary Association* **69**: 126-128.

Pretorius, A.M., Venter, T.P., Van der Ryst, E. and Kelly, P.J. 1999. A case report of possible human ehrlichiosis in the Free State Province of South Africa. *South African Medical Journal* **89**: 961.

Rar, V.A. and Golovljova, I. 2011. *Anaplasma*, *Ehrlichia*, and ‘‘Candidatus *Neoehrlichia’’* bacteria: Pathogenicity, biodiversity, and molecular genetic characteristics, a review. *Infections Genetics and Evolution* **11**: 1842-1861.

Rar, V.A., Livanova, N.N., Panov, V.V., Doroschenko, E.K., Pukhovskaya, N.M., Vysochina, N.P. and Ivanov, L.I. 2010. Genetic diversity of *Anaplasma* and *Ehrlichia* in the Asian part of Russia. *Ticks and Tick-borne Diseases* **1**: 57-65.

Rar, V.A., Epikhina, T.I., Pukhovskaya, N.M., Vysochina, N.P. and Ivanov, L.I. 2013. Genetic variability of Anaplasmataceae bacteria determined in *Haemaphysalis* spp. and *Dermacentor* sp. ticks on the territory of the Russian Far East. *Molecular Genetics, Microbiology and Virology* **28**: 56-63.

Richards, R., O’leary, B. and Mutsonziwa, K. 2007. Measuring quality of life in informal settlements in South Africa. *Social Indicators Research* **81**: 375-388.

Rymaszewska, A. and Grenda, S. 2008. Bacteria of the genus *Anaplasma* - characteristics of *Anaplasma* and their vectors: a review. *Veterinarni Medicina* **53**: 573-584.

Sanogo, Y.O., Davoust, B., Inokuma, H., Camicas, J., Parola, P. and Brouqui, P. 2003. First evidence of *Anaplasma platys* in *Rhipicephalus sanguineus* (Acari: Ixodida) collected from dogs in Africa. *Onderstepoort Journal of Veterinary Research* **70**: 205-212.

Sashika, M., Abe, G., Matsumoto, K. and Inokuma, H. 2011. Molecular survey of *Anaplasma* and *Ehrlichia* infections of feral raccoons (*Procyon lotor*) in Hokkaido, Japan. *Vector Borne Zoonotic Diseases* **11**: 349-354.

Schouls, L.M., Van De Pol, I., Rijpkema, S.G.T. and Schot, C.S. 1999. Detection and identification of *Ehrlichia, Borrelia burgdorferi* Sensu Lato, and *Bartonella* species in Dutch *Ixodes ricinus* ticks. *Journal of Clinical Microbiology* **37**: 2215-2222.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* **28**: 2731-2739.

Uhaa, I.J., MacLean, J.D., Greene, C.R. and Fishbein, D.B. 1992. A case of human ehrlichiosis acquired in Mali: Clinical and laboratory findings. *American Journal of Tropical Medicine and Hygiene* **46**: 161-164.

Vaughan, J.A., Tkach, V.V. and Greiman, S.E. 2012. Neorickettsial endosymbionts of the Digenea: diversity, transmission and distribution. *Advances in Parasitology* **79**: 253-297.

Yabsley, M.J., Murphy, S.M., Luttrell, M.P., Wilcox, B.R., Howerth, E.W. and Munderloh, U.G. 2008. Characterization of 'Candidatus *Neoehrlichia lotoris*'

(family Anaplasmataceae) from raccoons (*Procyon lotor*). *International Journal of Systematic and Evolutionary Microbiology* **58**: 2794-2798.

Yabsley, M.J. 2010. Natural History of *Ehrlichia chaffeensis*: Vertebrate hosts and tick vectors from the United States and evidence for endemic transmission in other countries. *Veterinary Parasitology* **167**: 136-148.

Chapter 6

General discussion

The primary objective of Chapter 2 was to accurately identify indigenous rodent species belonging to cryptic species complexes in order to assess their role in zoonotic disease transmission. The main aim of the subsequent chapters (Chapter 3-5) was to investigate the role of these indigenous rodents in spreading potentially zoonotic bacterial diseases, particularly those pathogens that could be spread through environmental contamination, as well as their function as reservoir hosts for vector-borne diseases belonging to the families Bartonellaceae and Anaplasmataceae.

Correct reservoir host identification is crucial during epidemiological studies (Mills and Childs 1998). When dealing with southern African rodents correct species identification can be problematic, especially when working with murid rodents. The family Muridae belongs to a species-rich group of mammals, the superfamily Muroidea (Musser and Carleton 2005). There is a large degree of uncertainty in the taxonomy of a number of African members of this family, exemplified by the description of several cryptic species complexes over the last three decades (Bronner *et al.* 2003; Skinner and Chimimba 2005). Species complexes usually contain two or more cryptic species that lack morphologically distinguishable characteristics and often have large overlaps in distributional ranges (Lecompte *et al.* 2005; Russo *et al.* 2006). The current study aimed to accurately identify all murid rodents that potentially belonged to cryptic species complexes by amplification and sequence analyses of the mitochondrial cytochrome *b* gene region. Phylogenetic analysis of

cytochrome *b* sequences revealed the presence of *Aethomys ineptus*, *Mastomys coucha* and *Otomys angoniensis* in the current sampling localities.

Additionally, the present study also aimed to identify which *Rhabdomys* mitochondrial lineages are present in the current sampling localities in an attempt to refine distributional data for the newly proposed species and subspecies within this genus. *Rhabdomys* was historically treated as a monotypic genus; however, recent cytogenetic and molecular data have indicated at least two species and two subspecies within this genus (Rambau *et al*. 2003; Castiglia *et al*. 2012). Phylogenetic analysis of *Rhabdomys* samples collected revealed the presence of one species and two subspecies in the region sampled during the current study. Cytochrome *b* phylogenetic analyses identified four *Rhabdomys* samples corresponding to the proposed subspecies *Rhabdomys dilectus chakae* and the remaining 32 belonging to the proposed subspecies *R. d. dilectus*. Both proposed subspecies within *R. dilectus* were sampled from Hammanskraal and from the University of Pretoria Experimental farm, providing tentative evidence of a contact zone. The true taxonomic status of the proposed taxa within *Rhabdomys* remains unclear and future studies should include more extensive sampling over the entire distributional range of the genus, sequencing of additional nuclear genes and complete morphological investigations.

Phylogenetic analyses revealed the presence of only one of the sibling species belonging to the cryptic species complexes of the murid genera *Aethomys*, *Mastomys*, *Otomys* and *Rhabdomys* in Hammanskraal and the University of Pretoria Experimental farm, Gauteng province, South Africa. Therefore the first null hypothesis (Chapter 1) is rejected and the alternative hypothesis is accepted. Given

the large diversity of murid rodents in southern Africa (Lecompte *et al*. 2008), future epidemiological studies should be mindful of the possible presence of cryptic sibling species when identifying reservoir host species.

Rodents are known to transmit a wide range of pathogens including viruses, bacteria, helminthes and protozoa to humans and domestic animals (Meerburg *et al*. 2009). Rodent-borne diseases are a cause of much morbidity and mortality in Africa (Gratz 1997). During the present study bacteria from two phyla (Proteobacteria and Firmicutes) and seven genera (*Bartonella*, *Anaplasma*, *Helicobacter*, *Burkholderia*, *Streptococcus*, *Aerococcus* and *Lactobacillus*) were identified molecularly in kidneys of indigenous rodents. Two of the identified genera (*Anaplasma* and *Bartonella*) contain bacteria that are transmitted primarily by haematophagus arthropod vectors (Billeter *et al*. 2008; Rar and Golovljova 2011). The remaining five genera contain bacteria that could be passed into the environment through rodent urine and faeces (Garrity *et al*. 2005; Vos *et al.* 2009). Although the overall initial PCR prevalence with broad-range 16S rRNA primers was very high (73.3 %; $^{74}/_{101}$) the overall sequence-confirmed estimates recovered a substantially lower prevalence (38.6 %; $^{39}/_{101}$). This was mainly due to the large proportion of bacterial mixtures of indiscernible species composition. This is an acknowledged drawback of molecular identification of bacteria using broad-range 16S rRNA primers (Clarridge 2004). However, the 16S rRNA gene region is highly conserved among all bacterial taxa and therefore provides a rapid, low risk, inexpensive means to screen a large number of samples for bacterial diversity (Clarridge 2004).

The bacterial genera detected in the kidneys of indigenous and commensal rodents contain a number of species with zoonotic potential. Furthermore, many of the bacterial genera identified contain pathogenic members which cause diseases which are more severe in individuals with compromised immune systems such as the very young, the elderly, malnourished individuals and those living with chronic illnesses such as HIV/AIDS (Harty *et al.* 1994; Coenye and LiPuma 2003; Comunian *et al.* 2006; Ismail *et al*. 2010; Chomel *et al.* 2012). Therefore, the null hypothesis that there are no bacteria of zoonotic potential associated with kidneys of indigenous commensal and natural rodents in South Africa is rejected and the alternative hypothesis is accepted. Although the true bacterial prevalence and diversity is probably under-estimated, this study still provides a valuable census of bacteria associated with indigenous rodents both in a commensal and natural setting. In future it will be useful to use traditional culturing and biochemical methods in combination with molecular methods such as genus-specific bacterial primers to screen indigenous rodents for potentially harmful bacteria.

The subsequent two chapters focussed on vector-borne bacteria identified in commensal and wild indigenous rodents. Rodents play host to a variety of ectoparasites and vector-borne diseases and are frequently implicated in the spread of these parasites and pathogens to humans and domestic animals (Morand *et al*. 2006; Meerburg *et al.* 2009). Vector-borne diseases contribute to almost 30 % of emerging infectious diseases (Jones *et al.* 2008) and this percentage is predicted to rise in future due to various factors, but especially due to global climate change (Mills *et al.* 2010; Panic and Ford 2013).

The genus *Bartonella* contains 33 valid species to date, with more than half of these having known or suspected zoonotic potential (Buffet *et al.* 2013). *Bartonella* infections have been found to be highly prevalent in a wide variety of rodent species worldwide (Buffet *et al.* 2013). In South Africa, a number of studies have recently demonstrated that indigenous murid rodents harbour a diverse collection of known and potentially novel *Bartonella* strains at prevalence rates as high as 96 % in some species (Pretorius *et al.* 2004; Bastos 2007; Brettschneider 2010; Brettschneider *et al.* 2012a). Bartonellae have also been detected in ectoparasites collected from indigenous rodents (Brettschneider 2010). Furthermore, it appeared from these studies that commensal indigenous rodents in South Africa may have a higher incidence of *Bartonella* infection than wild populations (Pretorius *et al.* 2004; Bastos 2007). The present study revealed that in a single murid species, the southern multimammate mouse (*Mastomys coucha*), commensal populations had a significantly higher *Bartonella* prevalence rate (92.9 %) as compared to a wild population (56.9 %). The null hypothesis is rejected and the second alternative hypothesis, that there is a difference in *Bartonella* prevalence and diversity between commensal and natural populations of *M. coucha* in South Africa, is accepted.

In addition to the difference in infection prevalence, Chapter 4 also investigated whether the difference in ectoparasite loads could be a possible factor affecting the difference in *Bartonella* infection between a commensal and a wild population of *M. coucha*. Although ectoparasite loads were higher for the commensal *M. coucha*, there was no statistically significant difference between infection status and ectoparasite indices. Unfortunately, the current study did not identify or screen ectoparasites collected from *M. coucha* samples and therefore did not investigate the difference

between *Bartonella* prevalence and specific ectoparasite species. It is possible that there could be a difference between the prevalence of certain *Bartonella* strains and specific ectoparasite species; however, this requires further investigation. Future studies should focus on the identification and bacterial screening of ectoparasites, especially fleas as it has previously been shown that they play an important role in the transmission of rodent-borne *Bartonella* (Brettschneider *et al.* 2012b).

Apart from several novel *Bartonella* strains identified in both *M. coucha* populations, a species of known zoonotic potential (*B. elizabethae*) was also identified in both commensal and wild populations. *Bartonella elizabethae* has been detected in humans worldwide and causes severe endocarditis, particularly in immune-compromised patients (Jacomo *et al*. 2002). The high *Bartonella* prevalence and presence of a pathogenic *Bartonella* species is therefore of particular importance in South Africa, where there are a large proportion of individuals living with HIV/AIDS (Cobbing *et al.* 2014). Future studies of *Bartonella* should attempt to isolated and fully describe the novel strains detected not only in *M. coucha* but also in other rodent species in southern Africa. Assessments of *Bartonella* prevalence in humans should also be conducted in future in order to establish whether strains detected in rodents are directly responsible for infections in humans.

The present study represents one of the first to screen indigenous commensal rodents for tick-borne members of the bacterial family Anaplasmataceae. Tick-borne members of this family (*Anaplasma* and *Ehrlichia*) are known to cause important diseases in humans and domestic animals (Rar and Golovljova 2011). The main aim of Chapter 5 was to determine the prevalence and diversity of tick-borne

Anaplasmataceae bacteria in commensal indigenous rodents sampled from Hammanskraal and the University of Pretoria Experimental Farm, Gauteng province, South Africa. *Anaplasma bovis*-like bacteria were detected in five of the six rodent species sampled (*Aethomys ineptus, Lemniscomys rosalia, Mastomys coucha, Otomys angoniensis* and *Rhabdomys dilectus*) at an overall prevalence of 39.2 % and *Ehrlichia ewingii* was detected in *M. coucha* samples only at a prevalence of 5.3 %. The null hypothesis that there is no prevalence and diversity of Anaplasmataceae bacteria in indigenous commensal rodents in South Africa is rejected and the alternative hypothesis is accepted.

Anaplasma bovis causes a mild febrile illness in ruminants and is primarily transmitted by *Amblyomma variegatum* and *Rhipicephalus appendiculatus* (Rymaszewska and Grenda 2008). *Anaplasma bovis* DNA has also been detected in small mammals and *Haemaphysalis* ticks from the USA and Japan suggesting that small mammals may also be involved as reservoirs of this infection (Goethert and Telford 2003; Sashika *et al.* 2011). The recent detection of *A. bovis* DNA in eastern rock sengis (*Elephantulus myurus*) and an undescribed Rhipicephaline tick species from South Africa further suggests the role of small mammals as reservoirs of this bacterial species (Harrison *et al.* 2011; Harrison *et al*. 2013). However, the veterinary and medical importance of the *A. bovis*-like bacteria detected in commensal rodents during the present study remains unclear. The present study represents the first report of *E. ewingii* DNA in rodents from South Africa. *Ehrlichia ewingii* is the etiological agent of Human Ewingii Ehrlichiosis (HEE). *Ehrlichia ewingii* DNA has been detected in *Rhipicephalus sanguineus* ticks and dogs from Cameroon and in *H. longicornis* ticks and voles from South Korea (Ndip *et al*. 2005; Kim *et al.* 2006).

However, confirmed human cases of *E. ewingii* infection have only been reported in the U.S.A. and the majority of confirmed HEE cases being reported from immune-compromised patients (Rar and Golovljova 2011).

No Anaplasmataceae were detected in rodent samples from the University of Pretoria Experimental farm and this could be due to established tick-control programs that may be in place in this area. It has previously been demonstrated in South Africa that antibodies to ehrlichial infections were higher in animals from resource-poor areas, where there is usually a lack of effective tick-control programs (Pretorius and Kelly 1998). Additionally, the present study found a relationship between the tick load and the infection prevalence in commensal rodents. Similar results have been demonstrated for *A. bovis* infection in feral raccoons in Japan, where the rate of infection in animals with ticks was significantly higher (Sashika *et al.* 2011). Unfortunately, the present study did not identify or screen the ticks collected from these commensal rodent species. Prospective studies should identify tick specimens collected from these commensal rodents in order to identify possible vectors of the detected Anaplasmataceae bacteria. This is especially important in the case of *E. ewingii*, as the majority of confirmed human infections have been detected from individuals with compromised immune systems such as those living with HIV/AIDS (Rar and Golovljova 2011). South Africa has a large number of individuals living with HIV/AIDS, especially in informal settlements (Richards *et al*. 2007; Cobbing *et al*. 2014). Therefore, these commensal rodents and their ticks could represent an overlooked disease threat to these individuals.

By the year 2025 it is estimated that more than half of the world's population will live in urban areas and that the majority of this urban population growth will take place in developing countries like South Africa (Gratz 1999). This rapid urbanisation frequently leads to a large number of individuals living in informal settlements characterised by inadequate housing and sanitation infrastructure, a situation that inevitably favours the increase of commensal rodent populations (Taylor *et al*. 2008). Increased rodent populations not only increase structural damage and competition, but also increase the risk of zoonotic disease transfer (Taylor *et al.* 2008). Furthermore, global climate change and changes in human behaviour and land use patterns may lead to increased contact with wild rodents and their parasites (McMichael 2004). All these factors highlight the importance of continual disease surveillance in commensal and natural indigenous rodents as this information will assist with the planning of health, environmental and conservation management practices that will mitigate the effect of emerging and re-emerging rodent-borne diseases.

References

Bastos, A.D.S., 2007. *Bartonella* incidence and diversity in endemic South African Murid rodents occurring commensally with humans. *The Southern African Society for Veterinary Epidemiology and Preventive Medicine* pp 76-81.

Billeter, S.A., Levy, M.G., Chomel, B.B. and Breitschwerdt, E.B. 2008. Vector transmission of *Bartonella* species with emphasis on the potential for tick transmission. *Medical and Veterinary Entomology* **22**: 1-15.

Brettschneider, H. 2010. *Bartonella* dynamics in indigenous and invasive murid rodents from South Africa: Prevalence, diversity and mathematical modelling. PhD Thesis, University of Pretoria, Pretoria, South Africa.

Brettschneider, H., Bennett, N.C., Chimimba, C.T. and Bastos, A.D.S. 2012a. Bartonellae of the Namaqua rock mouse, *Micaelamys namaquensis* (Rodentia: Muridae) from South Africa. *Veterinary Microbiology* **157**: 132-136.

Brettschneider, H., Anguelov, R., Chimimba, C.T. and Bastos, A.D.S. 2012b. A mathematical epidemiological model of gram-negative *Bartonella* bacteria: does differential ectoparasite load fully explain the differences in infection prevalence of *Rattus rattus* and *Rattus norvegicus*? *Journal of Biological Dynamics* **6**: 763-781.

Bronner, G.N., Hoffman, M., Taylor, P.J., Chimimba, C.T., Best, P.B., Matthee, C.A. and Robinson, T.J. 2003. A revised systematic checklist of the extant mammals of the southern African subregion. *Durban Museum Novitates* **28**: 56-106.

Buffet, J., Kosoy, M. and Vayssier-Taussat, M. 2013. Natural history of *Bartonella*-infecting rodents in light of new knowledge on genomics, diversity and evolution. *Future Microbiology* **8**: 1117-1128.

Castiglia, R., Solano, E., Makundi, R.H., Hulselmans, J., Verheyen, E. and Colangelo, P. 2012. Rapid chromosomal evolution in the mesic four-striped grass rat *Rhabdomys dilectus* (Rodentia, Muridae) revealed by mtDNA phylogeographic analysis. *Journal of Zoological Systematics and Evolutionary Research* **50**: 165-172.

Chomel, B.B., McMillan-Cole, A.C., Kasten, R.W., Stuckey, M.J., Sato, S., Maruyama, S., Diniz, P.P.V.P. and Breitschwerdt, E.B. 2012. Candidatus *Bartonella merieuxii*, a potential new zoonotic *bartonella* species in canids from Iraq. *PLoS Neglected Tropical Diseases* **6**: e1843.

Clarridge, J.E. 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews* **17**: 840-862.

Cobbing, S., Hanass-Hancock, J. and Deane, M. 2014. Physiotherapy rehabilitation in the context of HIV and disability in KwaZulu-Natal, South Africa. *Disability and Rehabilitation* DOI: 10.3109/09638288.2013.872199.

Coenye, T. and LiPuma, J.J. 2003. Molecular epidemiology of *Burkholderia* species. *Frontiers in Bioscience* **8**: 55-67.

Comunian, L.B., Moura, S.B., Paglia, A.P., Nicoli, J.R., Guerra, J.B., Rocha, G.A. and Queiroz, D.M.M. 2006. Detection of *Helicobacter* species in the gastrointestinal tract of wild rodents from Brazil. *Current Microbiology* **53**: 370-373.

Garrity, G.M., Bell, J.A. and Lilbur, T. 2005 Class V. Epsilonproteobacteria class. nov. In: *Bergey's Manual of Systematic Bacteriology, Volume Two: The*

Proteobacteria, (eds) G.M. Garrity, D.J. Brenner, N.R. Krieg and J.R. Staley. Springer-Verlag, New York.

Goethert, H.K. and Telford, S.R. 2003. Enzootic transmission of *Anaplasma bovis* in Nantucket cottontail rabbits. *Journal of Clinical Microbiology* **41**: 3744-3747.

Gratz, N.G. 1997. The burden of rodent-borne diseases in Africa south of the Sahara. *Belgian Journal of Zoology* **127**: 71-84.

Gratz, N.G. 1999. Urbanization, arthropod and rodent pests and human health. In: *Proceedings of the 3rd International Conference on Urban Pests* pp 51-59.

Harrison, A., Bown, K.J. and Horak, I.G., 2011. Detection of *Anaplasma bovis* in an undescribed tick species collected from the eastern rock sengi *Elephantulus myurus. Journal of Parasitology* **97**: 1012-1016.

Harrison, A., Bastos, A.D.S. Medger, K. and Bennett, N.C. 2013. Eastern rock sengis as reservoir hosts of *Anaplasma bovis* in South Africa. *Ticks and Tick-borne Diseases* **4**: 503-505.

Harty, D.W.S., Oakey, H.J., Patrikakis, M., Hume, E.B.H. and Knox, K.W. 1994. Pathogenic potential of lactobacilli. *International Journal of Food Microbiology* **24**: 179-189.

Ismail, N., Bloch, K.C. and McBride, J.W. 2010. Human ehrlichiosis and anaplasmosis. *Clinical Laboratory Medicine* **30**: 261-292.

Jacomo V., Kelly P.J. and Raoult D. 2002. Natural history of *Bartonella* infections (an exception to Koch's postulate). *Clinical and Diagnostic Laboratory Immunology* **9**: 8-18.

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L. and Daszak, P. 2008. Global trends in emerging infectious diseases. *Nature* **451**: 990-993.

Kim, C.M., Yi, Y.H., Yu, D.H., Lee, M.J., Cho, M.R., Desai, A.R., Shringi, S., Klein, T.A., Kim, H.C., Song, J.W., Baek, L.J., Chong, S.T., O'Guinn, M.L., Lee, J.S., Lee, I.Y., Park, J.H., Foley, J. and Chae, J.S. 2006. Tick-borne rickettsial pathogens in ticks and small mammals in Korea. *Applied Environmental Microbiology* **72**: 5766-5776.

Lecompte, E., Brouat, C., Duplantier, U-M., Galan, M., Granjon, L., Louiseau, A., Mouline, K. and Cosson, J. 2005. Molecular identification of four cryptic species of *Mastomys* (Rodentia, Murinae). *Biochemistry and Systematics Ecology* **33**: 681-689.

Lecompte, E., Aplin, K., Denys, C., Catzeflis, F., Chades, M. and Chevret, P. 2008. Phylogeny and biogeography of African Murinae based on mitochondrial and nuclear gene sequences, with a new tribal classification of the subfamily. *BMC Evolutionary Biology* **8**: 199.

McMichael, A. J. 2004. Environmental and social influences on emerging infectious diseases: past, present and future. *Philosophical Transactions: Biological Sciences* **359**: 1049-1058

Meerburg, B.G., Singleton, G.R. and Kijlstra, A. 2009. Rodent-borne diseases and their risks for public health. *Critical Reviews in Microbiology* **35**: 221-270.

Mills, J.N. and Childs, J.E. 1998. Ecologic studies of rodent reservoirs: their relevance for human health. *Emerging Infectious Diseases* **4**: 529-537.

Mills, J.N, Gage, K.L. and Khan, A.S. 2010. Potential influence of climate change on vector-borne and zoonotic diseases: a review and proposed research plan. *Environmental Health Perspectives* **118**: 1507-1514.

Morand, S. Krasnov, B.R. and Poulin, R. 2006. *Micromammals and Macroparasites: From Evolutionary Ecology to Management*. Springer-Verlag, Tokyo.

Musser, G.G. and Carleton, M.D. 2005. Superfamily Muroidea. In: *Mammal Species of the World a Taxonomic and Geographic Reference*, (eds) D.E. Wilson and D.M. Reeder. Johns Hopkins University Press, Baltimore.

Ndip, L.M., Ndip, R.N., Esemu, S.N., Dickmu, V.L., Fokam, E.B., Walker, D.H. and McBride, J.W. 2005. Ehrlichial infection in Cameroonian canines by *Ehrlichia canis* and *Ehrlichia ewingii*. *Veterinary Microbiology* **111**: 59-66.

Panic, M. and Ford, J.D. 2013. A review of national-level adaptation planning with regards to the risks posed by climate change on infectious diseases in 14 OECD nations. *International Journal of Environmental Research and Public Health* **10**: 7083-7109.

Pretorius, A.M. and Kelly, P.J. 1998. Serological survey for antibodies reactive with *Ehrlichia canis* and *E. chaffeensis* in dogs from the Bloemfontein area, South Africa. *Journal of South African Veterinary Association* **69**: 126-128.

Pretorius, A., Beati, L. and Birtles, R.J. 2004. Diversity of Bartonellae associated with small mammals inhabiting Free State province, South Africa. *International Journal of Systematic and Evolutionary Microbiology* **54**: 1959-1967.

Rambau, R.V., Robinson, T.J. and Stanyo, R. 2003. Molecular genetics of *Rhabdomys pumilio* subspecies boundaries: mtDNA phylogeography and karyotypic analysis

by fluorescence in situ hybridization. *Molecular Phylogenetics and Evolution* **28**: 564-575.

Rar, V.A. and Golovljova, I. 2011. *Anaplasma*, *Ehrlichia*, and ‘‘Candidatus *Neoehrlichia’’* bacteria: Pathogenicity, biodiversity, and molecular genetic characteristics, a review. *Infections Genetics and Evolution* **11**: 1842-1861.

Richards, R., O’leary, B. and Mutsonziwa, K. 2007. Measuring quality of life in informal settlements in South Africa. *Social Indicators Research* **81**: 375-388.

Russo, I.R., Chimimba, C.T. and Bloomer, P. 2006. Mitochondrial DNA differentiation between two species of *Aethomys* (Rodentia: Muridae) from southern Africa. *Journal of Mammalogy* **87**: 545-553.

Rymaszewska, A. and Grenda, S. 2008. Bacteria of the genus *Anaplasma* - characteristics of *Anaplasma* and their vectors: a review. *Veterinarni Medicina* **53**: 573-584.

Sashika, M., Abe, G., Matsumoto, K. and Inokuma, H. 2011. Molecular survey of *Anaplasma* and *Ehrlichia* infections of feral raccoons (*Procyon lotor*) in Hokkaido, Japan. *Vector Borne Zoonotic Diseases* **11**: 349-354.

Skinner, J.D. and Chimimba C.T. 2005. *The Mammals of the Southern African Subregion*. Cambridge University Press, Cambridge.

Taylor, P.J., Arntzen, L., Hayter, M., Iles, M., Frean, J. and Belmain, S. 2008. Understanding and managing sanitary risks due to rodent zoonoses in an African city: beyond the Boston Model. *Integrative Zoology* **3**: 38-50.

Vos, P., Garrity, G., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.H. and Whitman, W.B. 2009. *Bergey's Manual of Systematic Bacteriology: Volume 3: The Firmicutes*. Springer-Verlag, New York.

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Appendix I

#	Sample Code	Locality	GPS		Sampling Date	Species	Sex	Weight (g)	Identification method
1	H1-1	Hammanskraal	25.36918S	28.27704E	12082010	*Rhabdomys dilectus*	M	Not weighed	Cyt *b* Sequence
2	H1-2	Hammanskraal	25.36918S	28.27704E	12082010	*Mastomys coucha*	M	17.94	Cyt *b* Sequence
3	H1-3	Hammanskraal	25.36918S	28.27704E	12082010	*Rhabdomys dilectus*	M	25.22	Cyt *b* Sequence
4	H1-4	Hammanskraal	25.36918S	28.27704E	12082010	*Mastomys coucha*	M	29.56	Cyt *b* Sequence
5	H1-5	Hammanskraal	25.36918S	28.27704E	12082010	*Mastomys coucha*	M	20.88	Multiplex PCR
6	H1-6	Hammanskraal	25.36918S	28.27704E	12082010	*Mastomys coucha*	M	28.66	Multiplex PCR
7	H1-7	Hammanskraal	25.36918S	28.27704E	12082010	*Mastomys coucha*	M	16.15	Cyt *b* Sequence
8	HW1-8	Hammanskraal	25.36918S	28.27704E	12082010	*Rhabdomys dilectus*	M	33.15	Cyt *b* Sequence
9	HRES1-9	Red Eagle Security Hammanskraal	25.22700S	28.11260E	13082010	*Rhabdomys dilectus*	M	23.59	Cyt *b* Sequence
10	HW1-11	Hammanskraal	25.36918S	28.27704E	13082010	*Mastomys coucha*	M	25.09	Multiplex PCR
11	HW1-12	Hammanskraal	25.36918S	28.27704E	13082010	*Mastomys coucha*	M	20.47	Multiplex PCR
12	HW1-13	Hammanskraal	25.36918S	28.27704E	14082010	*Mastomys coucha*	M	31.1	Multiplex PCR
13	HW1-14	Hammanskraal	25.36918S	28.27704E	14082010	*Mastomys coucha*	M	20.71	Multiplex PCR
14	HW1-15	Hammanskraal	25.36918S	28.27704E	14082010	*Rhabdomys dilectus*	M	19.69	Cyt *b* Sequence
15	HW1-16	Hammanskraal	25.36918S	28.27704E	14082010	*Mastomys coucha*	M	30.38	Multiplex PCR
16	HH1-17	Hammanskraal	25.36918S	28.27704E	14082010	*Mastomys coucha*	M	25.62	Cyt *b* Sequence
17	HH1-18	Hammanskraal	25.36918S	28.27704E	14082010	*Mastomys coucha*	M	18.84	Cyt *b* Sequence
18	HH1-19	Hammanskraal	25.36918S	28.27704E	14082010	*Mastomys coucha*	M	40.31	Multiplex PCR
19	HH1-20	Hammanskraal	25.36918S	28.27704E	14082010	*Mastomys coucha*	M	26.5	Multiplex PCR
20	HPR2-1	Prestige College Hammanskraal	25.42605S	28.27209E	21092010	*Mastomys coucha*	M	30.5	Cyt *b* Sequence
21	HPB2-2	Prestige College Hammanskraal	25.42605S	28.27209E	22092010	*Mastomys coucha*	M	33.31	Cyt *b* Sequence
22	HPB2-3	Prestige College Hammanskraal	25.42605S	28.27209E	22092010	*Rhabdomys dilectus*	F	45.6	Cyt *b* Sequence
23	HPB3-1	Prestige College Hammanskraal	25.42605S	28.27209E	26102010	*Lemniscomys rosalia*	M	34.43	NA
24	HPB3-2	Prestige College Hammanskraal	25.42605S	28.27209E	26102010	*Rhabdomys dilectus*	F	35.92	Cyt *b* Sequence
25	HPB3-3	Prestige College Hammanskraal	25.42605S	28.27209E	27102010	*Rhabdomys dilectus*	F	31.35	Cyt *b* Sequence
26	HPB3-4	Prestige College Hammanskraal	25.42605S	28.27209E	28102010	*Mastomys coucha*	M	40.11	Cyt *b* Sequence
27	HPB3-5	Prestige College Hammanskraal	25.42605S	28.27209E	29102010	*Rhabdomys dilectus*	M	34.98	Cyt *b* Sequence
28	UPEF4-1	UP Experimental Farm	25.75112S	28.26298E	3122010	*Otomys angoniensis*	F	102.75	Cyt *b* Sequence
29	UPEF4-2	UP Experimental Farm	25.75112S	28.26298E	3122010	*Otomys angoniensis*	M	128.9	Cyt *b* Sequence
30	HPB4-3	Prestige College Hammanskraal	25.42605S	28.27209E	8122010	*Rhabdomys dilectus*	M	42.37	Cyt *b* Sequence
31	HPB4-4	Prestige College Hammanskraal	25.42605S	28.27209E	8122010	*Mastomys coucha*	F	23.07	Multiplex PCR
32	HPB4-5	Prestige College Hammanskraal	25.42605S	28.27209E	8122010	*Mastomys coucha*	F	30.84	Cyt *b* Sequence
33	HPB4-6	Prestige College Hammanskraal	25.42605S	28.27209E	8122010	*Rhabdomys dilectus*	F	34.86	Cyt *b* Sequence
34	UPEF4-7	UP Experimental Farm	25.75112S	28.26298E	8122010	*Mastomys coucha*	F	30.42	Multiplex PCR
35	UPEF4-8	UP Experimental Farm	25.75112S	28.26298E	8122010	*Rhabdomys dilectus*	M	35.15	Cyt *b* Sequence
36	UPEF4-9	UP Experimental Farm	25.75112S	28.26298E	8122010	*Rhabdomys dilectus*	M	16.13	Cyt *b* Sequence
37	UPEF4-10	UP Experimental Farm	25.75112S	28.26298E	8122010	*Otomys angoniensis*	F	99.87	Cyt *b* Sequence
38	UPEF4-11	UP Experimental Farm	25.75112S	28.26298E	8122010	*Rhabdomys dilectus*	F	47.36	Cyt *b* Sequence
39	HPB4-12	Prestige College Hammanskraal	25.42605S	28.27209E	9122010	*Rhabdomys dilectus*	F	41.9	Cyt *b* Sequence
40	HPB4-13	Prestige College Hammanskraal	25.42605S	28.27209E	9122010	*Rhabdomys dilectus*	M	12.28	Cyt *b* Sequence
41	UPEF4-14	UP Experimental Farm	25.75112S	28.26298E	9122010	*Otomys angoniensis*	M	74.22	Cyt *b* Sequence
42	UPEF4-15	UP Experimental Farm	25.42605S	28.27209E	10122010	*Mastomys coucha*	M	53.54	Multiplex PCR
43	HUP5-1	UP Hammanskraal Campus	25.40482S	28.26268E	25012011	*Rhabdomys dilectus*	F	38.77	Cyt *b* Sequence
44	HUP5-2	UP Hammanskraal Campus	25.40482S	28.26268E	25012011	*Aethomys ineptus*	M	97.08	Cyt *b* Sequence
45	HUP5-3	UP Hammanskraal Campus	25.40482S	28.26268E	25012011	*Mastomys coucha*	M	65.56	Cyt *b* Sequence
46	HUP5-4	UP Hammanskraal Campus	25.40482S	28.26268E	25012011	*Aethomys ineptus*	M	101.43	Cyt *b* Sequence
47	HUP5-5	UP Hammanskraal Campus	25.40482S	28.26268E	25012011	*Aethomys ineptus*	F	106.89	Cyt *b* Sequence
48	HUP5-6	UP Hammanskraal Campus	25.40482S	28.26268E	25012011	*Aethomys ineptus*	M	88.68	Cyt *b* Sequence
49	HUP5-7	UP Hammanskraal Campus	25.40482S	28.26268E	25012011	*Aethomys ineptus*	M	75.35	Cyt *b* Sequence
50	HUP5-8	UP Hammanskraal Campus	25.40482S	28.26268E	25012011	*Mastomys coucha*	M	80.69	Cyt *b* Sequence
51	HUP5-9	UP Hammanskraal Campus	25.40482S	28.26268E	26012011	*Saccostomys campestris*	M	58.84	NA
52	HUP5-10	UP Hammanskraal Campus	25.40482S	28.26268E	27012011	*Aethomys ineptus*	M	70.1	Cyt *b* Sequence
53	HUP5-11	UP Hammanskraal Campus	25.40482S	28.26268E	27012011	*Aethomys ineptus*	F	71.85	Cyt *b* Sequence
54	HUP5-12	UP Hammanskraal Campus	25.40482S	28.26268E	27012011	*Aethomys ineptus*	M	87.69	Cyt *b* Sequence
55	HUP5-13	UP Hammanskraal Campus	25.40482S	28.26268E	27012011	*Mastomys coucha*	F	43.06	Cyt *b* Sequence
56	HPB6-1	Prestige College Hammanskraal	25.42605S	28.27209E	23022011	*Otomys angoniensis*	F	64.02	Cyt *b* Sequence
57	HUPI6-2	UP Hammanskraal Campus	25.40482S	28.26268E	23022011	*Aethomys ineptus*	M	23.7	Cyt *b* Sequence

58	HUPI6-3	UP Hammanskraal Campus	25.40482S	28.26268E	23022011	*Aethomys ineptus*	M	29.73	Cyt *b* Sequence
59	HUPI6-4	UP Hammanskraal Campus	25.40482S	28.26268E	23022011	*Mastomys coucha*	F	44.13	Multiplex PCR
60	HUPI6-5	UP Hammanskraal Campus	25.40482S	28.26268E	23022011	*Aethomys ineptus*	M	70.41	Cyt *b* Sequence
61	HUPI6-6	UP Hammanskraal Campus	25.40482S	28.26268E	23022011	*Aethomys ineptus*	F	83.79	Cyt *b* Sequence
62	HUPI6-7	UP Hammanskraal Campus	25.40482S	28.26268E	23022011	*Aethomys ineptus*	F	86.38	Cyt *b* Sequence
63	HUPII6-8	UP Hammanskraal Campus	25.40482S	28.26268E	23022011	*Rhabdomys dilectus*	M	38.07	Cyt *b* Sequence
64	HUPII6-9	UP Hammanskraal Campus	25.40482S	28.26268E	23022011	*Mastomys coucha*	M	28.62	Multiplex PCR
65	HUPII6-10	UP Hammanskraal Campus	25.40482S	28.26268E	23022011	*Aethomys ineptus*	F	109.56	Cyt *b* Sequence
66	HUPII6-11	UP Hammanskraal Campus	25.40482S	28.26268E	23022011	*Mastomys coucha*	F	53.06	Multiplex PCR
67	HUPII6-12	UP Hammanskraal Campus	25.40482S	28.26268E	23022011	*Saccostomys campestris*	M	34.73	NA
68	HUPII6-13	UP Hammanskraal Campus	25.40482S	28.26268E	24022011	*Rhabdomys dilectus*	F	35.9	Cyt *b* Sequence
69	HUPII6-14	UP Hammanskraal Campus	25.40482S	28.26268E	24022011	*Rhabdomys dilectus*	F	44.69	Cyt *b* Sequence
70	HUPII6-15	UP Hammanskraal Campus	25.40482S	28.26268E	24022011	*Mastomys coucha*	F	44.77	Multiplex PCR
71	HUPI6-16	UP Hammanskraal Campus	25.40482S	28.26268E	24022011	*Aethomys ineptus*	M	31	Cyt *b* Sequence
72	HUPI6-17	UP Hammanskraal Campus	25.40482S	28.26268E	24022011	*Mastomys coucha*	F	34.07	Multiplex PCR
73	HUPI6-18	Prestige College Hammanskraal	25.42605S	28.27209E	24022011	*Aethomys ineptus*	M	75.4	Cyt *b* Sequence
74	HPB6-19	Prestige College Hammanskraal	25.42605S	28.27209E	24022011	*Mastomys coucha*	M	37	Multiplex PCR
75	HPB6-20	Prestige College Hammanskraal	25.42605S	28.27209E	24022011	*Aethomys ineptus*	F	69.02	Cyt *b* Sequence
76	HPB6-21	Prestige College Hammanskraal	25.42605S	28.27209E	25022011	*Mastomys coucha*	M	30.43	Multiplex PCR
77	HPB6-22	Prestige College Hammanskraal	25.42605S	28.27209E	25022011	*Mastomys coucha*	M	13.53	Multiplex PCR
78	HPB6-23	Prestige College Hammanskraal	25.42605S	28.27209E	25022011	*Mastomys coucha*	F	40.17	Multiplex PCR
79	HPB6-24	Prestige College Hammanskraal	25.42605S	28.27209E	25022011	*Mastomys coucha*	M	61.85	Multiplex PCR
80	HUPI6-25	UP Hammanskraal Campus	25.40482S	28.26268E	25022011	*Mastomys coucha*	M	21.86	Multiplex PCR
81	HUPI6-26	UP Hammanskraal Campus	25.40482S	28.26268E	25022011	*Mastomys coucha*	M	9.41	Multiplex PCR
82	HPB6-27	Prestige College Hammanskraal	25.42605S	28.27209E	25022011	*Mastomys coucha*	F	29.96	Multiplex PCR
83	HUP7-1	UP Hammanskraal Campus	25.40482S	28.26268E	23032011	*Aethomys ineptus*	M	80.59	Cyt *b* Sequence
84	HUP7-2	UP Hammanskraal Campus	25.40482S	28.26268E	23032011	*Mastomys coucha*	M	23.11	Multiplex PCR
85	HUP7-3	UP Hammanskraal Campus	25.40482S	28.26268E	23032011	*Mastomys coucha*	F	46.57	Multiplex PCR
86	HUP7-4	UP Hammanskraal Campus	25.40482S	28.26268E	23032011	*Mastomys coucha*	M	18.15	Multiplex PCR
87	HUP7-5	UP Hammanskraal Campus	25.40482S	28.26268E	23032011	*Mastomys coucha*	M	43.83	Multiplex PCR
88	HUP7-6	UP Hammanskraal Campus	25.40482S	28.26268E	23032011	*Aethomys ineptus*	F	87.45	Cyt *b* Sequence
89	HUP7-7	UP Hammanskraal Campus	25.40482S	28.26268E	23032011	*Mastomys coucha*	F	40.36	Multiplex PCR
90	EF7-8	UP Experimental Farm	25.75112S	28.26298E	23032011	*Mastomys coucha*	F	30.22	Multiplex PCR
91	EF7-9	UP Experimental Farm	25.75112S	28.26298E	23032011	*Mastomys coucha*	F	29.19	Multiplex PCR
92	EF7-10	UP Experimental Farm	25.75112S	28.26298E	23032011	*Mastomys coucha*	M	31.27	Multiplex PCR
93	EF7-11	UP Experimental Farm	25.75112S	28.26298E	24032011	*Mastomys coucha*	M	34.64	Multiplex PCR
94	HUP7-12	UP Hammanskraal Campus	25.40482S	28.26268E	24032011	*Aethomys ineptus*	M	28.98	Cyt *b* Sequence
95	HUP7-13	UP Hammanskraal Campus	25.40482S	28.26268E	25032011	*Mastomys coucha*	M	19.69	Multiplex PCR
96	HUP7-14	UP Hammanskraal Campus	25.40482S	28.26268E	25032011	*Aethomys ineptus*	F	101.31	Cyt *b* Sequence
97	EF8-1	UP Experimental Farm	25.75112S	28.26298E	19042011	*Mastomys coucha*	F	36.47	Multiplex PCR
98	HUP8-2	UP Hammanskraal Campus	25.40482S	28.26268E	19042011	*Mastomys coucha*	M	31.62	Multiplex PCR
99	HUP8-3	UP Hammanskraal Campus	25.40482S	28.26268E	19042011	*Mastomys coucha*	M	23.99	Multiplex PCR
100	HUP8-4	UP Hammanskraal Campus	25.40482S	28.26268E	19042011	*Mastomys coucha*	M	23.86	Multiplex PCR
101	HUP8-5	UP Hammanskraal Campus	25.40482S	28.26268E	19042011	*Aethomys ineptus*	F	49.84	Cyt *b* Sequence
102	HUP8-6	UP Hammanskraal Campus	25.40482S	28.26268E	19042011	*Mastomys coucha*	M	31.83	Multiplex PCR
103	HUP8-7	UP Hammanskraal Campus	25.40482S	28.26268E	19042011	*Mastomys coucha*	M	24.27	Multiplex PCR
104	HUP8-8	UP Hammanskraal Campus	25.40482S	28.26268E	19042011	*Aethomys ineptus*	M	82.74	Cyt *b* Sequence
105	HUP8-9	UP Hammanskraal Campus	25.40482S	28.26268E	19042011	*Mastomys coucha*	M	28.85	Multiplex PCR
106	HUP8-10	UP Hammanskraal Campus	25.40482S	28.26268E	20042011	*Aethomys ineptus*	F	77.54	Cyt *b* Sequence
107	HUP8-11	UP Hammanskraal Campus	25.40482S	28.26268E	20042011	*Mastomys coucha*	M	19.67	Multiplex PCR
108	EF8-12	UP Experimental Farm	25.75112S	28.26298E	20042011	*Mastomys coucha*	M	55.09	Multiplex PCR
109	EF8-13	UP Experimental Farm	25.75112S	28.26298E	20042011	*Mastomys coucha*	M	8.4	Multiplex PCR
110	EF8-14	UP Experimental Farm	25.75112S	28.26298E	21042011	*Rhabdomys dilectus*	F	23.5	Cyt *b* Sequence
111	EF8-15	UP Experimental Farm	25.75112S	28.26298E	21042011	*Otomys angoniensis*	F	97.27	Cyt *b* Sequence
112	HUP8-16	UP Hammanskraal Campus	25.40482S	28.26268E	21042011	*Mastomys coucha*	M	17.73	Multiplex PCR
113	HUP8-17	UP Hammanskraal Campus	25.40482S	28.26268E	21042011	*Aethomys ineptus*	M	52.55	Cyt *b* Sequence
114	HUP8-18	UP Hammanskraal Campus	25.40482S	28.26268E	21042011	*Mastomys coucha*	M	28.85	Multiplex PCR
115	HUPI9-1	UP Hammanskraal Campus	25.40482S	28.26268E	1062011	*Aethomys ineptus*	F	89.73	Cyt *b* Sequence
116	HUPI9-2	UP Hammanskraal Campus	25.40482S	28.26268E	1062011	*Mastomys coucha*	M	27.7	Cyt *b* Sequence
117	HUPI9-3	UP Hammanskraal Campus	25.40482S	28.26268E	1062011	*Mastomys coucha*	M	33.88	Multiplex PCR

118	HUPI9-4	UP Hammanskraal Campus	25.40482S	28.26268E	1062011	*Mastomys coucha*	F	40.91	Multiplex PCR
119	HUPI9-5	UP Hammanskraal Campus	25.40482S	28.26268E	1062011	*Mastomys coucha*	M	25.72	Multiplex PCR
120	HUPI9-6	UP Hammanskraal Campus	25.40482S	28.26268E	1062011	*Mastomys coucha*	M	29.02	Multiplex PCR
121	HUPI9-7	UP Hammanskraal Campus	25.40482S	28.26268E	1062011	*Aethomys ineptus*	F	80.88	Cyt *b* Sequence
122	HUPI9-8	UP Hammanskraal Campus	25.40482S	28.26268E	1062011	*Mastomys coucha*	M	22.19	Multiplex PCR
123	HUPII9-9	UP Hammanskraal Campus	25.40482S	28.26268E	1062011	*Rhabdomys dilectus*	M	18.24	Cyt *b* Sequence
124	HUPII9-10	UP Hammanskraal Campus	25.40482S	28.26268E	1062011	*Rhabdomys dilectus*	M	28.68	Cyt *b* Sequence
125	HUPII9-11	UP Hammanskraal Campus	25.40482S	28.26268E	1062011	*Rhabdomys dilectus*	F	25.27	Cyt *b* Sequence
126	HUPII9-12	UP Hammanskraal Campus	25.40482S	28.26268E	1062011	*Rhabdomys dilectus*	M	26.16	Cyt *b* Sequence
127	HUPII9-13	UP Hammanskraal Campus	25.40482S	28.26268E	1062011	*Mastomys coucha*	M	20.85	Multiplex PCR
128	EF9-14	UP Experimental Farm	25.75112S	28.26298E	1062011	*Otomys angoniensis*	M	127.07	Cyt *b* Sequence
129	EF9-15	UP Experimental Farm	25.75112S	28.26298E	1062011	*Mastomys coucha*	M	19.8	Cyt *b* Sequence
130	EF9-16	UP Experimental Farm	25.75112S	28.26298E	1062011	*Mastomys coucha*	M	15.25	Multiplex PCR
131	EF9-17	UP Experimental Farm	25.75112S	28.26298E	1062011	*Otomys angoniensis*	F	57.86	Cyt *b* Sequence
132	EF9-18	UP Experimental Farm	25.75112S	28.26298E	1062011	*Mastomys coucha*	M	28.06	Cyt *b* Sequence
133	EF9-19	UP Experimental Farm	25.75112S	28.26298E	1062011	*Mastomys coucha*	M	20.58	Multiplex PCR
134	EF9-20	UP Experimental Farm	25.75112S	28.26298E	1062011	*Mastomys coucha*	M	32.66	Cyt *b* Sequence
135	EF9-21	UP Experimental Farm	25.75112S	28.26298E	1062011	*Mastomys coucha*	M	37.86	Multiplex PCR
136	EF9-22	UP Experimental Farm	25.75112S	28.26298E	1062011	*Mastomys coucha*	M	15.9	Multiplex PCR
137	HUPII9-23	UP Hammanskraal Campus	25.40482S	28.26268E	2062011	*Rhabdomys dilectus*	M	24.77	Cyt *b* Sequence
138	HUPII9-24	UP Hammanskraal Campus	25.40482S	28.26268E	2062011	*Rhabdomys dilectus*	M	22.39	Cyt *b* Sequence
139	HUPII9-25	UP Hammanskraal Campus	25.40482S	28.26268E	2062011	*Rhabdomys dilectus*	M	22.14	Cyt *b* Sequence
140	HUPI9-26	UP Hammanskraal Campus	25.40482S	28.26268E	2062011	*Mastomys coucha*	M	33.61	Multiplex PCR
141	HUPI9-27	UP Hammanskraal Campus	25.40482S	28.26268E	2062011	*Rhabdomys dilectus*	M	30.91	Cyt *b* Sequence
142	HUPI9-28	UP Hammanskraal Campus	25.40482S	28.26268E	2062011	*Rhabdomys dilectus*	M	24.26	Cyt *b* Sequence
143	HUPI9-29	UP Hammanskraal Campus	25.40482S	28.26268E	2062011	*Aethomys ineptus*	M	49.04	Cyt *b* Sequence
144	HUPI9-30	UP Hammanskraal Campus	25.40482S	28.26268E	2062011	*Mastomys coucha*	M	22.18	Multiplex PCR
145	HUPI9-31	UP Hammanskraal Campus	25.40482S	28.26268E	2062011	*Rhabdomys dilectus*	M	19.9	Cyt *b* Sequence
146	HUPI9-32	UP Hammanskraal Campus	25.40482S	28.26268E	2062011	*Mastomys coucha*	M	36.84	Multiplex PCR
147	EF9-33	UP Experimental Farm	25.75112S	28.26298E	2062011	*Mastomys coucha*	M	17.21	Multiplex PCR
148	EF9-34	UP Hammanskraal Campus	25.40482S	28.26268E	2062011	*Otomys angoniensis*	M	61.9	Cyt *b* Sequence
149	EF9-35	UP Experimental Farm	25.75112S	28.26298E	2062011	*Mastomys coucha*	M	14.6	Cyt *b* Sequence
150	HUPII9-36	UP Hammanskraal Campus	25.40482S	28.26268E	3062011	*Rhabdomys dilectus*	M	14.75	Cyt *b* Sequence
151	HUPI9-37	UP Hammanskraal Campus	25.40482S	28.26268E	3062011	*Mastomys coucha*	M	37.11	Multiplex PCR
152	HUPI9-38	UP Hammanskraal Campus	25.40482S	28.26268E	3062011	*Rhabdomys dilectus*	M	16.91	Cyt *b* Sequence
153	HUPI9-39	UP Hammanskraal Campus	25.40482S	28.26268E	3062011	*Mastomys coucha*	M	28.85	Multiplex PCR
154	HUP10-1	UP Hammanskraal Campus	25.40482S	28.26268E	29062011	*Rhabdomys dilectus*	M	20.79	Cyt *b* Sequence
155	HUP10-2	UP Hammanskraal Campus	25.40482S	28.26268E	29062011	*Rhabdomys dilectus*	M	18.67	Cyt *b* Sequence
156	HUP10-3	UP Hammanskraal Campus	25.40482S	28.26268E	29062011	*Mastomys coucha*	M	21.06	Multiplex PCR
157	HUP10-4	UP Hammanskraal Campus	25.40482S	28.26268E	29062011	*Mastomys coucha*	M	34.04	Multiplex PCR
158	HUP10-5	UP Hammanskraal Campus	25.40482S	28.26268E	29062011	*Otomys angoniensis*	M	79.08	Cyt *b* Sequence
159	HUP10-6	UP Hammanskraal Campus	25.40482S	28.26268E	29062011	*Otomys angoniensis*	M	85.97	Cyt *b* Sequence
160	HUP10-7	UP Hammanskraal Campus	25.40482S	28.26268E	29062011	*Otomys angoniensis*	F	99.4	Cyt *b* Sequence
161	HUP10-8	UP Hammanskraal Campus	25.40482S	28.26268E	29062011	*Otomys angoniensis*	M	47.52	Cyt *b* Sequence
162	HUP10-9	UP Hammanskraal Campus	25.40482S	28.26268E	29062011	*Otomys angoniensis*	M	72.44	Cyt *b* Sequence
163	HUP10-10	UP Hammanskraal Campus	25.40482S	28.26268E	29062011	*Otomys angoniensis*	F	94.26	Cyt *b* Sequence
164	HUP10-11	UP Hammanskraal Campus	25.40482S	28.26268E	29062011	*Otomys angoniensis*	M	68.98	Cyt *b* Sequence
165	HUP10-12	UP Hammanskraal Campus	25.40482S	28.26268E	29062011	*Otomys angoniensis*	F	97.59	Cyt *b* Sequence
166	HUP10-13	UP Hammanskraal Campus	25.40482S	28.26268E	29062011	*Otomys angoniensis*	M	111.68	Cyt *b* Sequence
167	HUP10-14	UP Hammanskraal Campus	25.40482S	28.26268E	29062011	*Otomys angoniensis*	M	20.96	Cyt *b* Sequence
168	HUP10-15	UP Hammanskraal Campus	25.40482S	28.26268E	29062011	*Otomys angoniensis*	M	56.51	Cyt *b* Sequence
169	HUP10-16	UP Hammanskraal Campus	25.40482S	28.26268E	29062011	*Otomys angoniensis*	M	23.61	Cyt *b* Sequence
170	HUP10-17	UP Hammanskraal Campus	25.40482S	28.26268E	29062011	*Otomys angoniensis*	M	20.05	Cyt *b* Sequence
171	HUP10-18	UP Hammanskraal Campus	25.40482S	28.26268E	29062011	*Rhabdomys dilectus*	M	26.75	Cyt *b* Sequence
172	HUP10-19	UP Hammanskraal Campus	25.40482S	28.26268E	30062011	*Lemniscomys rosalia*	M	34.3	NA
173	HUP10-20	UP Hammanskraal Campus	25.40482S	28.26268E	30062011	*Mastomys coucha*	M	37.33	Multiplex PCR
174	HUP10-21	UP Hammanskraal Campus	25.40482S	28.26268E	1072011	*Mastomys coucha*	F	22.15	Multiplex PCR
175	HUP10-22	UP Hammanskraal Campus	25.40482S	28.26268E	1072011	*Mastomys coucha*	M	31.22	Multiplex PCR