UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

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*

By

Maria Elizabeth Mostert

Submitted in partial fulfilment of the requirements for the degree
Master of Science (Zoology)

Faculty of Natural and Agricultural Sciences
University of Pretoria
Pretoria
South Africa

October 2009

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

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*

By

Maria Elizabeth Mostert*

Supervisors: **Prof A.D.S. Bastos**
Mammal Research Institute (MRI)
Department of Zoology and Entomology
University of Pretoria
Pretoria
0002 South Africa
E-mail: adbastos@zoology.up.ac.za

Prof C.T. Chimimba
DST-NRF Centre of Excellence for Invasion Biology (CIB) &
Mammal Research Institute (MRI)
Department of Zoology and Entomology
University of Pretoria
Pretoria
0002 South Africa
E-mail: ctchimimba@zoology.up.ac.za

***Present address:**
DST-NRF Centre of Excellence for Invasion Biology (CIB) &
Mammal Research Institute (MRI)
Department of Zoology and Entomology
University of Pretoria
Pretoria
0002 South Africa
Email: memostert@zoology.up.ac.za

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Declaration

I, Maria Elizabeth Mostert 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……...

Disclaimer

This thesis 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.

General abstract

Invasive species are generally problematic where they occur, especially in terms of ecology, economy and disease. Members of the genus *Rattus* Fischer, 1803 particularly, are known as one of the most destructive invasive species to date since they cause widespread damage on terrestrial and island ecosystems. Two *Rattus* species have historically been reported as invasive species in South Africa, *Rattus rattus* Linnaeus, 1758, which has a widespread distribution throughout the country and *Rattus norvegicus* Berkenhout, 1769 which is primarily distributed along the coast of South Africa. A third species, *Rattus tanezumi* Temminck, 1844 (which forms part of the *R. rattus* species complex), a south-east Asian endemic, was first reported in 2005 to also occur in South Africa (and Africa). As this species is morphologically similar to *R. rattus*, its identification is reliant on molecular typing approaches.

In the current study, molecular, morphological and disease aspects of South African *Rattus* were assessed. The nature and extent of variation between the three species was investigated using cytochrome *b* sequences and extensive mitochondrial d-loop database for comparative purposes. D-loop data identified one, four and two haplotypes for *R. tanezumi, R. rattus* and *R. norvegicus*, respectively whereas cytochrome *b* data identified additional haplotypes for *R. rattus* and *R. tanezumi*. Pairwise sequence divergence was highest between *R. norvegicus* and *R. tanezumi* (12.5% for D-loop and 12.0% for cyt *b*). *Rattus norvegicus* was recovered in the central parts of South Africa for the first time and occurred sympatrically with *R. tanezumi* at one locality, whereas *Rattus rattus* and *R. tanezumi* occurred sympatrically at three localities.

The external and qualitative cranial morphology of all three species was compared in an attempt to find differences that could be used to morphologically differentiate between these *Rattus* species. Whereas *R. norvegicus* can easily be distinguished from *R. rattus* and *R. tanezumi*, there are no discernible morphological differences to distinguish *R. rattus* and *R. tanezumi*. A taxonomic synthesis and an identification key of the three species of *Rattus* based on qualitative morphology, molecular and cytogenetic data using genetically-identified individuals is provided.

Members of South African *Rattus* were also found to be carriers of the bacteria *Bartonella* Strong *et al*., 1915 and *Helicobacter* Goodwin *et al.*, 1989 emend. Vandamme *et al.*, 1991. *Bartonella elizabethae* (Daly *et al.,* 1993) Brenner *et al.,* 1993, occurring in

Rattus around the world was for the first time recovered from South African *Rattus*. This bacterium has been associated with infective endocarditis in humans and may pose a threat to immuno-compromised individuals in rural South African communities where *Rattus* occurs commensally. Two *Helicobacter* species, *H. rodentium* Shen *et al*., 1997 and *H. muridarum* Lee *et al.*, 1992, were identified neither of which have known zoonotic potential.

Apart from contributing to general small mammal studies in Africa, the present study may have implications in epidemiological, agricultural, biological conservation, and invasion biology research associated with problem rodents in the southern African subregion and beyond.

Key words: *Rattus*, invasive species, South Africa, phylogeny, zoonotic diseases, *Helicobacter*, *Bartonella*, qualitative morphology, cytochrome *b*, D-loop.

Acknowledgements

I am very grateful to my supervisors, Prof Armanda Bastos and Prof Chris Chimimba for their guidance, support and expertise without which this study would not have been possible. They have provided me with good advice and made valuable suggestions on various drafts of this dissertation.

I am indebted to the DST/NRF Centre of Excellence for Invasion Biology (CIB) under whose auspices this study was undertaken, for providing me with funding for this project. Life would have been a lot more difficult without its support.

To various people involved in the sampling of the rats, I want to express my gratitude: Frikkie Kirsten, Emil von Maltitz and all those that helped with the collection of samples. I am grateful to Martin Haupt and Simon Maake who assisted with animal care during the early stages of this project, and to Dr. Adrian Tordiffe for assistance in processing the animals.

I would like to express my thanks to Dr Steven Kleiboeker and Dr Schalk van der Merwe for providing me with *Helicobacter* DNA that helped with testing the lingeage-specificity of the primer sets designed for this project.

Various people helped me in a variety of ways. These include Dr Catherine Sole and Helene Brettschneider who provided some valuable comments and help with various aspects of the dissertation, and my special friends who provided me with a lot of help, support and motivation throughout this study. I would also like to thank all the members of the molecular lab in the Department of Zoology and Entomology, University of Pretoria for their help in various ways.

Last, but certainly not least, I would like to thank my family who I love dearly and that mean the world to me. Without their support and love I would not have been able to undertake the present study. I wish to specially express my sincere gratitude to my mom and dad for all they have done for me not only during this project but throughout my life. I am truly blessed!

Table of contents

List of figures

List of tables

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Generally, members of the genus *Rattus* Fischer, 1803 have a worldwide distribution (Musser & Carleton 2005; Fig. 1.1). These rodents have historically been problematic in agricultural systems where they cause extensive damage to crops, stored grain, and human-made infrastructure such as electrical installations (Putman 1989; Mills 1999; Amori & Clout 2003; Aplin *et al.* 2003). As carriers of various zoonotic diseases, they are also of medical significance (Hugh-Jones *et al.* 1995; Mills 1999; Aplin *et al.* 2003; Gratz 2006). In addition, their commonly invasive nature, make them a conservation threat to indigenous and endemic fauna (Aplin & Singleton 2003; Norris & Low 2005), ranging from birds to other rodents, especially on island ecosystems (Duplantier & Rakotondravony 1999; Thorsen *et al.* 2000; Amori & Clout 2003; Harris & Macdonald 2007; Harris 2009).

Although *Rattus* (and various other commensal rodents) are mostly associated with negative impacts on the environment as well as human livelihood, they also contribute positively as models for biomedical research especially after the complete sequencing of the rat genome (Van den Brandt *et al.* 2000; Schlick *et al.* 2006). Rats have also been used successfully to map historic human migration patterns (Matisoo-Smith & Robins 2004; Matisoo-Smith & Robins 2009).

Despite the generally global success in the survival of these rodents and their status as one of the most important vertebrate pests to date, there is still a need for more extensive studies on *Rattus* taxonomy and systematics, behaviour, ecology, habitat use, physiology, and genetics in order to develop successful management strategies for these problematic rodents (Singleton *et al.* 1999a and references therein). Because they live commensally with humans, there is also a need for knowledge on their disease vectoring capabilities, especially with regard to zoonotic diseases. Consequently, researchers have started reviewing rodent management strategies and ecological management approaches are currently being developed (Singleton *et al.* 1999b).

1.1.1 Taxonomy and systematics

The Order Rodentia represents the largest mammalian Order and consists of approximately 2277 species (Musser & Carleton 2005). The genus *Rattus* comprises

approximately 66 species allocated to the Subfamily Murinae within the Family Muridae (Musser & Carleton 2005). The Subfamily Murinae contains about 129 genera and 584 species (Musser & Carleton 2005). In spite of *Rattus* being one of the largest genera within the Class Mammalia, the taxonomy and systematics of the genus is extremely complex and poorly understood, and is in critical need of a systematic revision, particularly within the morphologically diverse *Rattus* species complex that includes *R. rattus* and *R. tanezumi* (Amori & Gippoliti 2003; Aplin *et al.* 2003; Musser & Carleton 2005).

Generally, the classification of members of the Family Muridae is uncertain largely because of the presence of sibling species and/or species complexes (Skinner & Chimimba 2005). Consequently, the family is in need of both morphological and genetic analyses (Baverstock 1983; Aplin *et al.* 2003; Musser & Carleton 2005) in order to assess both their systematics as well as their population structure (Amori & Clout 2003). Differentiating between many species of *Rattus* morphologically is difficult, not only for the layperson, but also for scientists (Robins *et al.* 2007).

Field studies of members of the genus *Rattus* are further complicated by the lack of workable identification keys based on external morphology to assist in the unequivocal identification of individuals of *R. rattus* and *R. tanezumi* (Aplin *et al.* 2003), particularly in areas where the two species occur in sympatry (Bastos *et al.* 2005; Chinen *et al.* 2005; Taylor *et al.* 2008; also see Chapter 2). Given the difficulty in distinguishing species within the *Rattus rattus* species complex based on external morphology alone, it is likely that many of the samples in natural history museum collections classified as *R. rattus* may also include *R. tanezumi*. There is thus a critical need to distinguish between these two species.

Various cytogenetic studies (Yosida 1980) have reported on the presence of four major groups within the *Rattus rattus* species complex (see Yosida 1980; Musser & Carleton 2005). These include:

1) A European group - *R. rattus* Linnaeus, 1758 with a chromosome number of either $2n = 38$ or $2n = 40$;

2) A Ceylonese group - *R. rattus* with a chromosome number of $2n = 40$;

3) A Mauritian group - *R. rattus* with a chromosome number of 2*n* = 42; and

4) An Asian group - *R. tanezumi* Temminck, 1844 with a chromosome number of 2*n* = 42.

1.1.2 Geographic distribution

Rattus rattus is native to India (Rosevear 1969; Musser & Carleton 2005) but is presently widespread and occurs as an invasive species worldwide (Yosida 1980; De Graaff 1981; Skinner & Smithers 1990; Chinen *et al.* 2005; Musser & Carleton 2005) especially in tropical and temperate regions (Yosida 1980; Skinner & Smithers 1990; Musser & Carleton 2005; Fig. 1.2). This current widespread distribution is attributed to historical trading routes and human movements between continents (Rosevear 1969; Chinen *et al.* 2005; Robins *et al.* 2007).

Rattus norvegicus originated from the colder regions of Palaearctic Asia and became more widespread as a result of human activities and now occurs worldwide as an introduced taxon (De Graaff 1981; Skinner & Smithers 1990; Musser & Carleton 2005; Fig. 1.3). It is more widespread in colder areas but can also be found in human-modified environments in warmer areas (De Graaff 1981; Skinner & Smithers 1990; Musser & Carleton 2005).

Rattus tanezumi is believed to be native to south-east Asia and also occurs as an introduced species in surrounding regions (Aplin *et al.* 2003; Musser & Carleton 2005; Fig. 1.4). Recently, based on mitochondrial DNA (mtDNA) evidence, a first record of the Asian *R. tanezumi* was found in Limpopo Province of South Africa, making it the first documented record of this species in South Africa and Africa (Bastos *et al.* 2005; Taylor *et al.* 2008). This new record therefore leads to the recognition of three species of *Rattus* in South Africa, all of which are invasive species that include: 1) *R. norvegicus*, which has traditionally been known to have a coastal distribution (Meester & Setzer 1971; De Graaff 1981; Meester *et al.* 1986; Skinner & Smithers 1990; Apps 2000; Fig. 1.5); 2) *R. rattus* which is considered to occur in most parts of South Africa (De Graaff 1981; Meester *et al.* 1986; Skinner & Smithers 1990; Apps 2000; Fig. 1.6); and 3) the newly recorded *R. tanezumi* which has so far been recorded to occur in Limpopo, Kwa-Zulu Natal and Gauteng Provinces of South Africa (Bastos *et al.* 2005; Taylor *et al.* 2008; Fig. 1.7), but may have a much wider distributional range.

1.1.3 Economic importance

Members of the genus *Rattus* have historically been problematic in agricultural systems where they cause extensive damage, resulting in great economic loss (Singleton *et al.* 1999a and references therein; Chinen *et al.* 2005; Kirsten & von Maltitz 2005) with members of the *R. rattus* species complex, in particular being responsible for crop damage (Aplin *et al.* 2003), especially *R. rattus* and *R. tanezumi* (Singleton 2003; Stenseth *et al.* 2007). In addition, members of the genus *Rattus* are in constant competition with humans for food especially in developing countries (Singleton *et al.* 1999b; Kirsten & von Maltitz 2005) such as Indonesia, where pre-harvest rice losses each year caused by rodents are estimated to be enough to feed approximately 25 million people per year (Singleton *et al.* 1999b).

In essence, members of the genus *Rattus* have a greater negative impact on human lives than that caused by any other wild vertebrate group (Aplin *et al.* 2003). Various crops such as cereals and fruits, and industrially cultivated crops such as cocoa and sugarcane are commonly targeted by members of the genus *Rattus*, resulting in significant losses (Buckle 1999). *Rattus rattus* and *R. norvegicus* are mainly responsible for the damage of stored products worldwide (Buckle 1999; Aplin *et al.* 2003; Khamphoukeo *et al.* 2003; Singleton 2003).

1.1.4 Disease transmission

Of concern is that members of the genus *Rattus* are also carriers of a number of zoonotic diseases (Chinen *et al.* 2005) that may potentially be harmful to humans and other animals and may even result in death (Mills 1999). Usually, pathogens that are virulent in humans are avirulent in their reservoir hosts (Begon 2003). Hugh-Jones *et al.* (1995) attributed at least 60 different zoonotic diseases to rodents and it is likely that the number of rodent-borne diseases with zoonotic potential has increased markedly since then. One of the best-known examples of rat-borne diseases is probably that of plague in which three major outbreaks (6th and 7th Century; 14th to 17th Century; and late 19th to early 20th Century) led to the deaths of millions of people (Begon 2003). The fleas carried by *R. rattus* are the hosts of the plague parasite, *Yersinia pestis* (Aplin *et al.* 2003). More recently, members of the genus *Rattus* have also been linked to diseases such as lyme disease (Richter *et al.* 1999), hepatitis E virus (Hirano *et al.* 2003), rat bite fever (Elliot 2007), viral haemorrhagic fever (Macdonald *et al.* 1999; Mills 1999) caused by the hanta- and arenaviruses (Mills 1999), and Lassa fever (Macdonald *et al.* 1999).

These diseases are not restricted to developing countries. For example, brown rats (*R. norvegicus*) captured from farms in southern England were infected with 13 zoonotic and 10 non-zoonotic parasites (Webster & Macdonald 1995). Zoonotic agents included ectoparasites (e.g., fleas, mites, lice), helminths (e.g., *Taenia*), bacteria (e.g., *Leptospira* spp.), protozoa (e.g., *Toxoplasma*), rickettsia (e.g., *Coxiella* that causes Q-fever) and viruses (e.g., Hantavirus) (Webster & Macdonald 1995). *Rattus norvegicus* is also a reservoir for the Seoul virus (Mills 1999). In South Africa, diseases that are known to be associated with rodents include leptospirosis, plague and toxoplasmosis (Taylor *et al.* 2008).

Rodent-borne diseases are most commonly spread via environmental aerosols or contamination with rodent fluids such as urine, saliva and faeces (Mills 1999). Transmission can also occur through a bite by an infected animal or when secretions or excretions come into contact with either open wounds or mucous membranes in humans (Mills 1999). In a study by Kirsten & von Maltitz (2005) in four villages in Limpopo Province, South Africa it was found that rats bite people during their sleep and even chew on their finger- and toe-nails.

The prevalence of two rodent-associated bacterial species of medical importance has not yet been evaluated for members of the genus *Rattus* in South Africa, namely *Helicobacter* (Goodwin *et al.,* 1989 emend. Vandamme *et al.,* 1991) and *Bartonella* (Strong *et al.,* 1915). Bacteria of the genus *Helicobacter* are gram-negative, helical curved and flagellated microaerophilic rods that infect the gastrointestinal tracts of various animals, including humans, where they cause diseases such as peptic ulcers, gastric adenocarcinoma, mucosa-associated lymphoid tissue lymphoma and chronic gastritis (Whary & Fox 2004). *Helicobacter pylori* is the type species of this genus and there are 26 formally recognised species (Ceelen *et al.* 2007). Several *Helicobacter* species have been cultured from the intestinal tracts of laboratory and wild rodents and some of these have known zoonotic potential (Handt *et al.* 1994; De Groote *et al.* 2000; Jalava *et al.* 2001; Waldenström *et al.* 2003). Members of the genus *Bartonella* are fastidious, gram-negative bacteria that infect the erythrocytes of their vertebrate hosts and are transmitted by hematophagous arthropod vectors (Birtles 2005). Of the 19 currently recognized species (Eremeeva *et al.* 2007), almost half have zoonotic potential and are associated with, amongst others, Carrion's disease, cat scratch disease, trench fever and endocarditis in humans (Anderson 1997; Fenollar & Raoult 2004; Birtles 2005).

1.1.5 Biological invasions

Rattus rattus, which has been classified as probably the most destructive rodent species on islands, has been accidentally introduced to a large number of islands by ships traveling from Europe (Amori & Clout 2003). *Rattus rattus*, *R. norvegicus* and *R. exulans*, all invasives, have all been reported to be present on all continents (except Antarctica) and on 80% of island groups worldwide (Atkinson 1985). Introductions are currently still on-going, leading to threats to the endemic fauna and flora of these island ecosystems (Amori & Clout 2003). *Rattus rattus* has also been reported to cause declines and/or extinctions of various land bird species, along with the extinctions of no less than one flightless weevil species and the last known population of the greater short-tailed bat (*Mystacina robusta*) on Big South Cape island of New Zealand in the early 1960s (Amori & Clout 2003).

It has been reported in New Zealand that the status of many threatened fauna is caused by invasive rodent species to such an extent that the eradication of rodents has led to the re-establishment of 12 lizard species as well as the protection of the threatened tuatara (Clout 2001; Towns *et al.* 2001). Hingston *et al.* (2005) also found *R. rattus* on Madagascar and it is believed these rodents originated from India through Egypt, spreading along major trade routes. Available evidence suggests a recent range expansion into southern Madagascar (Hingston *et al.* 2005), exacerbating the problem further. Apart from islands, *Rattus* is also a problematic pest species on mainlands. For example, Kirsten & von Maltitz (2005) showed that the largest number (57% and 69%) of rodents captured in two villages in northern Limpopo Province, South Africa was *R. rattus*, and rodent-associated damage was mainly caused to crops and food storage facilities in households.

1.1.6 Management

Various management strategies are presently available for the control of rodents, including ecological and physical management, the use of rodenticides and biological control (Buckle 1999; Singleton *et al.* 1999a; Singleton 2003). Rodenticides are still one of the most commonly used control methods for rodent pests (Buckle 1999). In terms of labour and expense, rodenticides are relatively cheap (Buckle 1999), hence their popularity. However, farmers in developing countries most commonly use physical methods to control rodent pests such as mechanical proofing of buildings, trapping and physical barriers that prevent access to certain areas (Singleton *et al.* 1999a).

When trying to control rodents in grasslands, an ecological approach is preferred due to the economic benefits of keeping rodenticide use to a minimum in order to avoid the contamination of the environment by chemicals whilst lowering the carrying capacity of rodents in order to have a long-term solution (Zhong *et al.* 1999). Ecological control is however highly reliant on detailed studies on the ecology (and life history) of the rodents before ecological management can be successfully applied (Zhong *et al.* 1999). Fertility control is used to manage pest populations and focuses on reducing reproduction instead of increasing the mortality of the pest animals (Chambers *et al.* 1999). Fertility control is recommended as a good control method in species with high reproductive potential (Chambers *et al.* 1999).

1.1.7 Aim of study

The present study is aimed at assessing the systematic status of *Rattus* in South Africa, evaluating its geographic distribution, and assessing zoonotic disease potential by focusing on two bacterial disease agents that these rodents harbour. The present study is based on a multidisciplinary approach that includes molecular analyses and qualitative cranial and external morphology.

1.1.8 Research questions

The following specific research questions are addressed in the present study:

1. What is the level of variation within and between species and populations based on molecular analyses?

2. Do the different species co-occur at certain localities?

3. How do the three *Rattus* species differ from each other based on qualitative cranial external morphology?

4. What is the current geographic distribution of the three invasive species of *Rattus* in South Africa?

5. Are these rodents carriers of the bacteria *Bartonella* and *Helicobacter* both of which have a zoonotic potential?

1.1.9 Justification of study

The systematics of particularly the *R. rattus* species complex (*R. rattus* and *R. tanezumi*) both of which occur in South Africa as invasive species, is poorly studied. These species are known to occur commensally with humans in South Africa where they cause disease and damage to crops, stored grain and human-made infrastructure, especially in rural communities. In spite of this, little is known about *Rattus* in South Africa in particular and the subregion and other parts of Africa in general. There is a need for zoonotic studies, especially in areas where these animals come into contact with humans, as well as information on their distribution, as available distributional records for South African *Rattus* are outdated. A multidisciplinary study has not been undertaken for these rodents in South Africa and could provide useful information (especially with regard to diseases and morphology) that could be used by the public and agricultural- and health authorities in areas that are affected by members of the genus *Rattus*. This study will also provide a comparison on the impacts of invasive species compared to indigenous species based on disease vectoring capabilities as well as impacts on human life with regard to agriculture, crop and infrastructure damage. This study could also serve as a model for similar studies on other invasive problem species of rodents.

1.1.10 Dissertation outline

The first part of the study (Chapter 2) will assess the nature and extent of variation within and between *Rattus* species occurring in South Africa based on a mitochondrial DNA analysis of the *d-loop* gene region. Results obtained will then be compared to those of a similar study based on the *cytochrome b* gene region in order to determine which gene region gives best resolution capabilities when studying closely related species groups.

Chapter 3 will assess qualitative external and cranial morphology, of genetically identified individuals from Chapter 2 in an attempt to identify taxonomically useful characters that could be used to differentiate between species of *Rattus* in South Africa. These characters may assist in the development of an identification key which will be useful to both laymen and scientists.

Chapters 4 and 5 are dedicated to the epidemiological part of the study and the assessment the bacterial prevalence of *Bartonella* (Chapter 3) and *Helicobacter* (Chapter 4), both of which have zoonotic potential. Samples are screened for bacterial presence in order to determine whether members of *Rattus* are carriers of these diseases and to identify whether the species of bacteria that are present pose a

significant zoonotic risk. The last chapter (Chapter 6) concludes the study with a general discussion of the major findings in this multidisciplinary analysis of the genus *Rattus* in South Africa.

1.2 REFERENCES

AMORI, G. & CLOUT, M. 2003. Rodents on islands: a conservation challenge. *In*: Rats, mice and people: rodent biology and management, (*eds*) G. R. Singleton, L. A. Hinds, C. J. Krebs & D. M. Spratt, Australian Centre for International Agricultural Research, Canberra. Pp. 63-68.

AMORI, G. & GIPPOLITI, S. 2003. How do rodent systematic affect conservation priorities? *In*: Rats, mice and people: rodent biology and management, (*eds*) G. R. Singleton, L. A. Hinds, C. J. Krebs & D. M. Spratt, Australian Centre for International Agricultural Research, Canberra. Pp. 112-114.

AMORI, G., HUTTERER, R., KRYŠTUFEK, B., YIGIT, N., MITSAIN, G. & MUÑOZ, L. J. P. 2008. *Rattus rattus*. *In*: IUCN 2009. IUCN Red List of Threatened Species. Version 2009.1. www.iucnredlist.org. Downloaded on 26 June 2009.

ANDERSON, B. E. & NEUMAN, M. A. 1997. Bartonella spp. as emerging human pathogens. *Clinical Microbiology Reviews* **10**: 203-219.

APLIN, K. P. & SINGLETON, G. R. 2003. Balancing rodent management and small mammal conservation in agricultural landscapes: challenges for the present and the future. *In*: Rats, mice and people: rodent biology and management, (*eds*) G. R. Singleton, L. A. Hinds, C. J. Krebs & D. M. Spratt, Australian Centre for International Agricultural Research, Canberra. Pp. 80-88.

APLIN, K. P., CHESSER, T., & TEN HAVE, J. 2003. Evolutionary biology of the genus *Rattus*: profile of an archetypal rodent pest. *In*: Rats, mice and people: rodent biology and management, (*eds*) G. R. Singleton, L. A. Hinds, C. J. Krebs & D. M. Spratt, Australian Centre for International Agricultural Research, Canberra. Pp. 487-498.

APPS, P. 2000. Smithers's mammals of Southern Africa. A field guide. Struik Publishers, Cape Town. p. 74.

ATKINSON, I. A. E. 1985. The spread of commensal species of *Rattus* to oceanic islands and their effects on island avifaunas. *In*: Conservation of island birds (*eds*) P. J. Moors, International Council for Bird Preservation Technical Publication No. 3, Cambridge, United Kingdom. Pp. 35-81.

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. *South African Society for Veterinary Epidemiology and Preventive Medicine*. Proceedings Pretoria, 11-12 August 2005. Pp. 78-83.

BAVERSTOCK, P. R., ADAMS, M., MAXSON, L. R. & YOSIDA, T. H. 1983. Genetic differentiation among karyotypic forms of the black rat, *Rattus rattus*. *Genetics* **105**: 969-983.

BEGON, M. 2003. Disease: health effects on humans, population effects on rodents. *In*: Rats, mice and people: rodent biology and management, (*eds*) G. R. Singleton, L. A. Hinds, C. J. Krebs & D. M. Spratt, Australian Centre for International Agricultural Research, Canberra. Pp. 13-24.

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

BUCKLE, A. P. 1999. Rodenticides – Their role in rodent pest management in tropical agriculture. *In*: Ecologically-based Rodent Management (*eds*) G. R. Singleton, H. A. Hinds, H. Leirs & Z. Zhang, Australian Centre for International Agricultural Research, Canberra. Pp. 163-177.

CEELEN, L. M., HAESEBROUCK, F., DUCATELLE, R. & DECOSTERE, A. 2007. The occurrence and clinical significance of enterohepatic *Helicobacter* species in laboratory rodents. *Vlaams Diergeneeskundig Tijdschrift*, **76**: 103- 116.

CHAMBERS, L. K., LAWSON, M. A. & HINDS, L. A. 1999. Biological control of rodents – the case for fertility control using immunocontraception. *In*: Ecologically-based Rodent Management (*eds*) G. R. Singleton, H. A. Hinds, H. Leirs & Z. Zhang, Australian Centre for International Agricultural Research, Canberra. Pp. 215-242.

CHINEN, A. A., SUZUKI, H., APLIN, K. P., TSUCHIYA, K. & SUZUKI, S. 2004. Preliminary genetic characterization of two lineages of black rats (*Rattus rattus* sensu lato) in Japan, with evidence for introgression at several localities. *Genes Genetic Systematics* **80**: 367–375.

CLOUT, M. 2001. Where protection is not enough: active conservation in New Zealand. *TRENDS in Ecology & Evolution* **16**: 415-416.

DE GRAAFF, G. 1981. The rodents of southern Africa: notes on their identification, distribution, ecology and taxonomy. Butterworth, Durban. Pp. 218-228.

DE GROOTE, D., DUCATELLE, R. & HAESEBROUCK, F. 2000. Helicobacters of possible zoonotic origin: A review. *Acta Gastroenterologica Belgica* **63**: 380 –387.

DUPLANTIER, J-M. & RAKOTONDRAVONY, D. 1999. The rodent problem in Madagascar: agricultural pest and threat to human health. *In*: Ecologically-based Rodent Management (*eds*) G. R. Singleton, H. A. Hinds, H. Leirs & Z. Zhang, Australian Centre for International Agricultural Research, Canberra. Pp. 441-459.

ELLIOT, S. P. 2007. Rat bit fever and *Streptobacillus moniliformis*. *Clinical Microbiology Reviews* **20**: 13-22.

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. & KOEHLER, J. E. 2007. Bacteremia, fever, and splenomegaly caused by a newly recognised *Bartonella* species. *The New England Journal of Medicine* **356**: 2381-2387.

FENOLLAR, F. & RAOULT, D. 2004. Molecular genetic methods for the diagnosis of fastidious microorganisms. *APMIS* **112**: 785-807.

GRATZ, N. G. 2006. Vector- and Rodent-Borne Diseases in Europe and North America: Distribution, Public Health Burden, and Control. Cambridge University Press, Cambridge. 410 pp.

HANDT, L. K., FOX, J. G., DEWHIRST, F. E., FRASER, G. J., PASTER, B. J., YAN, L. L., ROZMIAREK, H., RUFO, R. & STALIS, I. H. 1994. *Helicobacter pylori* isolated from the domestic cat: public health implications. *Infection and Immunity* **62**: 2367-2374.

HARRIS, D. B. 2009. Review of negative effects of introduced rodents on small mammals on islands. *Biological Invasions* **11**: 1611-1630.

HARRIS, C. B. & MACDONALD, D. W. 2007. Interference competition between introduced black rats and endemic Galápagos rice rats. *Ecology* **88**: 2330-2344.

HEANEY, L. & MOLUR, S. 2008. *Rattus tanezumi*. *In*: IUCN 2009. IUCN Red List of Threatened Species. Version 2009.1. www.iucnredlist.org. Downloaded on 26 June 2009.

HINGSTON, M., GOODMAN, S. M., GANZHORN, J. U. & SOMMER, S. 2005. Reconstruction of the colonization of southern Madagascar by introduced *Rattus rattus*. *Journal of Biogeography* **32**: 1549–1559.

HIRANO, M., DING, X., LI, T-C., TAKEDA, N., KAWABATA, H., KOIZUMI, N., KADOSAKA, T., GOTO, I., MASUZAWA, T., NAKAMURA, M., TAIRA, K., KUROKI, T., TANIKAWA, T., WATANABE, H. & ABE, K. 2003. Evidence for

widespread infection of hepatitis E virus among wild rats in Japan. *Hepatology Research* **27**: 1-5.

HUGH-JONES, B. E., HUBBERT, W. T., & HAGSTAD, H. V. 1995. Zoonoses: recognition, control, and prevention. Iowa State University Press, Ames. 384 pp.

IUCN RED DATA LIST 2008. Genus *Rattus*. www.iucnredlist.org. Downloaded on 26 June 2009.

JALAVA, K., ON, S. L. W., HARRINGTON, C. S., ANDERSEN, L. P., HÄNNINEN, M-L. & VANDAMME, P. 2001. A cultured strain of "*Helicobacter heilmannii*", a human gastric pathogen, identified as *H. bizzozeronii*: Evidence for zoonotic potential of *Helicobacter*. *Emerging Infectious Diseases* **7**: 1036-1038.

JALAVA, K., KAARTINEN, M., UTRIAINEN, M., HAPPONEN, I. & HÄNNINEN, M-L. 1997. *Helicobacter salomonis* sp. nov., a canine gastric *Helicobacter* sp. related to *Helicobacter felis* and *Helicobacter bizzozeronii*. *International Journal of Systematic Bacteriology* **47**: 975-982.

KHAMPHOUKEO, K., DOUANGBOUPHA, B., APLIN, K. P. & SINGLETON, G. R. 2003. Pest and non-pest rodents in the upland agricultural landscape of Laos: a progress report. *In*: Rats, mice and people: rodent biology and management, (*eds*) G. R. Singleton, L. A. Hinds, C. J. Krebs & D. M. Spratt, Australian Centre for International Agricultural Research, Canberra. Pp. 284-289.

KIRSTEN, F. & VON MALTITZ, E. 2005. Technology transfer and promotion of ecologically-based and sustainable rodent control strategies in South Africa. Final technical report. Agricultural Research Council – Plant Protection Research Institute, Private Bag X 134, Queenswood 0121, Republic of South Africa. http://www.fao.org/docs/eims/upload/agrotech/1980/R8190_FTR.pdf. Downloaded on 13 June 2009.

LONG, J. L. 2003. Introduced mammals of the world: their history, distribution and influence. CSIRO Publishing, Collingwood, Australia. 589 pp.

MACDONALD, D. W., MATHEWS, F. & BERDOY, M. 1999. The behaviour and ecology of *Rattus norvegicus*: from opportunism to Kamikaze tendencies. *In*: Ecologically-based Rodent Management (*eds*) G. R. Singleton, H. A. Hinds, H. Heirs & Z. Zhang, Australian Centre for International Agricultural Research, Canberra. Pp. 49-80.

MATISOO-SMITH, E. & ROBINS, J. H. 2004. Origins and dispersal of Pacific peoples: Evidence from mtDNA phylogenies of the Pacific rat. *Proceedings of the National Academy of Sciences of the USA* **101**: 9167-9172.

MATISOO-SMITH, E. & ROBINS, J. 2009. Mitochondrial DNA evidence for the spread of Pacific rats through Oceania. *Biological Invasions* **11**: 1521-1527.

MEESTER, J. & SETZER, H. W. 1971. The mammals of Africa: an identification manual. Smithsonian Institution Press, Washington DC. 505 pp.

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

MILLS, J. N. 1999. The role of rodents in emerging human disease: examples from the Hantaviruses and Arenaviruses. *In*: Ecologically-based Rodent Management (*eds*) G. R. Singleton, H. A. Hinds, H. Leirs & Z. Zhang, Australian Centre for International Agricultural Research, Canberra. Pp. 134-160.

MUSSER, G. G., & CARLETON, M. D. 2005. Family Muridae. *In*: Mammal Species of the World, (eds) D. E. Wilson & D. M. Reeder. The Johns Hopkins Univeristy Press, Baltimore. Pp 894–1531.

NORRIS, A. & LOW, T. 2005. Review of the management of feral animals and their impact on biodiversity in the Rangelands: A resource to aid NRM planning, Pest Animal Control CRC Report 2005, Pest Animal Control CRC, Canberra. Pp. 1 -239.

PUTMAN, R. J. 1989. Mammals as pests. Chapman & Hall Ltd, London. 288 pp.

RICHTER, D., ENDEPOLS, S., OHLENBUSCH, A., EIFFERT, H., SPIELMAN, A. & MATUSCHKA, F-R. 1999. Genospecies diversity of lyme disease spirochetes in rodent reservoirs. *Emerging Infectious Diseases* **5**: 291-296.

ROBINS, J. H., HINGSTON, M., MATISOO-SMITH, E. & ROSS, H. A. 2007. Identifying *Rattus* species using mitochondrial DNA. *Molecular Ecology Notes* **7**: 717-729.

ROSEVEAR, D.R. 1969. *The rodents of West Africa*. Trustees of the British Museum (Natural History), London, **677**: 1-604.

RUEDAS, L. 2008. *Rattus norvegicus*. *In*: IUCN 2009. IUCN Red List of Threatened Species. Version 2009.1. www.iucnredlist.org. Downloaded on 26 June 2009.

SCHLICK, N. E., JENSEN-SEAMAN, M. I., ORLEBEKE, K., KWITEK, A. E., JACOB, H. J. & LAZAR, J. 2006. Sequence analysis of the complete mitochondrial DNA in 10 commonly used inbred rat strains. *American Journal of Physiology and Cell Physiology*, **291**, 1183-1192.

SINGLETON, G., HINDS, L., LEIRS, H. & ZHANG, Z. (Eds). 1999a. *Ecologically based rodent management.* Australian Centre for International Agricultural Research, Canberra, 494 pp.

SINGLETON, G. R., LEIRS, H., HINDS, L. A., & ZHANG, Z. 1999b. Ecologically- based management of rodent pests – Re-evaluating our approach to an old problem. *In*: Ecologically-based Rodent Management (*eds*) G. R. Singleton, H. A. Hinds, H. Leirs & Z. Zhang, Australian Centre for International Agricultural Research, Canberra. Pp. 17-29.

SINGLETON, G. R., HINDS, L. A., KREBS, C. J. & SPRATT, D. M. 2003. *Rats, mice and people: Rodent biology and management*. Australian Centre for International Agricultural Research, Canberra. 564 pp.

SINGLETON, G. R. 2003. Impacts of rodents on rice production in Asia. http://www.irri.org/publications/discussion/pdfs/Singleton.pdf. Accessed on 18 August 2009.

SKINNER, J. D. & CHIMIMBA, C. T. 2005. *The Mammals of the Southern African subregion*. Cambridge University Press. Cape Town. 872 pp.

SKINNER, J. D. & SMITHERS, R. H. N. 1990. *The Mammals of the Southern African subregion*. CTP Book Printers, Cape Town. 736 pp.

STENSETH, N. C., LEIRS, H., SKONHOFT, A., DAVIS, 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. & WAN, X. 2007. Mice and rats: the dynamics and bio-economics of agricultural rodent pests. *Frontiers in Ecology and the Environment* **7**: 1-12.

TAYLOR, P. J., ARNTZEN, L., HAYTER, M., ILES, M., FREAN, J. & 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.

THORSEN, M., SHORTEN, R., LUCKING, R. AND LUCKING, V. 2000. Norway rats (*Rattus norvegicus*) on Fregate Island, Seychelles: the invasion; subsequent eradication attempts and implications for the island's fauna. *Biological Conservation* **96**: 133-138.

TOWNS, D. R., DAUGHERTY, C. H. & CREE, A. 2001. Raising the prospects for a forgotten fauna: a review of 10 years of conservation effort for New Zealand reptiles. *Biological Conservation* **99**: 3-16.

VAN DEN BRANDT, J., KOVÁCS, P. & KLÖTING, I. 2000. Wild rats (*Rattus norvegicus*) for mapping of disease-resistant genes. *Journal of Experimental Animal Science* **41**: 57-60.

WALDENSTRÖM, J. ON, S. L. W., OTTVALL, R., HASSELQUIST, D., HARRINGTON, C. S. & OLSEN, B. 2003. Avian reservoirs and zoonotic potential of the emerging human pathogen *Helicobacter canadensis*. *Applied and Environmental Microbiology* **69**: 7523-7526.

WEBSTER, J. P. & MACDONALD, D. W. 1995. Parasites of wild brown rats (*Rattus norvegicus*) on UK farms. *Parasitology* **111**: 247-255.

YOSIDA, T. H. 1980. Cytogenetics of the black rat: karyotype evolution and species differentiation. University of Tokyo Press, Tokyo. 256 pp.

ZHONG, W., WANG, M & WAN, X. 1999. Ecological management of Brandt's vole (*Microtus brandti*) in Inner Mongolia, China. *In*: Ecologically-based Rodent Management (*eds*) G. R. Singleton, H. A. Hinds, H. Leirs & Z. Zhang, Australian Centre for International Agricultural Research, Canberra. Pp. 199-214.

1.3 FIGURE LEGENDS

Fig. 1.1 A map showing the world distribution of the genus *Rattus* (black shading; adopted from Long 2003; IUCN Red List data 2008).

Fig. 1.2 A map showing the world distribution of *Rattus rattus* (black shading; adopted from Long 2003; IUCN Red List data 2008; Amori *et al.* 2008).

Fig. 1.3 A map showing the world distribution of *Rattus norvegicus* (black shading; adopted from Long 2003; IUCN Red List data 2008; Ruedas 2008).

Fig. 1.4 A map showing the world distribution of *Rattus tanezumi* (black shading; adopted from Long 2003; IUCN Red List data 2008; Heaney & Molur 2008).

Fig. 1.5 A map showing the historical distribution of *Rattus rattus* in southern Africa (black shading; adopted from Skinner & Smithers 1990).

Fig. 1.6 A map showing the historical distribution of *Rattus norvegicus* in southern Africa (black shading; adopted from Skinner & Smithers 1990).

Fig. 1.7 Map showing the four sampling localities in Limpopo Province, and one locality in Kwa-Zulu Natal, South Africa where *Rattus tanezumi* was recorded for the first time in South Africa (and Africa) (red dots; Bastos *et al.* 2005; Taylor *et al.* 2008).

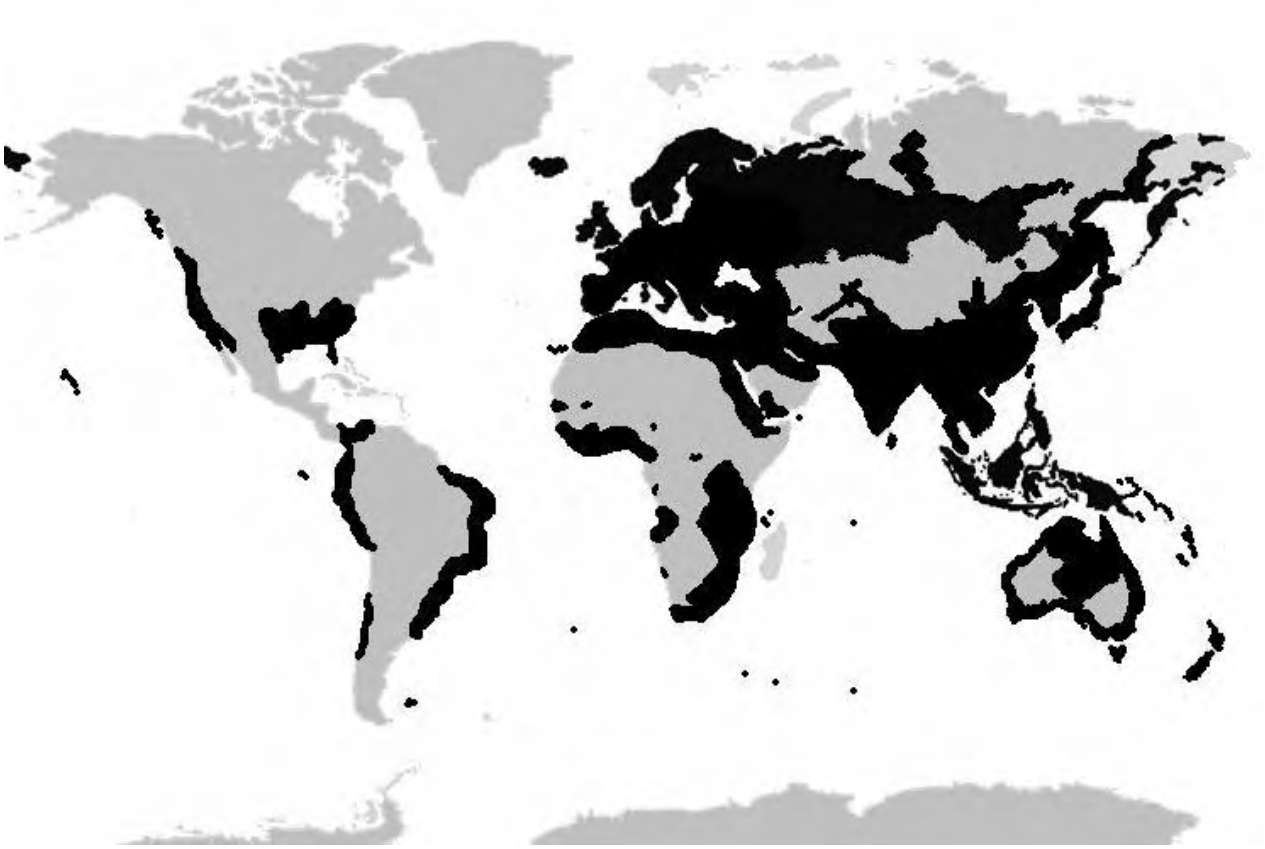

Fig. 1.1

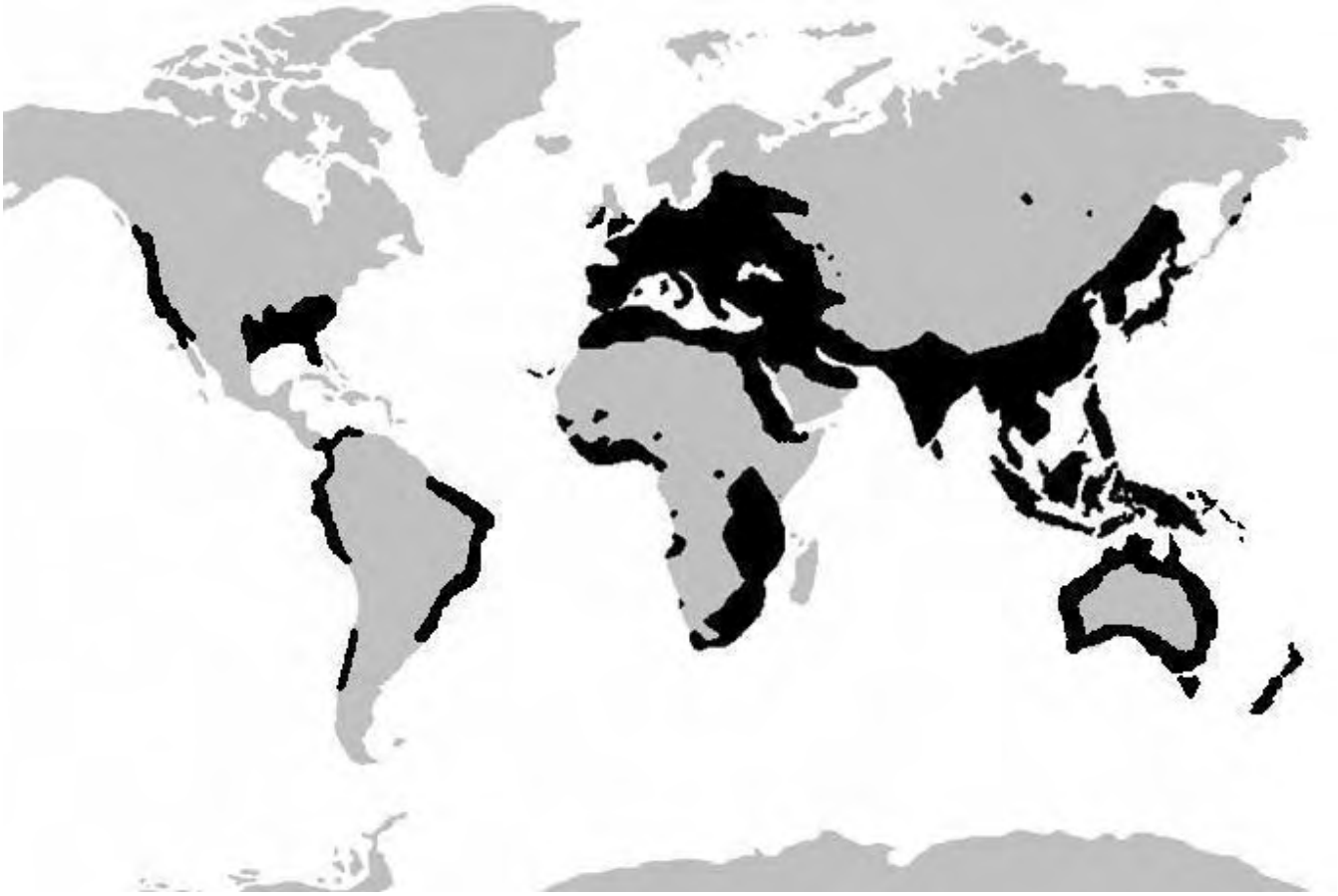

Fig. 1.2

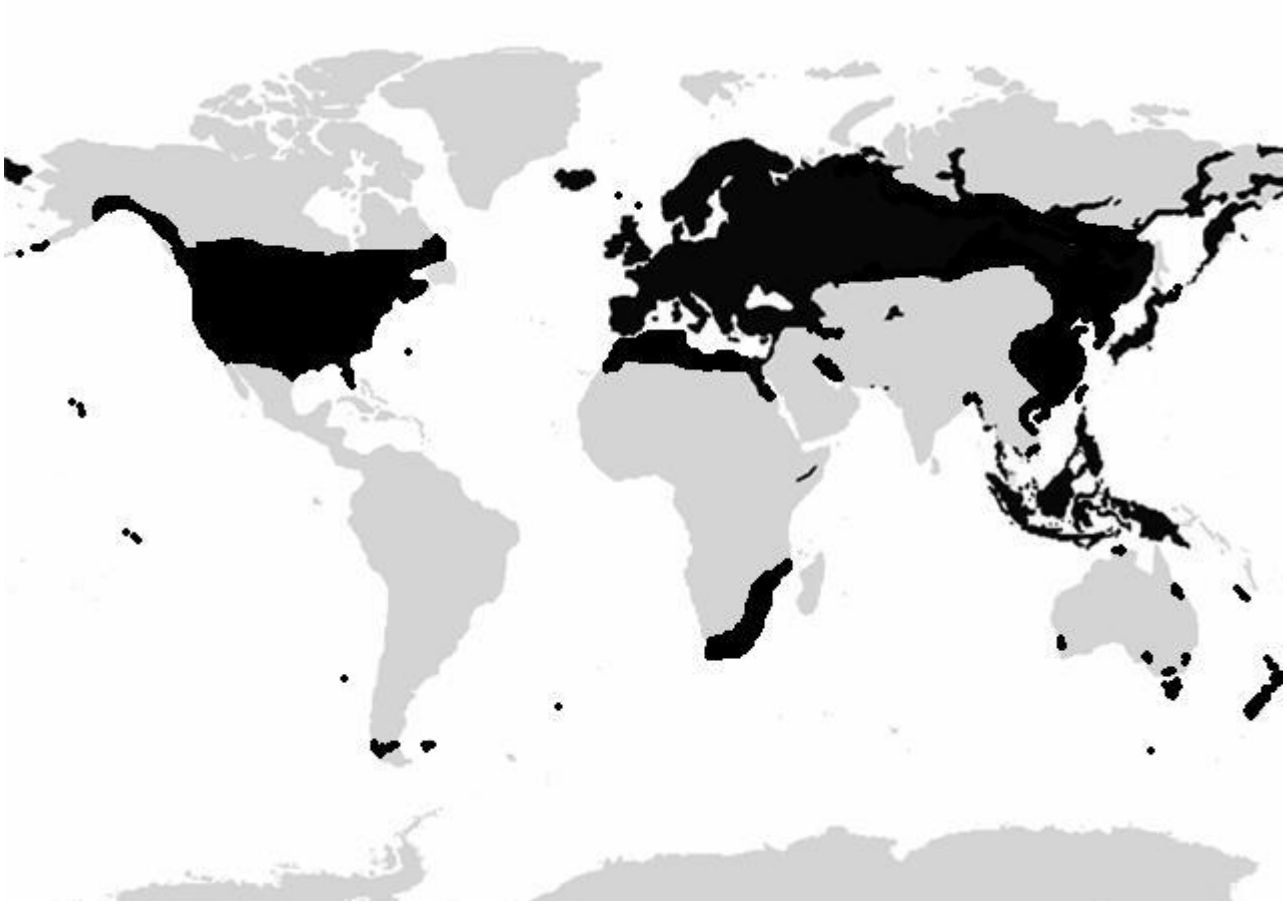

Fig. 1.3

Fig. 1.4

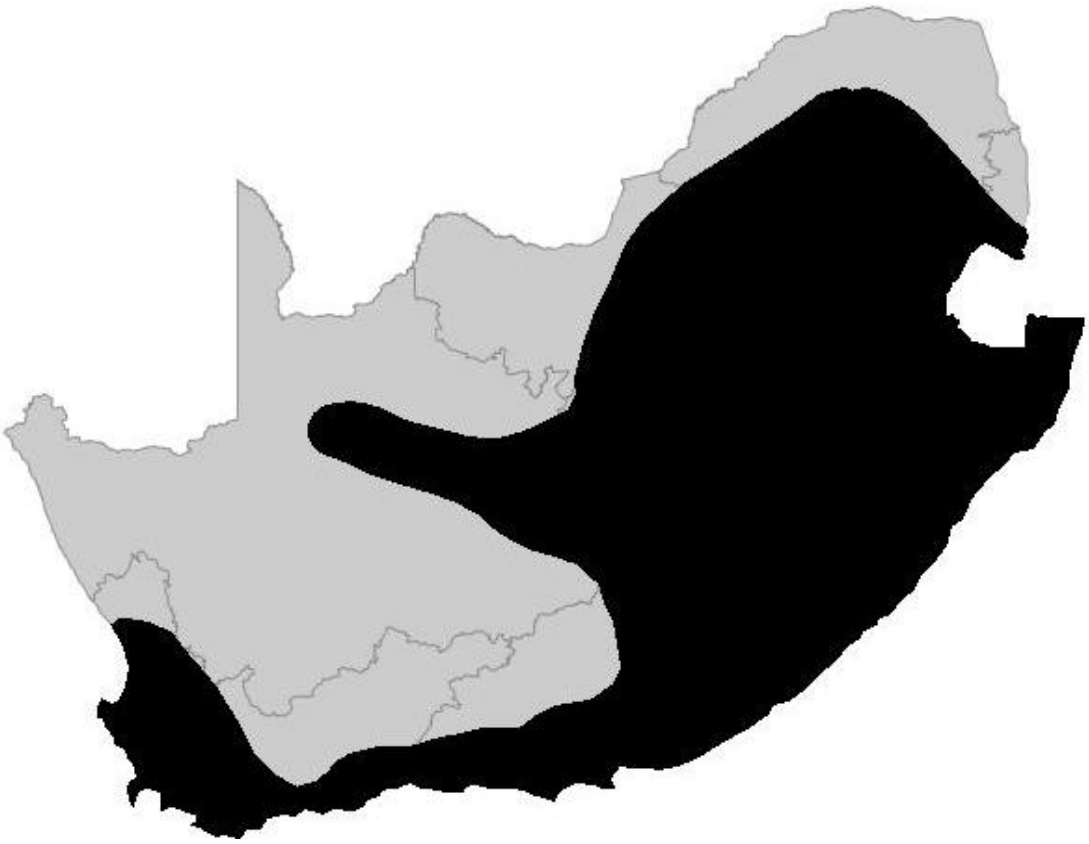

Fig. 1.5

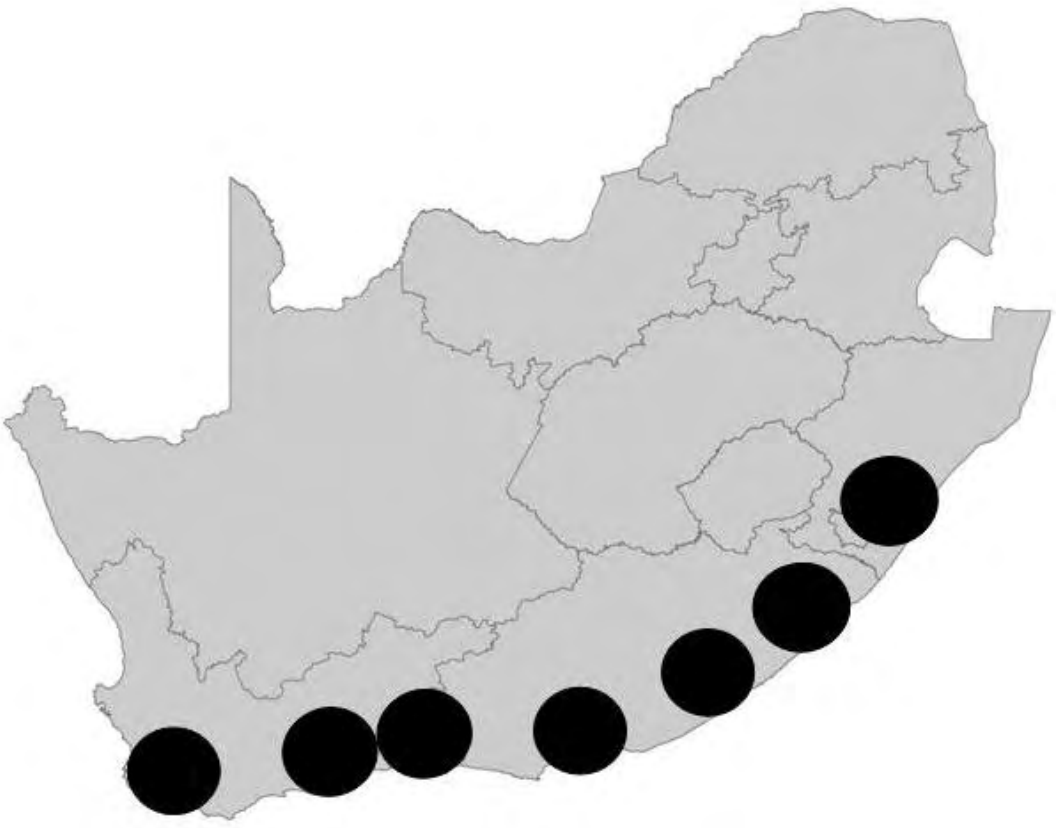

Fig. 1.6

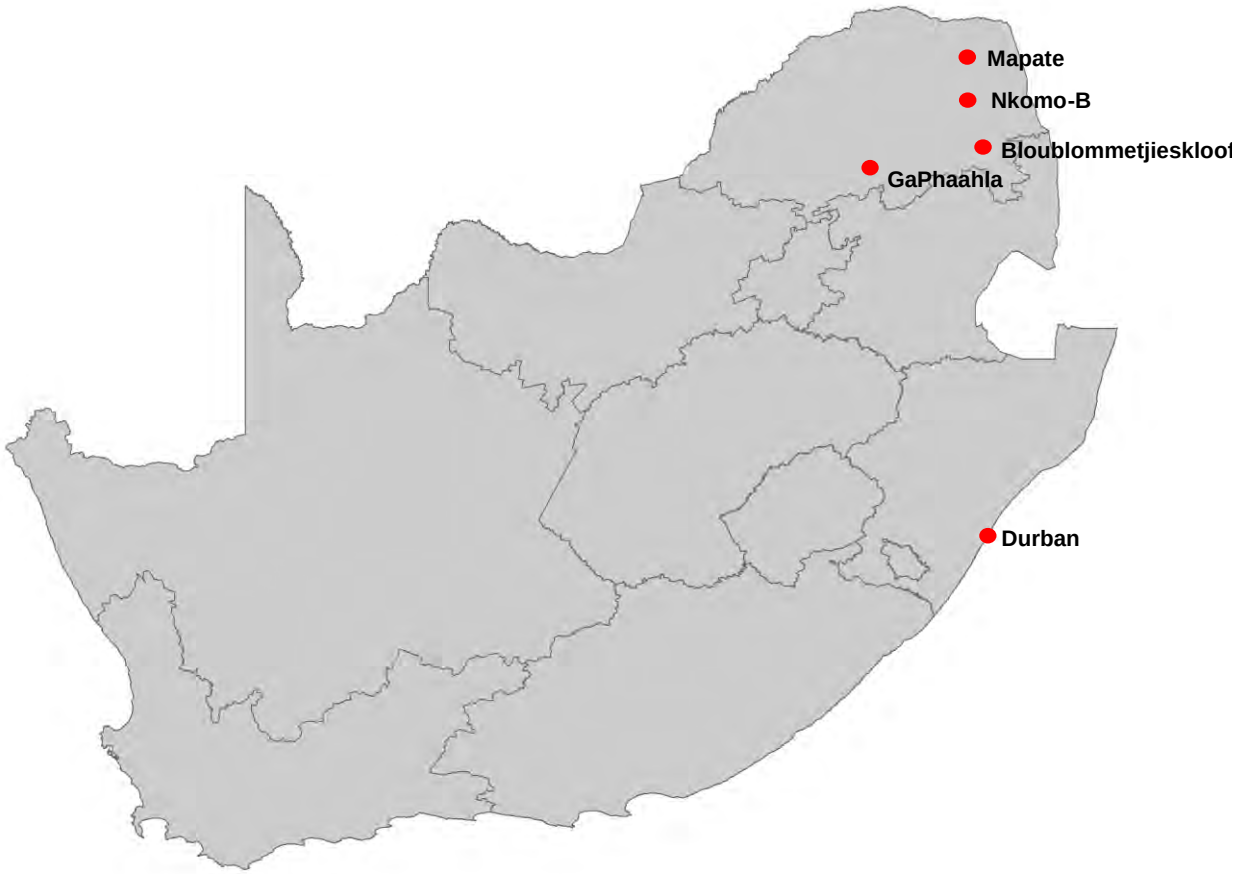

Fig. 1.7

CHAPTER 2

A MITOCHONDRIAL DNA ANALYSIS OF INVASIVE COMMENSAL *RATTUS* SPECIES (RODENTIA: MURIDAE) FROM SOUTH AFRICA

2.1 ABSTRACT

Members of the genus *Rattus* Fischer, 1803 are among the most successful invasive vertebrate pests, causing damage to agricultural products and infrastructure, worldwide. In South Africa, there are three invasive species, namely, *R. rattus* Linnaeus, 1758, *R. norvegicus* Berkenhout, 1769, and the recently recorded *R. tanezumi* Temminck, 1844, an otherwise south-east Asian endemic, which represents the first record of this species in South Africa (and Africa). Despite the group's widespread occurrence, *Rattus* taxonomy and systematics, patterns of geographic distribution, and population structure in many parts of the world, including South Africa, remain largely unknown. Consequently, the present study used the mitochondrially-encoded D-loop region in an attempt to gain an insight into these under-studied aspects of this group of invasive rodents in South Africa. To this end, partial D-loop sequences were generated for 149 *Rattus* specimens in this study. In addition, full-length cytochrome *b* (cyt *b*) gene sequences were also generated for 22 specimens, and complemented with previously generated cyt *b* data, so that comparative levels of intra-specific resolution could be assessed for the two mtDNA gene regions. The results revealed low levels of intra-specific D-loop variation (four haplotypes for *R. rattus*; one for *R. tanezumi* and two for *R. norvegicus*) suggesting a relatively recent introduction of this group of rodents into South Africa. Similar results were obtained from the cyt *b* gene analysis. However for *R. tanezumi*, two haplotypes, instead of just one were recovered. The results indicate that the traditionally recognized distributions of *R. rattus* and *R. norvegicus* in South Africa. In this study, *R. tanezumi* was found to occur sympatrically with *R. rattus* and with *R. norvegicus*, at three and one of the twelve sampling localities, respectively, however evidence for co-occurrence of *R. rattus* and *R. norvegicus* at any of the inland localities evaluated in this study was lacking.

Key words: *Rattus*; D-loop; South Africa; sympatry; invasive species

2.2 INTRODUCTION

The genus *Rattus* Fischer, 1803 is one of the largest mammalian genera (Aplin *et al.* 2003; Musser & Carleton 2005). Worldwide in distribution, the genus represents one of the most successful vertebrate pests that significantly affect human livelihood (Aplin *et al.* 2003). The genus which is estimated to have originated approximately 3.5 million years ago (Robins *et al.* 2008), have long been of medical concern due to the ability of *Rattus* to transmit zoonotic diseases such as bubonic plague, or the black death which caused the deaths of an estimated quarter to a third of the European population from 1347 to 1352 (Davis 1986). They also cause damage to agricultural products and infrastructure (Mills 1999).

Rodents overall but especially members of *Rattus* have however proved invaluable as models for the studies of historical human movements (Matisoo-Smith & Robins 2004; Matisoo-Smith & Robins 2009) and also as experimental animals for biomedical research (Schlick *et al.* 2006).

Nevertheless, this highly complex genus is also one of the least understood (Aplin *et al.* 2003), and has surprisingly received little attention from geneticists and taxonomists (Yosida 1980; Baverstock *et al.* 1983; Aplin *et al.* 2003; Musser and Carleton 2005). A high level of misidentification takes place within the genus as a result of members looking very similar and therefore not being distinguishable morphologically, with certainty. In order to manage rodents effectively, as well as the diseases that they transmit, it is very important to identify individuals genetically to confirm their specific status (Bastos *et al.* 2005). According to Aplin and co-workers (2003) the greatest need for taxonomic revision lies within the *Rattus rattus* species complex which consists mainly of *R. rattus* (Linnaeus, 1758) and *R. tanezumi* (Temminck, 1844). Attention should therefore focus not only on taxonomy, but also on phylogenetic associations of species and populations in order to clearly distinguish species and determine their taxonomic status (Amori and Clout 2003).

The presence of two invasive *Rattus* species in South Africa has long been acknowledged in literature (Meester & Setzer 1971; De Graaff 1981; Meester *et al.* 1986; Skinner & Smithers 1990), namely *R. rattus* which occurs throughout the country (De Graaff 1981; Skinner & Smithers 1990; Apps 2000; Fig. 2.1) and *R. norvegicus* which is considered to be confined to harbours and coastal towns (De Graaff 1981; Skinner & Smithers 1990; Apps 2000; Fig. 2.2). However, in 2005, the first report of a genetically

idenfied *R. tanezumi* was made in the Limpopo Province, and the species was subsequently found at additional localities in the northeastern and eastern parts of South Africa (Bastos *et al.* 2005; Taylor *et al.* 2008; Fig. 2.3), bringing the total number of invasive *Rattus* species in South Africa to three.

A number of features of mitochondrial DNA (mtDNA) make it particularly amenable to assessing evolutionary relationships among populations (Brown *et al.*1982; Irwin *et al.* 1991; Stewart & Baker 1994; Pesole *et al.* 1999; Gissi *et al.* 2000). Phylogenetic relationships, intra- and inter-specific variation and population structure of diverse taxa have been studied using mtDNA (Page & Holmes 1998; Pesole *et al.* 1999). Animal mitochondrial DNA is a maternally-inherited, non-recombining, circular molecule that mutates 5 to 10-fold faster than nuclear DNA (Brown *et al.* 1979; Brown *et al.* 1982; Moritz *et al.* 1987; Pesole *et al.* 1999; Gissi *et al.* 2000; Larizza *et al.* 2002). The non-coding D-loop region is particularly valuable for population-level studies as it is the most variable region in the mitochondrial genome due to the high rate at which substitutions, insertions, deletions and duplications occur (Aquadro & Greenberg 1982; Saccone *et al.* 1991). As the D-loop region has been used successfully to assess genetic diversity in a wide range of species (Nagata *et al.* 1998; Hirota *et al.* 2004; Belay & Mori 2006; Song-Jia *et al.* 2006), it will be used in this study to assess phylogenetic relationships among the three invasive species of *Rattus* in South Africa. The results obtained will be compared with those of a cytochrome *b* (cyt *b*) dataset and phylogeny generated in a separate study (Bastos *et al.* unpublished), and complemented with cytochrome *b* (cyt *b*) gene data generated in this study for individuals not characterised previously.

The cyt *b* gene has the advantage of having both slow and fast evolving regions (Irwin *et al.* 1991). These regions are both conserved and variable which in turn makes it possible to assess deep divergences as well as more recent ones. Cyt *b* is often used in combination with other gene regions in murid rodent studies to resolve phylogenetic relationships and has for example, been used to study *Peromyscus* (Tiemann-Boege *et al.* 2000), and has been useful in resolving relationships among 18 murid rodent species (Martin *et al.* 2000), as well as within groups such as *Praomys* (Lecompte *et al.* 2002), *Apodemus* (Michaux *et al.* 2002; Suzuki *et al.* 2003) and *Mus* (Suzuki *et al.* 2004). As cyt *b* is a coding gene, it more readily allows for the establishment of positional homology with unequivocal alignments and has also been shown to be suitable for studying evolutionary events that have taken place within the past 20 million years (MY) (Irwin *et al.* 1991).

This study expands on the earlier work by Bastos *et al.* (2005) and Bastos *et al.* (unpublished) by sampling *Rattus* from additional localities and by characterising a coding, as well as a non-coding gene region encoded within the mitochondrial genome, in order to assess the degree of inter- and intra-specific variation in *Rattus* species occurring in South Africa. This will be achieved by pooling cyt *b* gene data generated in the present study with that generated previously, and by characterising *de novo*, the non-coding D-loop region of all *Rattus* 149 specimens sampled. In so doing, valuable insights into the distribution of the three *Rattus* species across the country will be gained, particularly as up until recently, available data had been generated largely from opportunistic, rather than focussed sampling. Genetically-derived species identification will be valuable for identifying areas in which different *Rattus* species occur sympatrically.

2.3 MATERIALS AND METHODS

2.3.1 Study area and sampling

The specimens of *Rattus* (*n* = 149) examined in the present study were collected from 12 sampling localities encompassing a wide range of habitats, in three provinces in South Africa, namely the Limpopo, Gauteng, and KwaZulu-Natal Provinces. The collecting localities of all these specimens which included rural villages, townships, and some metropolitan areas within the cities of Pretoria and Johannesburg (Gauteng Province) are presented in Fig. 2.4, while sample sizes and geographic coordinates of these localities are summarised in Table 2.1. The specimens used in the mtDNA analysis in the present study are the same samples used in the qualitative cranial and external morphological (Chapter 3) and disease (Chapter 4 & 5) components of this multidisciplinary study of *Rattus* from South Africa.

Animals were obtained through a number of approaches as follows: 1) Live-trapping using Sherman traps (H.B. Sherman Traps Inc. Florida, U.S.A.) baited with a mixture of peanut butter, syrup, oatmeal and fish oil; 2) Samples obtained through a European Commission/DFID-funded community-participatory research project on rodents in southern Africa where 10 snap traps were placed in 10 community households in a number of villages and inspected daily; 3) Samples were obtained resourcefully from pest control companies during their routine extermination programmes at facilities such as the O.R. Tambo International Airport; and 4) Samples obtained opportunistically from the general public.

After capture, during transportation and in the laboratory, live-trapped animals were kept in polyurethane cages with wood shavings provided as bedding and mouse pellets and water provided *ad libitum* as per the guidelines of the American Society of Mammalogists (ASM; http://www.mammalogy.org/committees/index.asp. Halothane inhalation was used to euthanize live animals. Animals were dissected and the livers were removed and stored in either absolute ethanol or frozen at -20° C for subsequent molecular analysis. Voucher specimens were prepared using standard natural history museum procedures for mammal specimens and will be deposited in the mammal reference collection of the Transvaal Museum (TM) of the Northern Flagship Institute (NFI), Pretoria, South Africa and the Durban Natural Science Museum, South Africa.

2.3.2 DNA extraction and amplification

Total genomic DNA was extracted from 149 liver samples using either the Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minnesota, U.S.A.) or the Roche High Pure PCR Template Preparation Kit (Roche Applied Systems, Germany), according to the supplier-specified protocols for extraction from animal tissue. An overnight proteinase K digestion was performed at 55° C irrespective of which kit was used, and the DNA was eluted in a final volume of 50µl. The hypervariable region I (HVR I) of the displacement loop (D-loop) was targeted with primers L15925 5'-TACACTGGTCTTGTAAACC-3' and H16499 5'-CTTGAAGTAGGAACCAGAT-3' (modified from Kocher *et al.* 1989) and resulted in amplification of a region of approximately 501 base pairs (bp).

For the cyt *b* amplification, primers L14724 (5'-TGAYATGAAAAAYCATCGTTG-3'; Irwin *et al.* 1991) and H15915-Mus (5'-CATTTCAGGTTTACAAGAC-3'; Russo 2003) were used to amplify a ~1.2 kilo bp region. The D-loop gene region of all 149 samples was sequenced and the results were compared to cyt *b* sequences generated in a previous study (Bastos *et al*. unpublished) and complemented with additional cyt *b* gene sequences generated *de novo* in the current study for 22 samples for which no cyt *b* data were available.

Thermal cycling conditions for the D-loop region were: initial denaturation at 94° C for 5 minutes; followed by 35 cycles of 94° C for 30 s, annealing at 50° C for 30 s and extension at 72° C for 30 s. Final extension was done at 72° C for 7 minutes. For cyt *b*, a touch-down PCR was performed as follows: initial denaturation at 96° C, 20 s; three cycles of: 96° C, 12 s, 50° C, 20 s and 70° C, 70 s, followed by five cycles of:

denaturation 96° C, 12 s, annealing 48.5° C, 18 s, extension 70° C, 65 s and finally 32 cycles of: 96° C, 12 s, 47° C, 15 s and 70° C, 60 s. A final extension step was performed at 72° C for 60 s. Negative controls were always included to control for reagent contamination. Products were electrophoresed on a 1.5% low-melting point agarose gel (stained with either ethidium bromide or goldview) and then visualized under ultra-violet (UV) light. Polymerase chain reaction (PCR) products were purified using the Roche Purification Kit (according to manufacturers' specifications) and then cycle-sequenced using Big Dye terminator chemistry (Applied Biosystems [ABI]). Cycle sequencing products were precipitated, denatured and run on a Genetic Analyser 3130.

2.3.3 Phylogenetic analysis

2.3.3.1 D-loop analysis

Sequences were visualized and edited using Chromas version 1.43 (McCarthy 1996-1997), and the computer program DAPSA (Harley 1994) was used to align sequences. Closely related sequences deposited in the Genbank database were identified using the nucleotide BLAST search function (www.ncbi.nlm.nih.gov/blast) and included in the analyses in order to evaluate possible origins of the *Rattus* species introduced into South Africa.

Phylogenetic and molecular evolutionary D-loop and cyt *b* gene analyses were conducted using different inference methods and programs. MEGA version 4.0 (Tamura *et al.* 2007) was used to construct a midpoint rooted Neighbor-Joining (NJ) tree. Maximum likelihood (ML; Felsenstein 1981) and maximum parsimony analyses (MP) were performed in PAUP* version 4.0b10 (Swofford 2002), and Bayesian Inference (BI) analyses were performed using MrBayes version 3.1 (Huelsenbeck & Ronquist 2001). For the ML and MP analyses, stepwise addition with 1000 random addition replicates and tree-bisection-reconnection (TBR; Swofford 2002) branch-swapping was performed. Nonparametric bootstrap support (Felsenstein 1985) was determined following 1000 replications in order to assess confidence intervals. Modeltest version 3.06b (Posada & Crandall 1998) was used to determine the best-fit model for the dataset at hand, under the Akaike Information Criterion (AIC; Akaike 1974) and guided model selection for the NJ and ML analyses. Haplotype and nucleotide diversity were estimated in DNASP 5.0 (Librado & Rozas 2009).

Bayesian analyses of the D-loop sequence data were performed using MrBayes version 3.1 (Huelsenbeck & Ronquist 2001). Posterior parameter distribution was estimated using a Markov Chain Monte Carlo (MCMC) process, with four chains being

run simultaneously. To increase the chances of finding peaks in tree/parameter space, four Metropolis-coupled MCMC chains (three heated and one cold, and all at the default settings) were run simultaneously. Priors were those estimated from the best-fit model in Modeltest, namely the HKY model (Hasegawa *et al.* 1985) with invariable sites (I) and a gamma distribution (G). A burn-in of 25% of the run length was discarded following a run of 5 000 000 generations. In all phylogenetic analyses, *R. tiomanicus* (EF186399), *R. exulans* (EF186312) and *R. hoffmanni* (EF186327) were included and *Mus musculus musculus* (AB039262) was used as the outgroup.

2.3.3.2 Cytochrome b analysis

The cyt *b* dataset (22 samples; Fig. 2.7a) generated in this study was complemented with 29 homologous sequences from a parallel study (Bastos *et al.* unpublished) and imported into MEGA version 4.0 (Tamura *et al.* 2007) to construct a midpoint rooted Neighbor-Joining (NJ) tree for comparative purposes (Fig. 2.7b). Analyses were restricted to a phenetic analysis alone as more extensive analyses of the cyt *b* data conducted in a parallel study recovered no marked topological differences between inference methods (Bastos *et al*. unpublished). The NJ tree generated for the 51 cyt *b* samples was compared to a D-loop NJ tree of the same 51 samples in order to determine which gene region had the best resolution capabilities when determining phylogenetic relationships among members of the genus *Rattus*.

2.4 RESULTS

2.4.1 *D-loop sequence assessment*

D-loop haplotype sequences were generated for the *Rattus* samples and were then aligned with Genbank sequences resulting in a final dataset of 375 bp, containing 39 taxa (unique haplotypes) and representative of seven different species and of the geographical and genetic variation uncovered for *Rattus* from South Africa. The initial amplicon size was 453 bp (excluding primer sequences) of which a section had to be removed in order for the dataset to align with the Genbank data. However, the phylogenetic analysis of the smaller dataset gave similar results to the larger dataset (when the Genbank sequences were excluded), with the same number of haplotypes being generated for the different *Rattus* species. The final dataset (375 bp), which corresponds to positions 15389 to 15759 in the *Rattus norvegicus* reference genome (NC_001665) contained 254/375 (67.7%) conserved sites and 119/375 (31.7%) variable sites. Of these variable sites (Fig. 2.5), 71 (19.0%) were parsimony-informative and 47

(12.5%) were singletons. The average nucleotide composition was 33.3%, 9.6%, 22.8% and 34.3% for A, C, G and T, respectively, indicating a strong AT-bias (67.6%).

2.4.2 *Neighbor-joining (NJ), Maximum likelihood (ML), Maximum parsimony (MP) and Bayesian Inference (BI) analyses*

Under the AIC, the HKY+I+G model of evolution (Hasegawa *et al.* 1985) was selected in Modeltest version 3.06 (Posada 2000) as the best fit model for the D-loop dataset. The ML analysis yielded a single tree while the MP analysis yielded 132 possible trees (L = 323, CI = 0.586, RI = 0.764, RC = 0.448) from which a consensus tree was drawn. The transition: transversion ratio (Ti/Tv) was 2.8418, the proportion of invariable sites (I) was 0.3445 and the gamma distribution shape parameter was 0.3818.

The four trees, NJ, ML, MP and BI showed similar topologies, with the BI tree giving slightly better resolution of the *R. rattus* branches. A phylogenetic tree is presented in Fig. 2.6 which incorporates nodal support obtained with different analyses. Clade A (*R. rattus*) has bootstrap support values of 83% (ML); 99% (NJ) and 100% (MP); clade B (*R. tanezumi*) has 90% (ML); 97% (NJ); 100% (MP and BI) bootstrap support; and clade C (*R. norvegicus*) has 98% bootstrap support for the BI, 100% for MP, 54% for NJ and 76% for ML. In both the ML and the BI, the *R. rattus* haplotypes form a polytomy. The BI and MP however, separate the 13 *R. rattus* haplotypes recovered from Madagascar (Hingston *et al.* 2005) from the remainder of the *R. rattus* haplotypes.

Overall, the results indicated relatively low levels of variation in members of *Rattus* from South Africa, with no variation recorded for *R. tanezumi* in the D-loop region characterised. In the phylogenetic tree (Fig. 2.6), the first clade (red) represents 45 *R. rattus* individuals, which comprise four distinct haplotypes (*R. rattus* SA 1-4; corresponding to RR-C1-3). The second clade (blue) represents 62 *R. tanezumi* individuals which were identical to each other across the gene region sequenced, thus belonging to a single haplotype. The third clade (green) representing *R. norvegicus* formed two distinct haplotypes (*R. norvegicus* SA 1 & 2) and comprised 42 individuals. Hammanskraal represents the locality with the most variation with specimens from this locality falling within three of the four *R. rattus* haplotype lineages and within the *R. tanezumi* haplotype.

Mean p-distances calculated between species in Mega4 (Tamura *et al.* 2007) for the cyt *b* data were 4.0% for *R. tanezumi-R. rattus*, 12.0% for *R. tanezumi-R. norvegicus* and 11.4% for *R. rattus-R. norvegicus*. For the D-loop region, between species mean p-distances were 5.6% for *R. tanezumi-R. rattus*, 11.0% for *R. rattus-R. norvegicus* and 12.2% for *R. tanezumi-R. norvegicus*. The nucleotide diversities (pi) were 0.004 and 0.001 for *R. rattus* and *R. norvegicus* respectively with haplotype diversity being 0.48 for the former and 0.09 for the latter.

2.4.3 *D-loop comparisons of data from the present study and from Genbank*

When the D-loop data generated in this study were aligned with available D-loop data from the Genbank database it was clear that the four *R. rattus* lineages most likely reflect three separate introductions (Fig. 2.6). *Rattus rattus* SA lineage 1 includes two individuals from this study, one from Hillcrest, Pretoria and the other from Hammanskraal, north of Pretoria, both in Gauteng Province. These two sequences group together with Genbank haplotypes from French Polynesia, Samoa, New Zealand and Papua New Guinea, indicating a likely far-East origin for this lineage. *Rattus rattus* SA lineage 2 was represented by nine individuals from Hammanskraal and one from Moreleta Park in Pretoria, both in Gauteng Province. *Rattus rattus* SA lineage 3 comprised 29 individuals from Nkomo-B and two from Bloublommetjieskloof (Fig. 2.6), both located in Limpopo Province of South Africa. *Rattus rattus* SA lineage 4, is represented by two individuals from Hammanskraal, Gauteng Province.

The *R. tanezumi* data in the present study revealed no variation within the D-loop region, across the 10 localities sampled for this species in this study. These localities occur in three provinces that constitute approximately 25% of South Africa (Fig. 2.4). The *R. tanezumi* individuals are most similar to individuals recovered from Indonesia.

Forty-two *R. norvegicus* individuals were sampled at two localities, the O.R. Tambo International Airport and Tembisa, both in the East Rand region of Gauteng Province, South Africa. Only two haplotypes were recovered. The first of these, *R. norvegicus* SA lineage 1 is represented by one individual from Tembisa and one individual from O.R. Tambo International airport. These two individuals are most closely related to wild-caught specimens sampled in Great Britain and French Polynesia. *Rattus norvegicus* SA lineage 2 was identified from 37 individuals from O.R. Tambo International airport and three from Tembisa and is genetically identical to Genbank entries from Tokyo (Japan) and Milwaukee (U.S.A.). The two *R. norvegicus* haplotypes

again point to the likelihood of two separate introductions of this species into South Africa. *R. norvegicus* SA 1 was also identical to a lab strain, T2DN/Mcwi.

2.4.4 *Geographic distributions*

Localities have been identified at which members of different *Rattus* species occur sympatrically with one another (Table 2.1). This is also illustrated in the phylogenetic tree (Fig. 2.6) in which it it is shown that members of *R. rattus* and *R. tanezumi* occur sympatrically at three different localities, *viz*. Hammanskraal (Gauteng Province) (12 *R. rattus*; 10 *R. tanezumi*), Moreleta Park (Gauteng Province) (1 *R. rattus*; 1 *R. tanezumi*) and Nkomo-B (Limpopo Province) (29 *R. rattus*; 8 *R. tanezumi*). *Rattus norvegicus* was also found sympatrically with *R. tanezumi* at one locality, Tembisa (Gauteng Province) where four *R. norvegicus* and one *R. tanezumi*, were recovered.

2.4.5 *D-loop–Cytochrome b comparison*

The cyt *b* tree revealed the presence of two *R. tanezumi* lineages (RT-C1 & RT-C2; indicated in bold) compared to just one in the D-loop tree (RT-D1; Fig. 2.7). However, the second lineage is only represented by one sample (ER5 - Tembisa). Similarly, five *R. rattus* haplotypes were recovered in the cyt *b* tree (RR-C1-5) compared to four in the D-loop data (RR-D1-4). Cytochrome *b* also showed higher levels of variation between sequences HK9, PP01 and ARC170 (indicated in bold). A genetic pairwise comparison between the two gene regions is presented in Table. 2.2 with the D-loop values indicated in blue and cyt *b* in red. *R. norvegicus* had two cyt *b* haplotypes (RN-C1 & RN-C2) as well as two D-loop haplotypes (RN-D1 & RN-D2). The pairwise percentage sequence divergence was highest between the *R. norvegicus* and *R. tanezumi* with 12.5% sequence divergence for D-loop and 12.0% for cyt *b*.

2.5 DISCUSSION

The results indicate that the *R. rattus* individuals from South Africa may have a south-east Asian origin as the four Genbank locations in lineage 1 all clustered with samples from around the South Pacific. The most variable locality, Hammanskraal (Gauteng Province), is an informal settlement which might make it an ideal environment for *Rattus.*

Relatively low levels of variation were found in the South African individuals of *R. rattus.* The recovery of just four *R. rattus* haplotypes contrasts markedly with the report of 13 *R. rattus* haplotypes from Madagascar (Hingston *et al.* 2005; although 13 might be considered as low haplotype diversity), with none being recovered in South Africa. More

recently, Tollenaere *et al.* (2009) recovered 40 haplotypes in Madagascar with one haplotype being similar to a South African sample from Cape Town.

Madagascar was supposedly first colonised by humans at 2300 BP (Burney *et al.* 2004) or 350BC and it is believed that *R. rattus* may have reached the island along with these immigrators. This reflects a much earlier proposed presence of *R. rattus* into Madagascar than the first records of *R. rattus* in South Africa at 1000 AD in the Northern Transvaal region and the 8th century (701-800 BC) in the Natal Province (Avery 1985; reviewed by Long 2003). Low variation could therefore indicate a relatively recent (about 12 centuries ago) single introduction into South Africa with *in situ* diversification taking place. Tollenaere *et al.* (2009) suggests that rats grouping with the South African haplotype recovered in their study were distributed worldwide a few centuries ago and originated from western European populations.

It was found that the Madagascar population had a recent range expansion probably arising from deforestation pressure (Hingston *et al.* 2005). The Madagascar study also recorded a very low nucleotide diversity (only 2.6%; Hingston *et al.* 2005). This nucleotide diversity was however, still much higher than the 0.4% diversity for *R. rattus* individuals from this study. The individuals of *R. rattus* from Madagascar were found to be most closely related to *Rattus* from the Indian sub-continent (Hingston *et al.* 2005). The data also suggest that at least there are no current cross-introductions between South Africa and Madagascar and that the *R. rattus* populations in these two countries have different origins.

The lack of variation within *R. tanezumi* suggests a relatively recent, single introduction of this species into the inland provinces sampled in this study. However, if this homogeneity is country-wide then it would imply a recent, single introduction into South Africa, and may explain why this species was only recently (2005) recorded for the first time in South Africa (and Africa; Bastos *et al.* 2005; Taylor *et al.* 2008). However, it is also possible that the presence of this species in South Africa remained undetected for some time, and that some morphologically-identified specimens in museum holdings designated *R. rattus* may in fact include members of *R. tanezumi*, an aspect that is receiving attention in a parallel study (Chimimba *et al.* unpublished).

The origin of the South African *R. tanezumi* haplotype is not clear as few sequences are presently available worldwide, and those that are available, are restricted to samples from Indonesia, China, Japan and Vietnam. Bootstrap support values are also generally

low making it difficult to make firm inferences regarding the origin of the South African *R. tanezumi* (Fig. 2.6). The grouping of South African *R. tanezumi* with these Genbank sequences may confirm an origin in the Far East. In keeping with the apparently distinct histories of *Rattus* invasions into South African and Madagascar, no *R. tanezumi* has so far been recorded on the latter Indian Ocean Island (Hingston *et al.* 2005). The recent worldwide shift in attention to *Rattus*, and likely broader availability of sequences in future, may however, make the tracing of the origin of this newly-recorded invasive species in South Africa (and Africa), more accurate.

Rattus norvegicus has historically been considered to occur along the coastal regions and harbours of South Africa (De Graaff 1981; Apps 2000). However, sampling in the three inland provinces that were the focus of this study led to the unexpected discovery of members of *R. norvegicus* at localities in the inland Gauteng Province of South Africa. This represents a range expansion of *ca*. 500-600 kilometres beyond the traditionally-considered distributional range (De Graaff 1981; Meester *et al.* 1986; Skinner & Smithers 1990; Apps 2000).

It is difficult to determine exactly where the South African *R. norvegicus* individuals originated from as the Genbank sequences that match these individuals occur worldwide. When comparing coastal and inland *R. norvegicus* it seems as though there is not a lot of variation (Bastos *et al*. unpublished). It will however, be interesting to sample more intensively across South Africa in order to try and find more inland as well as coastal *R. norvegicus* samples to determine if there might be more genetic variants or haplotypes occurring between populations that differ in altitude and geography.

The total number of invasive *Rattus* species in South Africa has increased from two to three. New insights have been gained on the distribution patterns of these species in South Africa. *Rattus rattus* has been known to occur throughout the country (Fig. 2.1) and the present study has confirmed its presence in three provinces namely the KwaZulu Natal, Gauteng and Limpopo Provinces. *Rattus tanezumi* has not been recorded previously in South Africa (and Africa), but has now been sampled at 10 localities in South Africa (Fig. 2.3) and may most likely have a much broader distributional range. The traditionally considered coastal distributional range of *R. norvegicus* has now extended into inland South Africa (Fig. 2.2). This study therefore, represents the first record of *R. norvegicus* in the inland central region of South Africa (also see Mostert *et al.* 2007).

One of the aims of this study was also to determine whether any of these three species occur sympatrically with one another, which was confirmed for *R. rattus* and *R. tanezumi*, as well as *R. tanezumi* and *R. norvegicus.* This however, is not surprising as cases of natural hybridization have been recorded in other studies (Yosida *et al.* 1971; Chinen *et al.* 2005). F_1 hybrids have also been obtained between *R. rattus* (2*n* = 38) and *R. tanezumi* (2*n* = 42) under laboratory conditions, giving rise to offspring with a diploid number of 2*n* = 40. However, it is suggested that these offspring are semi-sterile (Yosida 1980).

A study by Bastos *et al.* (unpublished) examined the cyt *b* region of some of the individuals used in this study. The samples examined by Bastos *et al.* (unpublished) together with 22 additional cyt *b* sequences generated in the present study (Table 2.3), when compared to the D-loop sequences of the present study in an NJ tree (Fig. 2.7) showed higher levels of variation within the cyt *b* gene sequences and recovered two more *Rattus* haplotypes than the D-loop gene dataset. This suggests that the former region may be more appropriate for population level differentiation of *Rattus.*

2.6 REFERENCES

AKAIKE, H. 1974. A new look at statistical model identification. *IEEE Transactions on Automatic Control* **19**: 716–722.

AMORI, G. & CLOUT, M. 2003. Rodents on islands: a conservation challenge. *In*: Rats, mice and people: rodent biology and management (Singleton, G. R., Hinds, L. A., Krebs, C. J. & Spratt, D. M.), Australian Centre for International Agricultural Research, Canberra. Pp. 63-68.

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.

APLIN, K. P., CHESSER, T., & TEN HAVE, J. 2003. Evolutionary biology of the genus *Rattus*: profile of an archetypal rodent pest. *In*: Rats, mice and people: rodent biology and management (Singleton, G. R., Hinds, L. A., Krebs, C. J. & Spratt, D. M.), Australian Centre for International Agricultural Research, Canberra. Pp. 487-498.

APPS, P. 2000. Smithers's mammals of Southern Africa. A field guide. Struik Publishers, Cape Town. p. 74

AQUADRO, C. F. & GREENBERG, B. D. 1982. Human mitochondrial DNA variation and evolution: analysis of nucleotide sequences from seven individuals. *Genetics* **103**: 287-312.

AVERY, D. M. 1985. The dispersal of brown rats *Rattus norvegicus* and new specimens from the 19th century Cape Town. *Mammalia* **49**: 573-592.

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*. Pretoria, 11-12 August 2005, Pp. 78-83.

BAVERSTOCK, P. R., ADAMS, M., MAXSON, L. R. & YOSIDA, T. H. 1983. Genetic differentiation among karyotypic forms of the black rat, *Rattus rattus*. *Genetics* **105**: 969-983.

BELAY, G. & MORI, A. 2006. Intraspecific phylogeographic mitochondrial DNA (D-loop) variation of Gelada baboon, *Theropithecus gelada*, in Ethiopia. *Biochemical Systematics and Ecology* **34**: 554-561.

BROWN, W. M., GEORGE, M. & WILSON, A. C. 1979. Rapid evolution of animal mitochondrial DNA. *Proceedings of the National Academy of Sciences of the United States of America* **76**: 1967-1971.

BROWN, W. M., PRAGER, E. M., WANG, A. & WILSON, A. C. 1982. Mitochondrial sequences of primates: tempo and mode of evolution. *Journal of Molecular Evolution* **18**: 225-239.

BURNEY, D. A., BURNEY, L. P., GODFREY, L. R., JUNGERS, W. L., GOODMAN, S. M., WRIGHT, H. T. & JULL, A. J. T. 2004. A chronology for late prehistoric Madagascar. *Journal of Human Evolution* **47**: 25-63.

CHINEN, A. A., SUZUKI, H., APLIN, K. P., TSUCHIYA, K. & SUZUKI, S. 2004. Preliminary genetic characterization of two lineages of black rats (*Rattus rattus* sensu lato) in Japan, with evidence for introgression at several localities. *Genes Genetic Systematics* **80**: 367–375.

DAVIS, D. E. 1986. The scarcity of rats and the black death: an ecological history. *Journal of the Interdisciplinary History* **16**: 455-470.

DE GRAAFF, G. 1981. The rodents of southern Africa: notes on their identification, distribution, ecology and taxonomy. Butterworth, Durban. Pp. 218-228.

FELSENSTEIN, J. 1981. Evolutionary trees from DNA sequences: A maximum likelihood approach. *Journal of Molecular Evolution* **17**: 368-376.

FELSENSTEIN, J. 1985. Confidence limits of phylogenies: an approach using the bootstrap. *Evolution* **39**: 783-791.

GISSI, C., REYES, A., PESOLE, G. & SACCONE, C. 2000. Lineage-specific evolutionary rate in mammalian mtDNA. *Molecular Biology and Evolution* **17**: 1022-1031.

HARLEY, E. H. 1994. DAPSA, DNA and Protein Sequence Analysis, version 2.9. Department of Chemical Pathology, University of Cape Town.

HASEGAWA, M., KISHINO, H. & YANO, T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution*, **22**: 160-174.

HINGSTON, M., GOODMAN, S. M., GANZHORN, J. U. & SOMMER, S. 2005. Reconstruction of the colonization of southern Madagascar by introduced *Rattus rattus. Journal of Biogeography* **32**: 1549–1559.

HIROTA, T., HIROHATA, T., MASHIMA, H., SATOH, T. & OBARA, Y. 2004. Population structure of the large Japanese field mouse, *Apodemus speciosus* (Rodentia: Muridae), in suburban landscape, based on mitochondrial D-loop sequences. *Molecular Ecology* **13**: 3275–3282.

HUELSENBECK, J. P. & RONQUIST, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Oxford University Press **17**: 754-755.

IRWIN, D. M., KOCHER, T. D. & WILSON, A. C. 1991. Evolution of cytochrome-b in mammals. *Journal of Molecular Evolution* **32**: 128-144.

KOCHER, T. D., THOMAS, W. K., MEYER, A., EDWARDS, S. V., PÄÄBO, S., VILLABLANCA, F. X. & WILSON, A. C. 1989. Dynamics of mitochondrial DNA evolution in animals. Amplification and sequencing with conserved primers. *Proceedings of the National Academy of Science*, *USA* **86**: 6196-6200.

LARIZZA, A., PESOLE, G., REYES, A., SBISA, E. & SACCONE, C. 2002. Lineagespecificity of the evolutionary dynamics of the mtDNA d-loop region in rodents. *Journal of Molecular Evolution* **54**: 145-155.

LECOMPTE, É., GRANJON, L., PETERHANS, J. K. & DENYS, C. 2002. Cytochrome b-based phylogeny of the *Praomys* group (Rodentia, Murinae): a new African radiation? *Evolution* **325**: 827-840.

LIBRADO, P. & ROZAS, J. 2009. DnaSP v5: A software for comprehensive analaysis of DNA polymorphism data. *Bioinformatics* **25**: 1451-1452 | doi: 10.1093/bioinformatics/btp187.

LONG, J. L. 2003. Introduced mammals of the world: their history, distribution and influence. CSIRO Publishing, Collingwood, Australia. 589 pp.

McCARTHY, C. 1996-1997. Chromas, version 1.43. School for Biomolecular and Biomedical Science. Griffith University, Australia.

MARTIN, Y., GERLACH, G., SCHLÖTTERER, C. & MEYER, A. 2000. Molecular phylogeny of European muroid rodents based on complete cytochrome *b* sequences. *Molecular Phylogenetics and Evolution* **16**: 37-47.

MATISOO-SMITH, E. & ROBINS, J. H. 2004. Origins and dispersal of Pacific peoples: Evidence from mtDNA phylogenies of the Pacific rat. *Proceedings of the National Academy of Sciences of the USA* **101**: 9167-9172.

MATISOO-SMITH, E. & ROBINS, J. 2009. Mitochondrial DNA evidence for the spread of Pacific rats through Oceania. *Biological Invasions* **11**: 1521-1527.

MEESTER, J. & SETZER, H. W. 1971. The mammals of Africa: an identification manual. Smithsonian Institution Press, Washington DC. 505 pp.

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

MICHAUX, J. R., CHEVRET, P., FILIPPUCCI, M.-G. & MACHOLAN, M. 2002. Phylogney of the genus *Apodemus* with a special emphasis on the subgenus *Sylvaemus* using the nuclear IRBP gene and two mitochondrial markers: cytochrome *b* and 12S rRNA. *Molecular Phylogenetics and Evolution* **23**: 123-136.

MILLS, J. N. 1999. The role of rodents in emerging human disease: examples from the Hantaviruses and Arenaviruses. *In*: Ecologically-based Rodent Management (*Singleton, G. R., Hinds, L. A., Leirs H. & Zhang, Z. eds*), Australian Centre for International Agricultural Research, Canberra. Pp. 134-160.

MORITZ, C., DOWLING, T. E. & BROWN, W. M. 1987. Evolution of animal mitochondrial DNA: Relevance for population biology and systematics. *Annual Review of Ecology and Systematics* **18**: 269-292.

MOSTERT, M. E., CHIMIMBA, C. T. & BASTOS, A. D. S. 2007. *Helicobacter* and *Bartonella* prevalence in invasive rodents of the *Rattus* species complex (Rodentia: Muridae) in South Africa. Proceedings of the 7th South African Society for Veterinary Epidemiology and Preventive Medicine Conference, Somerset-West, South Africa, pp. 167.

MUSSER, G. G., & CARLETON, M. D. 2005. Family Muridae. *In*: Mammal Species of the World, (eds) D. E. Wilson & D. M. Reeder. The Johns Hopkins Univeristy Press, Baltimore. Pp 894–1531.

NAGATA, J., MASUDA, R., KAJI, K., KANEKO, M. & YOSHIDA, M. C. 1998. Genetic variation and population structure of the Japanese sika deer (*Cervus nippon*) in Hokkaido Island, based on mitochondrial D-loop sequences. *Molecular ecology* **7**: 871-877.

PAGE, R. D. M. & HOLMES, E. C. 1998. Molecular evolution. A phylogenetic approach. Blackwell Science Ltd, London. 352 pp.

PESOLE, G. GISSI, C., De CHIRICO, A. & SACCONE, C. 1999. Nucleotide substitution rate of mammalian mitochondrial genomes. *Journal of Molecular Evolution* **48**: 427-434.

POSADA, D. & CRANDALL, K. A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817-818.

POSADA, D. 2000. Testing models of evolution – Modeltest version 3.06. Brigham Young University, USA.

ROBINS, J. H., MCLENACHAN, P. A., PHILLIPS, M. J., CRAIG, L., ROSS, H. A. & MATISOO-SMITH, E. 2008. Dating of divergences within the *Rattus* genus phylogeny using whole mitochondrial genomes. *Molecular Phylogenetics and Evolution* **49**: 460-466.

RUSSO, I. M. 2003. Molecular systematics of southern African *Aethomys* (Rodentia: Muridae). *MSc Thesis*, University of Pretoria, Pretoria. Pp. 60-61

SACCONE, C., PESOLE, G. & SBISÁ, E. 1991. The main regulatory region of mammalian mitochondrial DNA: structure-funcion model and evolutionary pattern. *Journal of Molecular Evolution* **33**: 83-91.

SCHLICK, N. E., JENSEN-SEAMAN, M. I., ORLEBEKE, K., KWITEK, A. E., JACOB, H. J. & LAZAR, J. 2006. Sequence analysis of the complete mitochondrial DNA in 10 commonly used inbred rat strains. *American Journal of Physiology and Cell Physiology*, **291**, 1183-1192.

SKINNER, J. D. & SMITHERS, R. H. N. 1990. *The Mammals of the Southern African Subregion*. CTP Book Printers, Cape Town. 736 pp.

SONG-JIA, L., YI-PING, L., YAN-XING, L., XUE-WEI, L. & YONG-GANG, Y. 2006. Genetic diversity and origin of Chinese cattle revealed by mtDNA d-loop sequence variation. *Molecular Phylogenetics and Evolution* **38**: 146 –154.

STEWART, D. T. & BAKER, A. J. 1994. Patterns of sequence variation in the mitochondrial D-loop region of shrews. *Molecular Biology and Evolution* **11**: 9-21.

SUZUKI, H., SATO, J. J., TSUCHIYA, K., LUO, J., ZHANG, Y-P., WANG, Y-X. & JIANG, X-L. 2003. Molecular phylogeny of wood mice (*Apodemus*, Muridae) in East Asia. *Biological Journal of the Linnean Society* **80**: 469-481.

SUZUKI, H., SHIMADA, T., TERASHIMA, M., TSUCHIYA, K. & APLIN, K. 2004. Temporal, spatial, and ecological modes of evolution of Eurasian *Mus* based on mitochondrial and nuclear gene sequences. *Molecular Phylogenetics and Evolution* **33**: 626-646.

SWOFFORD, D. L. 2002. PAUP*: Phylogenetic Analysis using Parsimony (* and other methods), Version 4.0b10. Sinauer Associates, Sutherland, MA.

TAMURA, K., DUDLEY, J., NEI, M. & KUMAR, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**: 1596-1599.

TAYLOR, P. J., ARNTZEN, L., HAYTER, M., ILES, M., FREAN, J. & 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.

TIEMANN-BOEGE, I., KILPATRICK, C. W. & SCHMIDLY, D. J. 2000. Molecular phylogenetics of the *Peromyscus boylii* species group (Rodentia: Muridae) based on mitochondrial cytochrome *b* sequences. *Molecular Phylogenetics and Evolution* **16**: 366-378.

TOLLENAERE, C., BROUAT, C., DUPLANTIER, J-M., RAHALISON, L., RAHELINIRINA, S., PASCAL, M., MONÉ, H., MOUAHID, G., LEIRS, H. & COSSON, J-F. 2009. Phylogeography of the introduced species *Rattus rattus* in the western Indian Ocean, with special emphasis on the colonization history of Madagascar. *Journal of Biogeography* **37**: 398-410.

WILKINSON, G. S. & CHAPMAN, A. M. 1991. Length and sequence variation in evening bat D-loop mtDNA. *Genetics* **128**: 607-617.

YOSIDA, T. H., TSUCHIYA, K. & MORIWAKI, K. 1971. Karyotypic differences of black rats, *Rattus rattus*, collected in various localities of East and Southeast Asia and Oceania. *Chromosoma* **33**: 252-267.

YOSIDA, T. H. 1980. Cytogenetics of the black rat: karyotype evolution and species differentiation. University of Tokyo Press, Tokyo. 256 pp.

2.7 FIGURE LEGENDS

Fig. 2.1 A map showing previous (indicated in black) and revised (red dots) distributions of *Rattus rattus* in South Africa. Pretoria (2) represents two localities around Pretoria, namely, Moreleta Park and Hillcrest.

Fig. 2.2 The traditionally known coastal (and harbour town) geographic distribution of *Rattus norvegicus* in South Africa (indicated by the circles) and the new inland records of the species in Gauteng Province (red dots) found in the present study.

Fig. 2.3 The distribution map of *Rattus tanezumi* so far recorded in South Africa in the present study (red) and in a study by Taylor *et al*. 2008 (black). Pretoria (3) represents three localities around Pretoria, namely Rietondale, Waterkloof and Moreleta Park.

Fig. 2.4 A map of South Africa showing localities in three provinces (Limpopo, Gauteng and Kwa-Zulu Natal) where members of *Rattus* were collected for this study. Pretoria (4) represents the four localities around Pretoria, namely Waterkloof, Moreleta Park, Rietondale and Hillcrest.

Fig. 2.5 Nucleotide sequence alignment of the D-loop region of members of *Rattus* characterized in this study indicating variable sites only.

Fig. 2.6 A phylogenetic tree of the D-loop gene region representing members of *Rattus.* Bootstrap values > 50 obtained in the Maximum parsimony (MP; indicated in black above the line), Maximum likelihood (ML; indicated in green below the line) and Neighbor-Joining (NJ; indicated in red below the line) analyses are shown, as well as posterior probability values obtained from the Bayesian Inference (BI; indicated in blue above the line) analysis. The four *R. rattus* lineages are indicated in red, *R. tanezumi* designated by blue and the two *R. norvegicus* lineages are presented in green. The Madagascar haplotypes from the study by Hingston *et al.* 2005 are indicated in pink.

Fig. 2.7 Cytochrome *b* (Cyt *b*) phylogeny (i) of samples representing the three *Rattus* species occurring in South Africa. The same individuals are represented in a D-loop phylogeny (ii) in order to determine which gene region provides the best resolution capabilities. Taxa that can be distinguished in the cyt *b* phylogeny are indicated in bold.

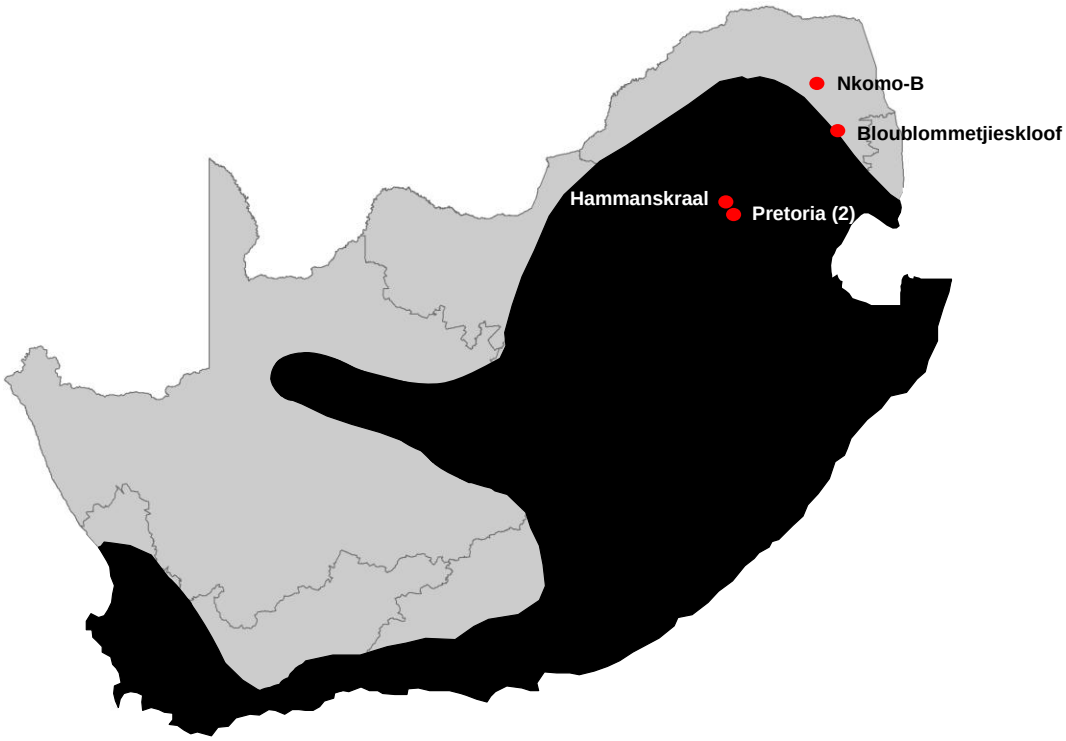
Nkomo-B
Bloublommetjieskloof
Hammanskraal
Pretoria (2)

Fig. 2.1

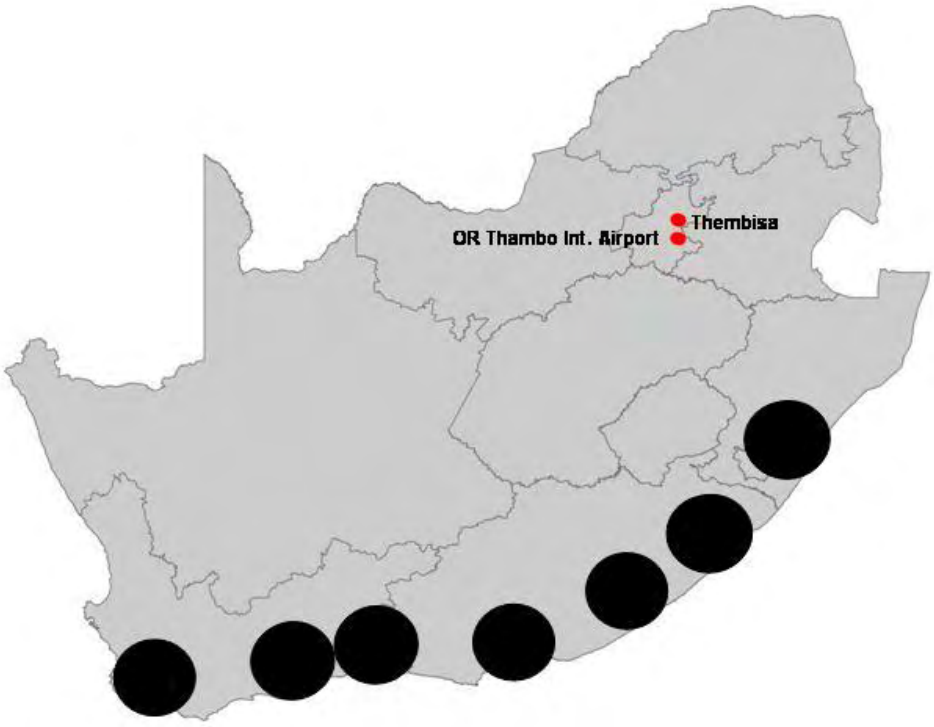
OR Thambo Int. Airport
Thembisa

Fig. 2.2

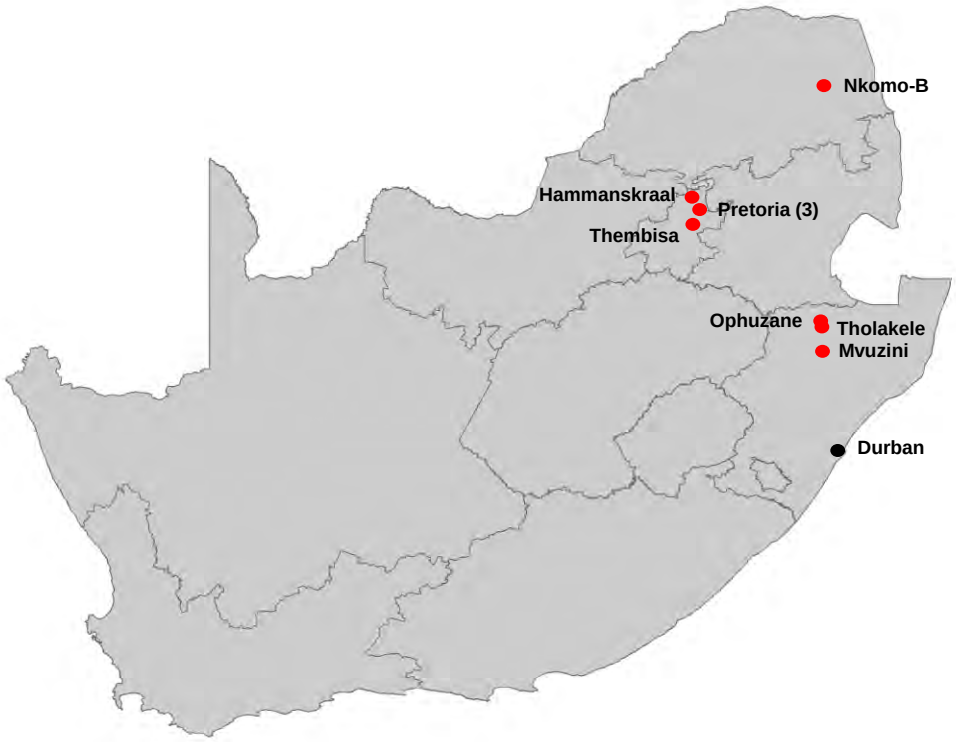
Nkomo-B
Hammanskraal
Pretoria (3)
Thembisa
Ophuzane
Tholakele
Mvuzini
Durban

Fig. 2.3

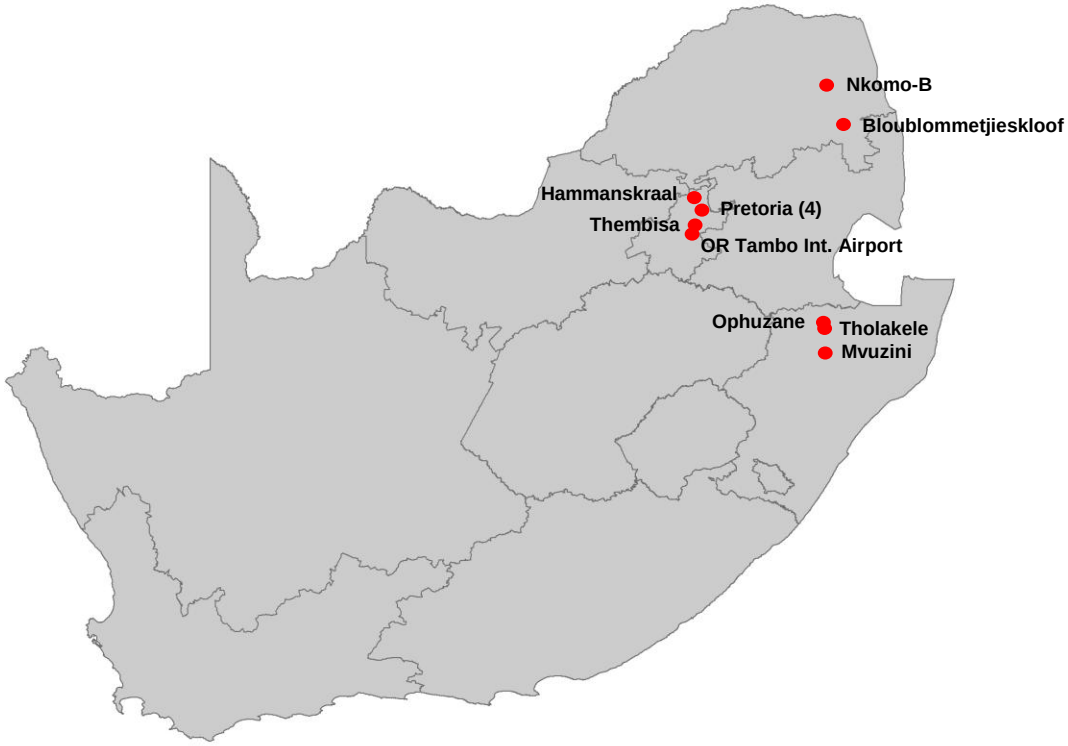
Nkomo-B
Bloublommetjieskloof
Hammanskraal
Pretoria (4)
Thembisa
OR Tambo Int. Airport
Ophuzane
Tholakele
Mvuzini

Fig. 2.4

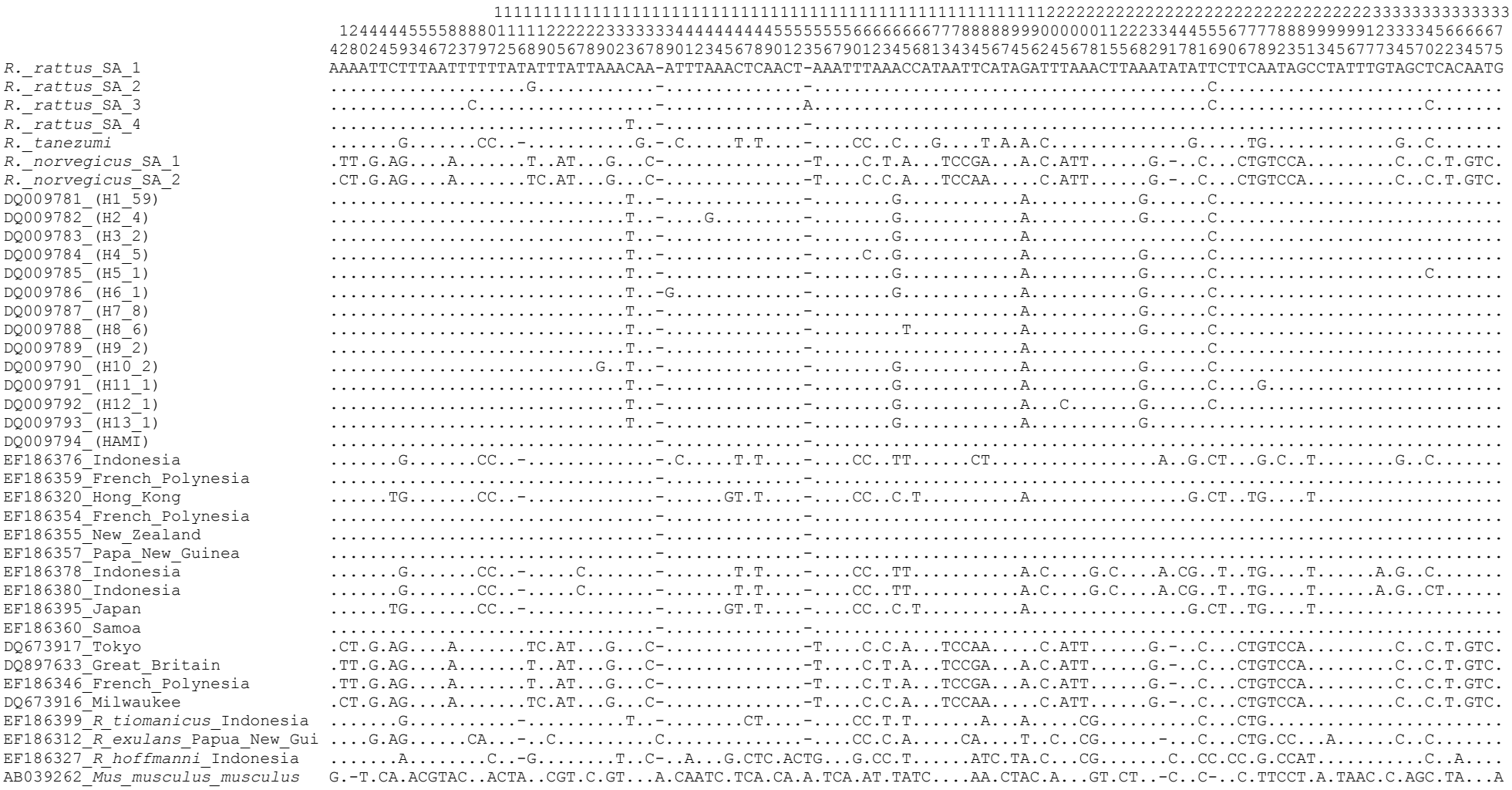
R._rattus_SA_1
R._rattus_SA_2
R._rattus_SA_3
R._rattus_SA_4
R._tanezumi
R._norvegicus_SA_1
R._norvegicus_SA_2
DQ009781_(H1_59)
DQ009782_(H2_4)
DQ009783_(H3_2)
DQ009784_(H4_5)
DQ009785_(H5_1)
DQ009786_(H6_1)
DQ009787_(H7_8)
DQ009788_(H8_6)
DQ009789_(H9_2)
DQ009790_(H10_2)
DQ009791_(H11_1)
DQ009792_(H12_1)
DQ009793_(H13_1)
DQ009794_(HAMI)
EF186376_Indonesia
EF186359_French_Polynesia
EF186320_Hong_Kong
EF186354_French_Polynesia
EF186355_New_Zealand
EF186357_Papa_New_Guinea
EF186378_Indonesia
EF186380_Indonesia
EF186395_Japan
EF186360_Samoa
DQ673917_Tokyo
DQ897633_Great_Britain
EF186346_French_Polynesia
DQ673916_Milwaukee
EF186399_R_tiomanicus_Indonesia
EF186312_R_exulans_Papua_New_Gui
EF186327_R_hoffmanni_Indonesia
AB039262_Mus_musculus_musculus

Fig. 2.5

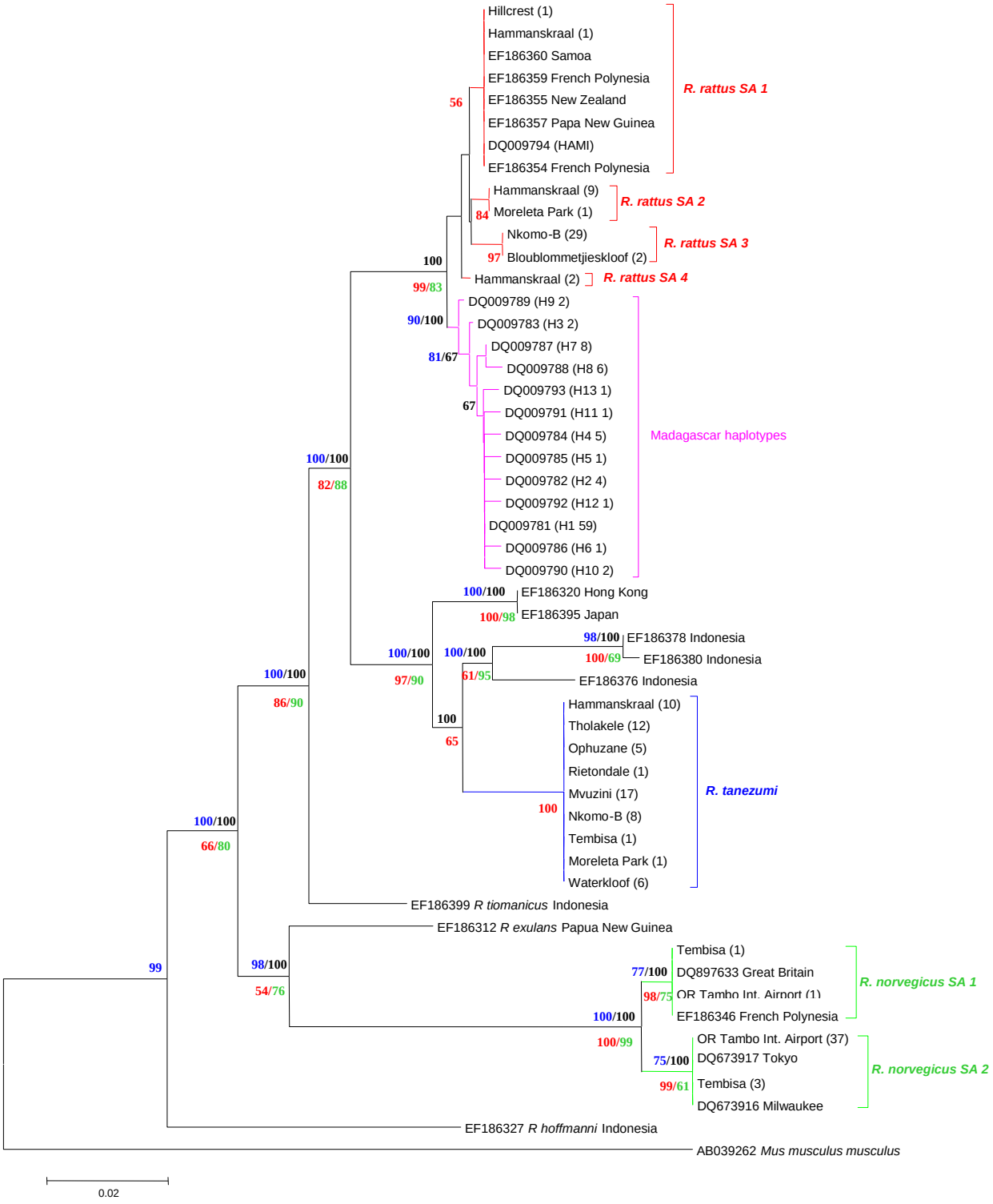
Hillcrest (1)
Hammanskraal (1)
EF186360 Samoa
EF186359 French Polynesia
EF186355 New Zealand
EF186357 Papa New Guinea
DQ009794 (HAMI)
EF186354 French Polynesia
R. rattus SA 1
Hammanskraal (9)
Moreleta Park (1)
R. rattus SA 2
Nkomo-B (29)
Bloublommetjieskloof (2)
R. rattus SA 3
Hammanskraal (2)
R. rattus SA 4
DQ009789 (H9 2)
DQ009783 (H3 2)
DQ009787 (H7 8)
DQ009788 (H8 6)
DQ009793 (H13 1)
DQ009791 (H11 1)
DQ009784 (H4 5)
DQ009785 (H5 1)
DQ009782 (H2 4)
DQ009792 (H12 1)
DQ009781 (H1 59)
DQ009786 (H6 1)
DQ009790 (H10 2)
Madagascar haplotypes
EF186320 Hong Kong
EF186395 Japan
EF186378 Indonesia
EF186380 Indonesia
EF186376 Indonesia
Hammanskraal (10)
Tholakele (12)
Ophuzane (5)
Rietondale (1)
Mvuzini (17)
Nkomo-B (8)
Tembisa (1)
Moreleta Park (1)
Waterkloof (6)
R. tanezumi
EF186399 R tiomanicus Indonesia
EF186312 R exulans Papua New Guinea
Tembisa (1)
DQ897633 Great Britain
OR Tambo Int. Airport (1)
EF186346 French Polynesia
R. norvegicus SA 1
OR Tambo Int. Airport (37)
DQ673917 Tokyo
Tembisa (3)
DQ673916 Milwaukee
R. norvegicus SA 2
EF186327 R hoffmanni Indonesia
AB039262 Mus musculus musculus
0.02

Fig. 2.6

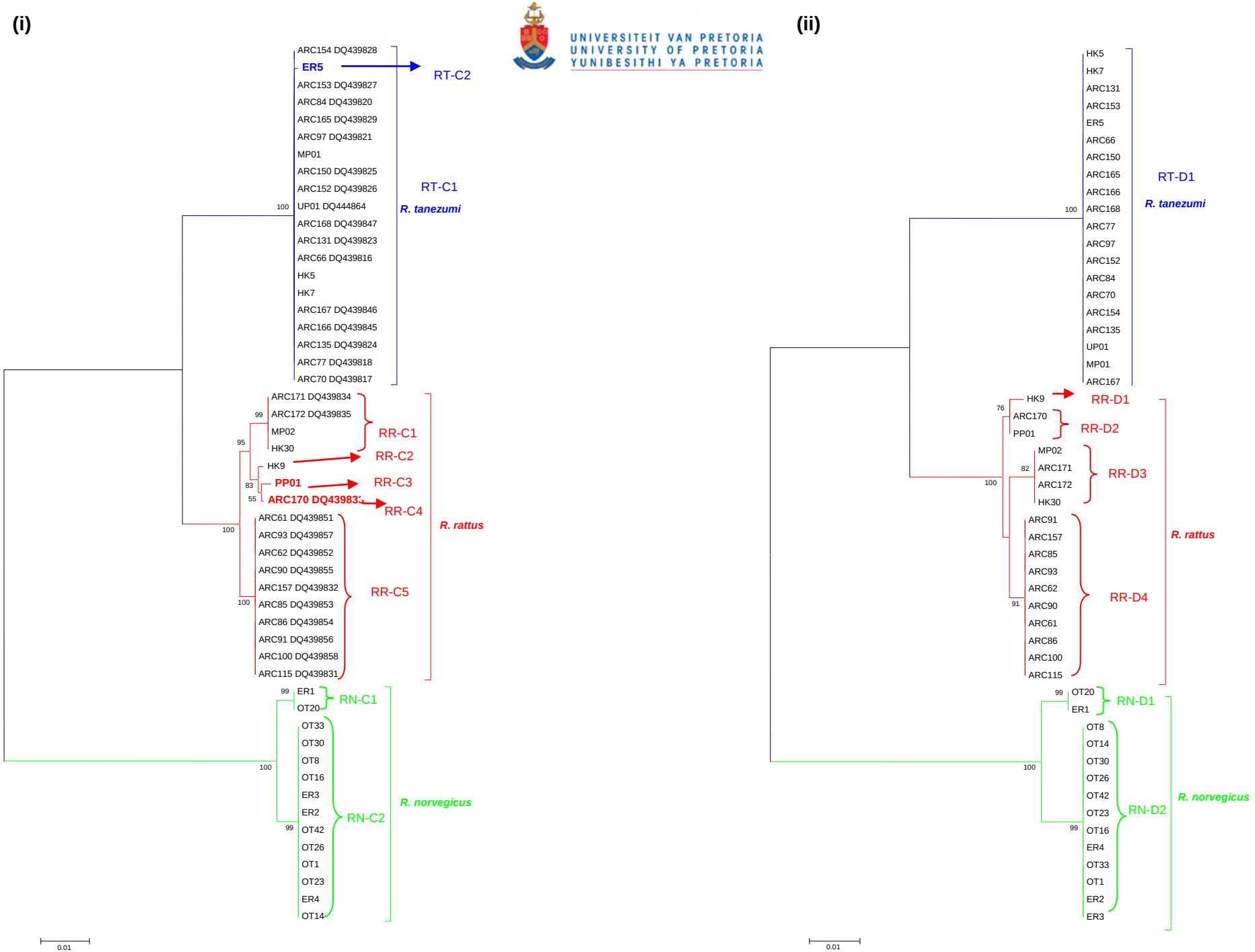
(i)
ARC154 DQ439828
ER5
ARC153 DQ439827
ARC84 DQ439820
ARC165 DQ439829
ARC97 DQ439821
MP01
ARC150 DQ439825
ARC152 DQ439826
UP01 DQ444864
ARC168 DQ439847
ARC131 DQ439823
ARC66 DQ439816
HK5
HK7
ARC167 DQ439846
ARC166 DQ439845
ARC135 DQ439824
ARC77 DQ439818
ARC70 DQ439817
RT-C2
RT-C1
R. tanezumi
ARC171 DQ439834
ARC172 DQ439835
MP02
HK30
HK9
PP01
ARC170 DQ439833
RR-C1
RR-C2
RR-C3
RR-C4
ARC61 DQ439851
ARC93 DQ439857
ARC62 DQ439852
ARC90 DQ439855
ARC157 DQ439832
ARC85 DQ439853
ARC86 DQ439854
ARC91 DQ439856
ARC100 DQ439858
ARC115 DQ439831
RR-C5
R. rattus
ER1
OT20
RN-C1
OT33
OT30
OT8
OT16
ER3
ER2
OT42
OT26
OT1
OT23
ER4
OT14
RN-C2
R. norvegicus
0.01
(ii)
HK5
HK7
ARC131
ARC153
ER5
ARC66
ARC150
ARC165
ARC166
ARC168
ARC77
ARC97
ARC152
ARC84
ARC70
ARC154
ARC135
UP01
MP01
ARC167
RT-D1
R. tanezumi
HK9
ARC170
PP01
RR-D1
RR-D2
MP02
ARC171
ARC172
HK30
RR-D3
ARC91
ARC157
ARC85
ARC93
ARC62
ARC90
ARC61
ARC86
ARC100
ARC115
RR-D4
R. rattus
OT20
ER1
RN-D1
OT8
OT14
OT30
OT26
OT42
OT23
OT16
ER4
OT33
OT1
ER2
ER3
RN-D2
R. norvegicus
0.01

Fig. 2.7

Table 2.1 Geographic coordinates of sampled localities *R. rattus*, *R. tanezumi* and *R. norvegicus* from South Africa. Sample size and the province of the localities are also shown.

	Sampled locality	Province	Sample size	Geographic Coordinates	Species identified at the sampling site
1	Bloublommetjieskloof	Limpopo	2	24°18.66 S; 29°46.17 E	*R. rattus*
2	Hammanskraal	Gauteng	22	25°24.45 S; 28°17.13 E	*R. rattus, R. tanezumi*
3	Moreleta Park, Pretoria	Gauteng	2	25°49.67 S; 28°17.30 E	*R. rattus, R. tanezumi*
4	Mvuzini	Kwa-Zulu Natal	17	28°00.50 S; 30°40.52 E	*R. tanezumi*
5	Nkomo-B	Limpopo	37	23°24.96 S; 30°47.13 E	*R. rattus, R. tanezumi*
6	Ophuzane	Kwa-Zulu Natal	5	27°29.16 S; 30°56.03 E	*R. tanezumi*
7	O.R. Tambo International Airport	Gauteng	38	26°08.68 S; 28°13.57 E	*R. norvegicus*
8	Rietondale, Pretoria	Gauteng	1	25°26.10 S; 28°07.85 E	*R. tanezumi*
9	Tembisa	Gauteng	5	26°00.11 S; 28°12.78 E	*R. norvegicus, R. tanezumi*
10	Tholakele	Kwa-Zulu Natal	12	27°26.05 S; 30°59.28 E	*R. tanezumi*
11	Hillcrest, Pretoria	Gauteng	1	25°45.05 S; 30°59.28 E	*R. rattus*
12	Waterkloof, Pretoria	Gauteng	6	25°49.13 S; 28°15.30 E	*R. tanezumi*

Table 2.2 Genetic pairwise sequence comparison for the different haplotypes of members of *Rattus* represented in Fig. 2.7 with the D-loop genetic distances being given in the top right of the matrix (black) and the cyt *b* in the bottom left (orange). Haplotypes are indicated in the colours that represent each of the different species as follows: *R. tanezumi* (blue), *R. rattus* (red); *R. norvegicus* (green) and are as indicated in Fig. 2.7.

	1	2	3	4	5	6	7	8	9
1. ER5		0.000	0.059	0.057	0.054	0.054	0.057	0.120	0.125
2. ARC70	0.001		0.059	0.057	0.054	0.054	0.057	0.120	0.125
3. ARC171	0.042	0.041		0.008	0.005	0.005	0.008	0.111	0.111
4. HK9	0.041	0.040	0.006		0.003	0.003	0.011	0.111	0.111
5. PP01	0.042	0.041	0.009	0.004		0.000	0.008	0.108	0.108
6. ARC170	0.041	0.040	0.006	0.002	0.003		0.008	0.108	0.108
7. ARC61	0.039	0.038	0.009	0.008	0.011	0.008		0.111	0.111
8. ER1	0.120	0.119	0.114	0.115	0.115	0.115	0.110		0.014
9. ER2	0.121	0.120	0.115	0.116	0.116	0.116	0.111	0.008	

Table 2.3 Summary of all specimens (and their sampling localities), for which D-loop sequences were generated in this study, with cytochrome *b* (cyt *b*) gene sequence data of *Rattus* generated in this and in a previous study (Bastos *et al.* unpublished), being indicated. ORT Int. Airport = O.R. Tambo International Airport; '--' indicates those specimens for which D-loop data is available, but for which no corresponding cyt *b* data were generated; N/A: Not applicable

Sample number	Sampling locality	Genbank accession no. Cyt *b*	Reference
ARC45	Nkomo-B, Limpopo	--	N/A
ARC49	Nkomo-B, Limpopo	--	N/A
ARC61	Nkomo-B, Limpopo	DQ439851	Bastos *et al.* unpubli.
ARC62	Nkomo-B, Limpopo	DQ439852	Bastos *et al.* unpubli.
ARC63	Nkomo-B, Limpopo	--	N/A
ARC64	Nkomo-B, Limpopo	--	N/A
ARC65	Nkomo-B, Limpopo	--	N/A
ARC66	Nkomo-B, Limpopo	DQ439816	Bastos *et al*. unpubli.
ARC69	Nkomo-B, Limpopo	--	N/A
ARC70	Nkomo-B, Limpopo	DQ439817	Bastos *et al*. unpubli.
ARC71	Nkomo-B, Limpopo	--	N/A
ARC72	Nkomo-B, Limpopo	--	N/A
ARC76	Nkomo-B, Limpopo	--	N/A
ARC77	Nkomo-B, Limpopo	DQ439818	Bastos *et al*. unpubli.
ARC78	Nkomo-B, Limpopo	--	N/A
ARC80	Nkomo-B, Limpopo	--	N/A
ARC83	Nkomo-B, Limpopo	--	N/A
ARC84	Nkomo-B, Limpopo	DQ439820	Bastos *et al*. unpubli.
ARC85	Nkomo-B, Limpopo	DQ439853	Bastos *et al*. unpubli.
ARC86	Nkomo-B, Limpopo	DQ439854	Bastos *et al*. unpubli.
ARC87	Nkomo-B, Limpopo	--	N/A
ARC88	Nkomo-B, Limpopo	--	N/A
ARC89	Nkomo-B, Limpopo	--	N/A
ARC90	Nkomo-B, Limpopo	DQ439855	Bastos *et al*. unpubli.
ARC91	Nkomo-B, Limpopo	DQ439856	Bastos *et al*. unpubli.
ARC92	Nkomo-B, Limpopo	--	N/A
ARC93	Nkomo-B, Limpopo	DQ439857	Bastos *et al.* unpubli.
ARC94	Nkomo-B, Limpopo	--	N/A
ARC95	Nkomo-B, Limpopo	--	N/A
ARC96	Nkomo-B, Limpopo	--	N/A
ARC97	Nkomo-B, Limpopo	DQ439821	Bastos *et al*. unpubli.
ARC98	Nkomo-B, Limpopo	--	N/A
ARC99	Nkomo-B, Limpopo	--	N/A
ARC100	Nkomo-B, Limpopo	DQ439858	Bastos et al. unpubli.
ARC103	Nkomo-B, Limpopo	--	N/A
ARC115	Bloublommetjieskloof, Limpopo	DQ439831	Bastos et al. unpubli.
ARC121	Nkomo-B, Limpopo	--	N/A
ARC122	Nkomo-B, Limpopo	--	N/A
ARC123	Mvuzini, Kwa-Zulu Natal	--	N/A
ARC124	Tholakele, Kwa-Zulu Natal	--	N/A
ARC125	Mvuzini, Kwa-Zulu Natal	--	N/A

Sample number	Sampling locality	Genbank accession no. Cyt *b*	Reference
ARC126	Mvuzini, Kwa-Zulu Natal	--	N/A
ARC127	Tholakele, Kwa-Zulu Natal	--	N/A
ARC128	Tholakele, Kwa-Zulu Natal	--	N/A
ARC130	Tholakele, Kwa-Zulu Natal	--	N/A
ARC131	Tholakele, Kwa-Zulu Natal	DQ439823	Bastos et al. unpubli.
ARC132	Tholakele, Kwa-Zulu Natal	--	N/A
ARC133	Tholakele, Kwa-Zulu Natal	--	N/A
ARC134	Tholakele, Kwa-Zulu Natal	--	N/A
ARC135	Mvuzini, Kwa-Zulu Natal	DQ439824	Bastos et al. unpubli.
ARC136	Tholakele, Kwa-Zulu Natal	--	N/A
ARC137	Mvuzini, Kwa-Zulu Natal	--	N/A
ARC138	Mvuzini, Kwa-Zulu Natal	--	N/A
ARC139	Mvuzini, Kwa-Zulu Natal	--	N/A
ARC140	Mvuzini, Kwa-Zulu Natal	--	N/A
ARC141	Mvuzini, Kwa-Zulu Natal	--	N/A
ARC142	Mvuzini, Kwa-Zulu Natal	--	N/A
ARC144	Tholakele, Kwa-Zulu Natal	--	N/A
ARC145	Mvuzini, Kwa-Zulu Natal	--	N/A
ARC146	Mvuzini, Kwa-Zulu Natal	--	N/A
ARC147	Mvuzini, Kwa-Zulu Natal	--	N/A
ARC148	Tholakele, Kwa-Zulu Natal	--	N/A
ARC149	Mvuzini, Kwa-Zulu Natal	--	N/A
ARC150	Mvuzini, Kwa-Zulu Natal	DQ439825	Bastos *et al.* unpubli.
ARC151	Mvuzini, Kwa-Zulu Natal	--	N/A
ARC152	Tholakele, Kwa-Zulu Natal	DQ439826	Bastos *et al*. unpubli.
ARC153	Mvuzini, Kwa-Zulu Natal	DQ439827	Bastos *et al.* unpubli.
ARC154	Hammanskraal, Gauteng	DQ439828	Bastos *et al.* unpubli.
ARC157	Bloublommetjieskloof, Limpopo	DQ439832	Bastos *et al.* unpubli.
ARC165	Ophuzane, Kwa-Zulu Natal	DQ439829	Bastos *et al*. unpubli.
ARC166	Ophuzane, Kwa-Zulu Natal	DQ439845	Bastos *et al.* unpubli.
ARC167	Ophuzane, Kwa-Zulu Natal	DQ439846	Bastos *et al*. unpubli.
ARC168	Ophuzane, Kwa-Zulu Natal	DQ439847	Bastos *et al*. unpubli.
UP01	Rietondale (Pretoria), Gauteng	DQ444864	Bastos *et al*. unpubli.
ARC170	Hammanskraal, Gauteng	DQ439833	Bastos *et al*. unpubli.
ARC171	Hammanskraal, Gauteng	DQ439834	Bastos *et al*. unpubli.
ARC172	Hammanskraal, Gauteng	DQ439835	Bastos *et al*. unpubli.
KZN	Ophuzane, Kwa-Zulu Natal	--	N/A
WK1	Waterkloof (Pretoria), Gauteng	--	N/A
WK2	Waterkloof (Pretoria), Gauteng	--	N/A
WK3	Waterkloof (Pretoria), Gauteng	--	N/A
WK4	Waterkloof (Pretoria), Gauteng	--	N/A
WK6	Waterkloof (Pretoria), Gauteng	--	N/A
OT1	ORT Int. Airport (Johannesburg), Gauteng	--	This study
OT4	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT5	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT7	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT8	ORT Int. Airport (Johannesburg), Gauteng	--	This study
OT9	ORT Int. Airport (Johannesburg), Gauteng	--	N/A

Sample number	Sampling locality	Genbank accession no. Cyt *b*	Reference
OT11	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT12	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT13	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT14	ORT Int. Airport (Johannesburg), Gauteng	--	This study
OT15	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT16	ORT Int. Airport (Johannesburg), Gauteng	--	This study
OT17	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT18	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT19	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT20	ORT Int. Airport (Johannesburg), Gauteng	--	This study
OT21	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT22	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT23	ORT Int. Airport (Johannesburg), Gauteng	--	This study
OT24	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT25	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT26	ORT Int. Airport (Johannesburg), Gauteng	--	This study
OT27	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT28	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT29	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT30	ORT Int. Airport (Johannesburg), Gauteng	--	This study
OT31	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT32	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT33	ORT Int. Airport (Johannesburg), Gauteng	--	This study
OT34	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT35	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT36	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT37	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT38	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT39	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT40	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT41	ORT Int. Airport (Johannesburg), Gauteng	--	N/A
OT42	ORT Int. Airport (Johannesburg), Gauteng	--	This study
HK04	Hammanskraal, Gauteng	--	N/A
HK05	Hammanskraal, Gauteng	--	This study
HK06	Hammanskraal, Gauteng	--	N/A
HK07	Hammanskraal, Gauteng	--	This study
HK08	Hammanskraal, Gauteng	--	N/A
HK09	Hammanskraal, Gauteng	--	This study
HK10	Hammanskraal, Gauteng	--	N/A
HK11	Hammanskraal, Gauteng	--	N/A
HK12	Hammanskraal, Gauteng	--	N/A
HK13	Hammanskraal, Gauteng	--	N/A
HK14	Hammanskraal, Gauteng	--	N/A
HK15	Hammanskraal, Gauteng	--	N/A
HK16	Hammanskraal, Gauteng	--	N/A
HK17	Hammanskraal, Gauteng	--	N/A
HK19	Hammanskraal, Gauteng	--	N/A
HK20	Hammanskraal, Gauteng	--	N/A

Sample number	Sampling locality	Genbank accession no. Cyt *b*	Reference
HK21	Hammanskraal, Gauteng	--	N/A
HK22	Hammanskraal, Gauteng	--	N/A
HK24	Hammanskraal, Gauteng	--	N/A
HK25	Hammanskraal, Gauteng	--	N/A
HK26	Hammanskraal, Gauteng	--	N/A
HK27	Hammanskraal, Gauteng	--	N/A
HK28	Hammanskraal, Gauteng	--	N/A
HK29	Hammanskraal, Gauteng	--	N/A
HK30	Hammanskraal, Gauteng	--	This study
HK31	Hammanskraal, Gauteng	--	N/A
ER1	Thembisa, Gauteng	--	This study
ER2	Thembisa, Gauteng	--	This study
ER3	Thembisa, Gauteng	--	This study
ER4	Thembisa, Gauteng	--	This study
ER5	Thembisa, Gauteng	--	This study
PP01	Hillcrest (Pretoria), Gauteng	--	This study
MP01	Moreleta Park (Pretoria), Gauteng	--	This study
MP02	Moreleta Park (Pretoria), Gauteng	--	This study

CHAPTER 3

QUALITATIVE CRANIAL AND EXTERNAL MORPHOLOGICAL VARIATION, AN IDENTIFICATION KEY, AND A TAXONOMIC SYNTHESIS OF INVASIVE AND COMMENSAL *RATTUS* SPECIES (RODENTIA: MURIDAE) FROM SOUTH AFRICA

3.1 ABSTRACT

It is generally difficult to morphologically distinguish between members of the invasive and commensal genus *Rattus* (Fischer, 1803) both cranially and externally, particularly between members of the cryptic and sympatric *R. rattus* species complex, *R. rattus* (Linnaeus, 1758) and *R. tanezumi* (Temminck, 1844), an otherwise south-east Asian endemic that has for the first time recently been recorded to occur in South Africa (and Africa). However, the currently recognized species in South Africa, namely, *R. rattus*, *R. tanezumi*, and *R. norvegicus* (Berkenhout, 1769) can be distinguished using D-loop and cytochrome *b* gene sequence data generated from parallel studies, and diploid chromosome numbers from previously published data where *R. rattus* differs from *R. tanezumi* in having a 2*n* of either 38 or 40 and four haplotypes (*vs* 2*n* = 42 and a single haplotype for *R. tanezumi*; *R. norvegicus* has a 2*n* = 42 and two haplotypes). Consequently, genetically-identified individuals of these three species based on the two gene regions were used to assess qualitative cranial and external morphological differences between them. There are no cranial and external morphological differences between the cryptic *R. rattus* and *R. tanezumi*. Similarly, there are no discernible pelage colour differences between all the three species of *Rattus* because of high degrees of variation both within and between species that vary dorsally from brownish and blackish grey, brownish, to orange-yellowish, and ventrally from yellowish to completely white. Cranially, however, *R. rattus*/*R. tanezumi* differ from *R. norvegicus* in having: 1) a relatively smaller and less robust skull, a narrower brain case; 2) a more angular supraorbital ridge (*vs* a more curved one); a less robust, relatively thinner, and a more fragile jugal bone; 3) a posterior margin of the palatal foramen that is level with or exceeds the anterior margin of M^1 (*vs* one not reaching the anterior margin); and an antero-external cusp on M^1 that is not reduced (*vs* a reduced one). Externally, *R. rattus*/*R. tanezumi* differ from *R. norvegicus* in having a longer tail than the length of head and body, a relatively thinner tail at its base, a relatively longer and thinner front and hind toes, smaller feet, and larger ears. These morphological differences were in

turn used to devise an identification key for the three species of *Rattus* in South Africa. In addition, a taxonomic synthesis of the three species of *Rattus* based on the collation of morphological, molecular, and previously published data is presented.

Keywords: *Rattus rattus*, *R. norvegicus*, *R. tanezumi*, identification key, qualitative cranial/external morphology, southern Africa, taxonomic synthesis

3.2 INTRODUCTION

Rodents of the genus *Rattus* (Fischer, 1803) belong to the Order Rodentia, the largest mammalian Order, with *ca.* 2277 species representing *ca.* 42% of the world's mammalian species (Musser & Carleton 2005). The genus represents one of the largest mammalian genera containing *ca.* 66 species taxonomically allocated to the Subfamily Murinae within the Family Muridae (Musser & Carleton 2005). Among members of the genus *Rattus*, the house rat, *Rattus rattus* (Linnaeus, 1758) and the Norwegian rat, *R. norvegicus* (Berkenhout, 1769) occur worldwide as invasive commensal species (De Graaff 1981; Meester *et al.* 1986; Skinner & Smithers 1990; Aplin *et al.* 2003; Musser & Carleton 2005).

Until recently, only these two species of *Rattus* were known to occur in South Africa, with *R. rattus* known to be widely distributed (De Graaff 1981; Skinner & Smithers 1990; Apps 2000; Chapter 2; Fig. 2.1), and *R. norvegicus* being considered to occur along coastal regions and harbour towns of the country (De Graaff 1981; Skinner & Smithers 1990; Apps 2000; Chapter 2; Fig. 2.2). However, using cytochrome *b* gene sequence data, the otherwise Asian endemic, Oriental (or Tanezumi) rat, *R. tanezumi* (Temminck, 1844) was for the first time recorded in Limpopo Province representing a first record for South Africa (and Africa) (Bastos *et al.* 2005; Taylor *et al.* 2008; Chapter 2), whilst D-loop sequence data uncovered the first inland record of *R. norvegicus* (Mostert *et al.* 2007; Chapter 2). Subsequent to the initial records of *R. tanezumi* in Limpopo and Kwa-Zulu Natal Provinces, South Africa (Bastos *et al.* 2005; Taylor *et al.* 2008; Chapter 2; Fig. 2.3), the species was later recorded in Gauteng Province and in Swaziland (Chapter 2). *Rattus tanezumi* together with the morphologically indistinguishable cryptic and sympatric species, *R. rattus,* forms part of the *Rattus rattus* species complex (Aplin *et al.* 2003; Bastos *et al.* 2005; Musser & Carleton 2005; Chapter

2). While the cytogenetics of the three species currently known to occur in South Africa has yet to be comprehensively undertaken, extralimitally, *R. rattus* has been shown to comprise a diploid chromosome number (2*n*) of either 38 or 40, while *R*. *norvegicus* and R. *tanezumi* share a diploid number of 2*n* = 42.

Invasive commensal rodents of the genus *Rattus*, including the three species, that have been reported to occur in South Africa, cause extensive damage to agricultural products and human-made infrastructure such as electrical installations, and have also been reported to be a threat to a wide range of indigenous fauna and flora, particularly on island ecosystems (Putman 1989; Mills 1999; Aplin *et al.* 2003; Chinen *et al.* 2005; Bai 2007; Pimentel 2007). Like most rodent species, members of the genus *Rattus* are associated with the transmission of various zoonotic diseases (Lazarus 1989; Lodal & Lund 1989; Putman 1989; Hugh-Jones *et al.* 1995; Mills 1999; Aplin *et al.* 2003; Duplantier *et al.* 2003; Hirano *et al.* 2003; Chinen *et al.* 2005). However, *Rattus* and other problematic species can also be of value in the biomedical field where they are used as model species (Van den Brandt *et al.* 2000; Schlick *et al.* 2006). Rats have also been useful in the study of historical human movement patterns (Matisoo-Smith & Robins 2004; Matisoo-Smith & Robins 2009).

In spite of the importance of *Rattus* as a genus (both positively and negatively) the fundamental systematics and taxonomy, and the general natural history of this group of rodents is complex and not clearly understood at both the species and supraspecific levels worldwide (Yosida 1980; Baverstock 1983; Aplin *et al.* 2003; Musser & Carleton 2005), and is therefore, in critical need of systematic revisions (De Graaff 1981; Aplin *et al.* 2003). The systematics and taxonomic problems within the genus are exacerbated further by the presence of numerous morphologically (both cranially and externally) indistinguishable cryptic species (Baverstock 1981; Aplin *et al.* 2003; Robins *et al.* 2007).

While it has been considered to be relatively easy to morphologically distinguish between *R. rattus* and *R. norvegicus* from southern Africa, the identification key provided by Meester *et al.* (1986), and subsequently by Skinner & Smithers (1990) may have included individuals of the newly recorded *R. tanezumi*, and therefore, needs to be re-examined morphologically. This is particularly critical for *R. rattus* and *R. tanezumi,* the two morphologically indistinguishable cryptic and sympatric species of the *R. rattus*

species complex (Aplin *et al.* 2003; Bastos *et al.* 2005; Musser & Carleton 2005; Chapter 2).

To this end, the present chapter is aimed at assessing the nature and extent of morphological variation in the three species of *Rattus* currently known to occur in South Africa using classical qualitative cranial and external morphology in an attempt to identify morphological differences between them. The morphological assessment was based on genetically-identified individuals of *R. norvegicus*, *R. rattus*, and *R. tanezumi* that were identified using D-loop and cytochrome *b* gene sequence data generated in Chapter 2 and by Bastos *et al.* (unpublished). The use of positively-identified individuals in the present study may allow the morphological identification of material, particularly that of *R. rattus* and *R. tanezumi*, that may have accumulated over long periods of time in mammal reference collections worldwide that may also have been wrongly identified. By so doing, the generated data could in turn be used to map the geographic distributions of this medically and agriculturally-important group of rodents that may also be a conservation threat to indigenous fauna and flora.

Classical qualitative cranial and external morphology has previously been widely applied in rodent systematics and taxonomy worldwide in general (e.g., Carleton 1980; Mathias & Mira 1992; Yabe *et al.* 1998; Stefen & Rudolf 2007; Miljutin & Lehtonen 2008) and the southern African subregion in particular (e.g., Chimimba 1998; Chimimba *et al.* 1999). The identified differences between the three species of *Rattus* known to occur in South Africa using classical qualitative cranial and external morphology in the present chapter may be useful for devising simple and practical identification keys that may assist health, agricultural, and nature conservation authorities, and members of the public in the identification of members of this problematic group of invasive and commensal rodents. In addition, the present morphological assessment also provides a taxonomic synthesis of *R. norvegicus*, *R. rattus*, and *R. tanezumi* based on the collation of the morphological (present chapter), molecular (Bastos *et al.* 2005; Bastos *et al.* (unpublished); Chapter 2), and previously published data. The present study complements the molecular part of the study (Bastos *et al.* 2005; Bastos *et al.* (unpublished); Chapter 2) and an on-going parallel study on geometric morphometric data (Chimimba *et al.* unpublished) that forms part of a broader multidisciplinary characterization of members of the genus *Rattus* in South Africa.

3.3 MATERIALS AND METHODS

3.3.1 Study area and sampling

Specimens of *Rattus* (*n* = 50) used in the present morphological assessment are from five sampling localities in Gauteng Province, South Africa. The collecting localities of these specimens are shown in Fig. 3.1, while their geographic coordinates and sample sizes are summarised in Table 3.1. For direct comparison with genetic data, the specimens used in the present study represent morphologically undamaged individuals that were genetically-identified using D-loop and cytochrome *b* gene sequence data (Bastos *et al.* 2005; Bastos *et al.* (unpublished); Chapter 2) in the multidisciplinary characterization of the genus *Rattus* from South Africa.

Animals were obtained through a number of approaches as follows: 1) Live-trapping using Sherman traps (H.B. Sherman Traps Inc. Florida, U.S.A.) baited with a mixture of peanut butter, syrup, oatmeal and fish oil; 2) Samples obtained through a European Commission/DFID-funded community-participatory research project on rodents in southern Africa where 10 snap traps were placed in 10 community households in a number of villages and inspected daily; 3) Samples obtained opportunistically from pest control companies during their routine extermination programmes at facilities such as the O.R. Tambo International Airport; and 4) Samples obtained opportunistically from the general public.

After capture, during transportation and in the laboratory, live-trapped animals were kept in polyurethane cages with wood shavings provided as bedding and mouse pellets and water provided *ad libitum*. The guidelines of the American Society of Mammalogists (ASM; http://www.mammalogy.org/committees/index.asp; Animal Care and Use Committee 1998) and as approved by the Animal Ethics Committee of the University of Pretoria, Pretoria, South Africa were used to maintain live animals. Halothane inhalation was used to euthanize live animals. Animals were dissected and the livers were removed and stored in either absolute ethanol or frozen at -20° C for subsequent molecular analysis. Voucher specimens were prepared using standard natural history museum procedures for mammal specimens and will be deposited in the mammal reference collection of the Transvaal Museum (TM) of the Northern Flagship Institute (NFI), Pretoria, South Africa and the Durban Natural Science Museum, South Africa.

3.3.2 *Qualitative cranial and external morphological examination of specimens*

Specimens were examined by a single observer (MEM) using an M3C WILD dissecting microscope (Leitz, Heerbrugg, Switzerland), while photographs were taken using a D70 SLR digital camera (Nikon, Tokyo, Japan). The examination of qualitative external morphology included pelage colouration, and foot pad, tail and ear morphology, while the examination of qualitative cranial morphology included the dorsal, ventral, and lateral views of the cranium, the lateral views of the mandible, as well as maxillary and mandibular teeth morphology.

3.3.3 *Relative ageing of specimens and sexual dimorphism*

To reduce the potential effect of age variation, only adult specimens were used in the present chapter. Relative ageing of specimens was based on the degree of maxillary tooth eruption and wear defined and illustrated by Abdel-Rahman *et al.* (2008) who identified six toothwear classes. Only specimens of toothwear classes III–IV which were considered to be adult specimens by Abdel-Rahman (2008) were used in the present study. Abdel-Rahman considered animals of toothwear classes I and II to be juvenile and were therefore not included in the present morphological assessment. Similarly, very old individuals of toothwear class VI (with completely worn-out teeth; Abdel-Rahman 2008) that may be associated with morphological deformities (see Dippenaar & Rautenbach 1986) were also excluded in the present morphological assessment. Since the on-going parallel geometric morphometric study (Chimimba *et al.* unpublished) showed a lack of sexual dimorphism in the three species of *Rattus* from South Africa, the sexes were pooled in all classical qualitative cranial and external morphological assessments in the present chapter.

3.4 RESULTS

3.4.1 Qualitative external morphology

3.4.1.1 Rattus rattus versus R. tanezumi

There are no unequivocal qualitative external morphological differences that were consistent between *R. rattus* and *R. tanezumi*. Similarly, there are no consistent discernible differences in pelage colouration with both species showing high degrees of pelage colour variation both within and between species. Dorsal pelage colour in both species varies from brownish and blackish grey, brownish, to orange-yellowish, while ventral pelage colouration varies from yellowish to completely white.

3.4.1.2 Rattus rattus versus R. norvegicus

The overall body size of *R. Rattus* is relatively smaller (for dimensions see Identification key in section 3.6 below), with a relatively longer tail that is also relatively thinner at its base, relatively longer and thinner front and hind toes, and relatively smaller feet (Figs. 3.5 & 3.6), and relatively larger ears than in *R. norvegicus*. There are no discernible consistent differences in pelage colouration with both species showing high degrees of pelage colour variation both within and between species. Dorsal pelage colour in both species varies from brownish and blackish grey, brownish, to orange-yellowish, while ventral pelage colouration varies from yellowish to completely white.

3.4.1.3 Rattus tanezumi versus R. norvergicus

Similar to *R. rattus*, the overall body size of *R. tanezumi* is relatively smaller (for dimensions see Identification key in section 3.6 below), with a relatively longer tail that is also relatively thinner at its base, relatively longer and thinner front and hind toes, and relatively smaller feet (Figs. 3.5 & 3.6), and relatively larger ears than in *R. norvegicus*. There are no discernible consistent differences in pelage colouration with both species showing high degrees of pelage colour variation both within and between species. Dorsal pelage colour in both species varies from brownish and blackish grey, brownish, to orange-yellowish, while ventral pelage colouration varies from yellowish to completely white.

3.4.2 Qualitative cranial morphology

3.4.2.1 Rattus rattus versus R. tanezumi

There are no unequivocal consistent qualitative cranial morphological differences between *R. rattus* and *R. tanezumi*.

3.4.2.2 Rattus rattus versus R. norvegicus

Rattus norvegicus has a markedly large (for dimensions see Identification key in section 3.6 below), and more robust skull, with a markedly wider braincase than *R. rattus* (Fig. 3.2). While *R. norvegicus* has a more curved supraorbital ridge, it is more angular in *R. rattus* (Fig. 3.2a). *Rattus norvegicus* has a more robust, thick, and less fragile jugal bone than *R. rattus* (Fig. 3.2b). While the posterior margin of the palatal foramen in *R. norvegicus* does not reach the anterior margin of the first set of maxillary molars (M^1), it is level with or exceeds the anterior margin of the first set of maxillary molars in *R. rattus*

(Fig. 3.3). The antero-external cusp on M^1 in *R. norvegicus* is reduced but not in *R. rattus* (Fig. 3.4).

3.4.2.3 Rattus tanezumi versus R. norvegicus

The skull of *R. norvegicus* is markedly large (for dimensions see Identification key in section 3.6 below), and more robust, with a markedly wider braincase than *R. tanezumi* (Fig. 3.2). *Rattus norvegicus* has a more curved supraorbital ridge, while it is more angular in *R. tanezumi* (Fig. 3.2a). *Rattus norvegicus* has a more robust, thick, and less fragile jugal bone than *R. rattus* (Fig. 3.2b). The posterior margin of the palatal foramen in *R. norvegicus* does not reach the anterior margin of the first set of maxillary molars (M^1), while it is level with or exceeds the anterior margin of the first set of maxillary molars in *R. tanezumi* (Fig. 3.4). The antero-external cusp on M^1 in *R. norvegicus* is reduced but not in *R. tanezumi* (Fig. 3.4).

3.5 DISCUSSION

The aim of the present chapter was to assess qualitative cranial and external morphological differences between the three invasive and commensal species of *Rattus*, namely *R. rattus*, *R. tanezumi*, and *R. norvegicus* currently known to occur in South Africa that may be useful for devising an identification key for the three species. While morphological identification keys have previously been presented for *R. rattus* and *R. norvegicus* from southern Africa (e.g., Meester & Setzer 1971; De Graaff 1981; Meester *et al.* 1986; Skinner & Smithers 1990) these need to be re-examined, particularly in light of the recently discovered first record of *R. tanezumi* in South Africa (and Africa), a species that together with *R. rattus* belong to the morphologically indistinguishable *R. rattus* species complex (Aplin *et al.* 2003; Chinen *et al.* 2005; Robins *et al.* 2007).

The present chapter found neither cranial nor external morphological differences between the cryptic *R. rattus* and *R. tanezumi*. Similarly, there are no discernible pelage colour differences between *R. rattus*, *R. tanezumi* and *R. norvegicus* from South Africa. All three species show high degrees of pelage colour variation both within and between species. Dorsally, these vary from brownish and blackish grey, brownish, to orange-yellowish, and ventrally from yellowish to completely white.

Cranially, however, members of the *R. rattus/R. tanezumi* species complex were shown to differ from *R. norvegicus* in having: 1) a relatively smaller and less robust skull,

and a narrower brain; 2) a more angular supraorbital ridge that is more curved in *R. novergicus*); a less robust, relatively thinner, and a more fragile jugal bone; 3) a posterior margin of the palatal foramen that is level with or exceeds the anterior margin of M^1 (*vs* one that does not reach the anterior margin in *R. novergicus*); and an antero-external cusp on M^1 that is not reduced (*vs* one that is reduced in *R. norvegicus*). The extent of the posterior margin of the palatal foramen relative to the anterior margin of M^1 has previously been used to distinguish between *R. rattus* and *R. norvegicus* from southern Africa (e.g., Meester & Setzer 1971; De Graaff 1981; Meester *et al.* 1986; Skinner & Smithers 1990).

The morphology of the antero-external cusp on M^1 used to distinguish between *R. rattus*/*R. tanezumi* and *R. norvegicus* in the present chapter was also used in earlier work by Harrison & Bates (1991) and previous identification keys developed by De Graaff (1981) and Meester *et al.* (1986). It is possible that these dental morphological differences may be associated with food and habitat preferences of the three species of *Rattus* known to occur in South Africa. Myers *et al.* (1996) showed the influence of diet on the skull and dental morphology of rodents of the genus *Peromyscus*. It may be relevant to note that in the present study, apart from one individual of *R. tanezumi* that was collected together with *R. norvegicus*, the latter species were collected from more urban localities, while *R. rattus* and *R. tanezumi* were largely collected from semi-urban localities.

Externally, *R. rattus*/*R. tanezumi* have been shown to differ from *R. norvegicus* in having a relatively longer and thicker tail at its base, longer and thinner front and hind toes, smaller feet, and larger ears. Harrison & Bates (1991) and Yiğit et al. (1998) also recorded larger ears and longer tails in *R. rattus* than observed in *R. norvegicus*. Lawrence & Brown (1967) reported that the tail of *R. rattus* touches the ground when running. It is possible that the morphological characteristics of the tail (and its base), the front and hind toes, and the feet of *R. rattus* and *R. tanezumi* presented above may be attributed to the more arboreal life style of the two cryptic species of the *R. rattus* species complex. Similarly, Lawrence & Brown (1967) reported that *R. norvegicus* holds its tail above the ground when running. It is possible that the relatively shorter and thinner tail at its base, shorter and thicker front and hind toes, and relatively larger feet may be attributed to the more terrestrial life style of the species. Although Yabe *et al.* (1998)

reported paw pad differences between the more arboreal *R. rattus* (with thicker and more horn-like with deeper and narrower lamellae) and the more terrestrial *R. norvegicus* (with more superficial, broader lamellae that are thinner), these could not easily be discerned in the present morphological assessment, and require further investigation.

The three species of *Rattus* currently known to occur in South Africa can also be distinguished using D-loop and cytochrome *b* gene sequence data generated from parallel studies (Bastos *et al.* 2005; Chapter 2; Bastos *et al.* unpublished), and diploid chromosome numbers from previously published data (Yosida & Sagai 1973; Yosida 1980) where *R. rattus* was shown to differ from *R. tanezumi* in having a 2*n* of either 38 or 40 and four haplotypes (*vs* 2*n* = 42 and a single haplotype; *R. norvegicus* has a 2*n* = 42 and two haplotypes). The diploid numbers of South African *R. rattus* were 2*n* = 38 and 2*n* = 40 for two individuals from the same locality, and 2*n* = 40 for South African *R. tanezumi* (Bastos *et al*. unpublished). Similarly, given the high degree of complex intra-specific morphological variation found in the present chapter, there is a critical need to determine whether there is introgression between species at sympatric localities. A recent study by Chinen *et al.* (2005) reported on evidence of introgression between extralimital *R. rattus* and *R. tanezumi*. Questions relating to morphometric differences between the three species of *Rattus* currently known to occur in South Africa are being addressed in a parallel morphometric study (Chimimba *et al.* unpublished).

The present study, parallel and previous studies suggest a critical need for extensive sampling of members of the genus *Rattus* in South Africa and beyond in order to determine the systematics (including population genetics) and the centre of origin of the species in South Africa, and the extent of their geographic distributions. There is also a critical need to investigate the ecology (including the population dynamics) of the three species of *Rattus* known to occur in South Africa and how they interact with each other as well as with indigenous fauna in order to assess the nature and extent of zoonotic disease potential and damage to agricultural products. Parallel studies (Chapters 4 & 5) have identified the presence of to bacterial genera with known zoonotic potential, some of which are being reported in South Africa for the first time, and require further investigation (see remarks section of the taxonomic synthesis of each of the three species below).

The refinement of the identification key in the present study, and subsequent studies on morphological differences between *R. rattus* and *R. tanezumi* may facilitate the morphological identification of wrongly-identified material in reference collections worldwide that have accumulated over long periods of time. Such morphological identifications could be useful for mapping the geographic distributions of these medically and agriculturally-important group of rodents that may also be a conservation threat to indigenous fauna and flora. Apart from contributing to general small mammal studies in Africa, the present chapter may have implications in epidemiological, agricultural, biological conservation, and invasion biology research associated with problem rodents in the southern African subregion and beyond.

3.6 AN IDENTIFICATION KEY TO THE SOUTH AFRICAN SPECIES OF *RATTUS*

The identification key of the three species of *Rattus* currently known to occur in South Africa presented below is based on the collation of the morphological data from the present study, molecular data from Chapter 2, and previously published morphological data from De Graaff (1981), Meester *et al.* (1986), Skinner & Smithers (1990) and Yiğit *et al.* (1998), and cytogenetic data from Yosida & Sagai (1973) and Yosida (1980).

1) Body size relatively large (length of head and body 210 mm or more); tail shorter than length of head and body (less than 210 mm); large and robust skull (greatest length of skull 45 mm or more); braincase wide (width of skull 23-35 mm); supraorbital ridge curved (Fig. 3.2a); more robust, relatively thicker, and less fragile jugal bone (Fig. 3.2b); posterior margin of palatal foramen not reaching anterior margin of first set of maxillary molars (M^1) (Fig. 3.3); antero-external cusp on M^1 reduced (Fig. 3.4); diploid chromosome number of $2n = 42$; South African individuals with two haplotypes (based on D-loop data generated in the current study) ..... *R. norvegicus*

– Body size small (length of head and body 150 – 200 mm; tail longer than length of head and body (length of tail 185–245 mm); relatively smaller less robust skull (greatest length of skull 38 – 44 mm); relatively narrow braincase (width of skull 18.5–21.5 mm); supraorbital ridge angular (Fig. 3.2a); less robust, relatively thinner, and fragile jugal bone (Fig. 3.2b); posterior margin of palatal foramen

level or exceeding the anterior margin of first set of maxillary molars (M^1) (Fig. 3.4); antero-external cusp on M^1 not reduced; diploid chromosome numbers of either 2*n* = 38, 40, or 42; South African individuals with either a single or four haplotypes (based on D-loop data generated in the current study) 2

2) Diploid chromosome numbers of either 2*n* = 38 or 40; South African individuals with four haplotypes (based on D-loop data in the current study) .....*R. rattus*

– Diploid chromosome numbers of 2*n* = 42; South African individuals with a single haplotype (based on D-loop data in the current study) .....*R. tanezumi*

3.7 A TAXONOMIC SYNTHESIS OF SOUTH AFRICAN SPECIES OF *RATTUS*

The taxonomic synthesis presented below is based on the collation of the morphological data from the present study, molecular data from Chapter 2, and previously published data of the three species of *Rattus* that are currently known to occur in South Africa.

3.7.1 Genus *Rattus* Fischer, 1803

For a full synonymy see Ellerman and Morrison-Scott (1951: 581-587).

1775. *Rattus* Frisch, *Natursystem der vierfüssigen Thiere*: **7**. Not available (*Bulletin of Zoological Nomenclature* **4**: 549, 1950; *International Commission on Zoological Nomenclature*, Opinion No. **258**, 1954).

1803. *Rattus* Fischer, *Das Nationalmuseum der Naturgeschichte zu Paris* **2**: 128. Misspelt *Ruttus*, a *lapsus*. *Mus decumanus* Pallas = *Mus norvegicus* Berkenhout.

Type species: *Mus decumanus* Pallas, 1779

3.7.1.1 Rattus rattus (Linnaeus, 1758)

For a full synonymy see Ellerman and Morrison-Scott (1951: 581-587).

1758. *Mus rattus* Linnaeus, *Systema naturae* 10th ed. **1**: 61. Sweden.

Holotype – No information on the holotype is available. Names were coined prior to the convention of assigning type specimens (K. Aplin *pers. comm*.).

Geographic distribution – *Rattus rattus* is native to India, but is currently cosmopolitan as an invasive commensal species especially in tropical and temperate areas (Musser & Carleton 2005; Chapter 1; Fig. 1.2). Known to occur in most parts of South Africa except the Karoo region (De Graaff 1981; Skinner & Smithers 1990; Apps 2000; Musser & Carleton 2005; see Fig. 2.1 in Chapter 2). For worldwide distribution see Musser & Carleton 2005 and Fig. 1.2 in Chapter 1.

Diagnosis – *Rattus rattus* is cranially and externally morphologically similar to *R. tanezumi*, both of which belong to the *R. rattus* species complex. *Rattus rattus/R. tanezumi* have a relatively smaller and less robust skull, a relatively narrow brain case, and a relatively longer tail than the length of head and body that is also relatively thinner at its base than *R. norvegicus.* The supraorbital ridge is more angular in *R. rattus/tanezumi* than in *R. norvegicus* where it is more curved (Fig. 3.2a). The jugal bone of *R. rattus*/*R. tenezumi* is less robust, relatively thinner, and more fragile than that of *R. novergicus* (Fig. 3.2b). The posterior margin of the palatal foramen of *R. rattus*/*R.tanezumi* is level or exceeds the anterior margin of the first set of maxillary molars (M^1), while in *R. novergicus* it does not reach the anterior margin of the first set of maxillary molars (M^1) (Fig. 3.4). The antero-external cusp on M^1 of *R. rattus*/*R. tanezumi* is not reduced, while it is reduced in *R. novergicus* (Fig. 3.4). *Rattus rattus*/*R. tanezumi* have relatively longer and thinner front and hind toes, relatively smaller feet (Fig. 3.5 and Fig. 3.6), and relatively larger ears than *R. norvegicus*. There are no discernible consistent differences in pelage colouration with all three species of *Rattus* known to occur in South Africa showing high degrees of pelage colour both within and between species that dorsally varies from brownish and blackish grey, brownish, to orange-yellowish, and ventrally from yellowish to completely white.

Etymology – The scientific name is derived from the mediaeval Latin for the term ‘Black Rat’ (De Graaff 1981). The species is commonly referred to as either the house rat, black rat, ship rat, or roof rat (Meester & Setzer 1971; De Graaf 1981; Meester *et al.* 1986; Musser & Carleton 2005).

Ecology – *Rattus rattus* is a water-dependent commensal species (Meester & Setzer 1971), living in trees, house roofs, ships and seldom occupies underground burrows and aquatic environments (Davis 1986; Skinner & Smithers 1990; Amori & Clout 2003). Food and cover are necessary habitat constraints for *R. rattus* (Skinner & Smithers 1990). Unlike *R. norvegicus*, the tail of the species touches the ground when running (Lawrence & Brown 1967). This, together with the a relatively longer tail that is also relatively thinner at its base, relatively longer and thinner front and hind toes, and relatively smaller feet (as in *R. tanezumi*) may be attributed to the more arboreal life style of the species than the more terrestrial life style of *R. novergicus*, that has relatively shorter and thicker front and hind toes, and relatively larger feet.

Remarks – *Rattus rattus* has chromosome diploid numbers of either 2*n* = 38 (European), 2*n* = 40 (European & Ceylonese), or *2n* = 42 (Mauritian) (Yosida 1980). The diploid numbers of South African *R. rattus* were 2*n* = 38 and 2*n* = 40 for two individuals from the same locality (Bastos *et al*. unpublished). D-loop gene sequence data shows four haplotypes within the species, which may be indicative of three relatively recent introductions of the species into the country. However, the origin of the South African *R. rattus* is difficult to determine due to its cosmopolitan distribution. The species occurs sympatrically with *R. tanezumi* at a number of localities in South Africa (see Chapter 2). There is a critical need for extensive sampling in South Africa and beyond in order to determine the systematics (including population genetics), the centre of origin of the species in South Africa and its extent of geographic distribution. There is also a critical need to investigate the ecology (including the population dynamics) of the three species of *Rattus* known to occur in Africa and how they interact with each other as well as with indigenous fauna in order to assess the nature and extent of zoonotic disease potential and damage to agricultural products. As a carrier of zoonotic diseases, *R. rattus* has been associated with toxoplasmosis and leptospirosis in South Africa (Taylor *et al.* 2008). In South Africa, the species has also been shown to be a reservoir host for *Bartonella* (*B. coopersplainensis* and *B. elizabethae*; Chapter 4) and *Helicobacter* species (*H. rodentium*; Chapter 3) with *B. coopersplainensis* previously only known to occur in Australia and *B. elizabethae* being zoonotic and causing infective endocarditis in humans (Chapter 4).

3.7.1.2 Rattus tanezumi (Temminck, 1844)

Holotype - Type locality: Japan (Musser & Carleton 2005). Two syntypes (a young adult – museum no. 24206 and a juvenile – museum no. 24207 are in the Royal Museum of Natural History (RMNH) collection in Leiden, The Netherlands. Damaged skulls were removed and only mounted skins are represented (K. Aplin *pers. comm*.).

Geographic distribution – *Rattus tanezumi* is believed to be endemic to south-east Asia and as an introduced species in the surrounding parts of the region (Musser & Carleton 2005; Chapter 1; Fig. 1.4). However, it was not known to occur in South Africa (and Africa) until it was recently recorded for the first time in the country (and the continent) in Limpopo Province, South Africa (Bastos *et al.* 2005; Taylor *et al.* 2008), and subsequently in Gauteng and Kwa-Zulu Natal Provinces, South Africa (Chapter 2; Fig. 2.3), and in Swaziland (Bastos *et al.* unpublished).

Diagnosis – see the diagnosis of *R. rattus* (section 3.7.1.1 above), a species that is cranially and externally morphologically similar to *R. tanezumi*, both of which belong to the *R. rattus* species complex.

Etymology – The scientific name is derived from the words *ta* and *nezumi* which in Japanese meaning rice-field and rat, respectively (Masuda 1974). The species is commonly referred to as either the Oriental house rat, Asian house rat or the Tanezumi rat (Musser & Carleton 2005; Heaney & Molur 2008).

Ecology – Given its recent discovery in South Africa (and Africa), the ecology of *R. tanezumi* in South Africa (and Africa) is currently unknown. Extralimitally, it has been recorded to live in swampy/marshy areas and in close proximity to water. They mainly ingest rice and are abundant in rice-fields (Temminck, 1844). The species can adapt easily and often occurs in close proximity to villages and agricultural areas (Heaney & Molur 2008 and references therein). The relatively longer tail that is also somewhat thinner at its base, longer and thinner front and hind toes, and smaller feet (as in *R. rattus*) may be attributed to the more arboreal life style of the species than the more terrestrial life style of *R. novergicus* that has relatively shorter and thicker front and hind toes, and relatively larger feet.

Remarks – A chromosome diploid number of 2*n* = 42 has been recorded for *R. tanezumi* (Yoshida 1980). The diploid numbers of South African *R. tanezumi* has been reported to be 2*n* = 40 (Bastos *et al.* unpublished). The species, a supposedly south-east Asian endemic has been recorded for the first time in South Africa (and Africa) in Limpopo and Kwa-Zulu Natal Provinces of South Africa (Bastos *et al.* 2005; Taylor *et al.* 2008; see also Chapter 2 and Fig. 2.3), and subsequently in the Gauteng Province (Bastos *et al.* unpublished; Chapter 2; Fig. 2.3) and in Swaziland (Bastos *et al.* unpublished). D-loop sequence data indicate that *R. tanezumi* has a low degree of genetic variation in South Africa with only one haplotype recovered. This may be indicative of a single, recent introduction, with Indonesia being its possible centre of origin (Chapter 2). In South Africa, the species occurs sympatrically with *R. rattus* at a number of localities, and with *R. norvegicus* at one locality (Chapter 2; Figs. 2.2 & 2.3). There is a critical need for extensive sampling in South Africa and beyond in order to determine the systematics (including population genetics), ascertain the centre of origin of the species in South Africa and to determine the extent of its geographic distribution. There is also a critical need to investigate the ecology (including the population dynamics) of the three species of *Rattus* known to occur in South Africa and how they interact with each other as well as with indigenous fauna in order to assess the nature and extent of zoonotic disease potential and damage to agricultural products. The species is a known carrier of zoonotic diseases including *Bartonella elizabethae* which causes infective endocarditis in humans (Chapter 4).

3.7.1.3 Rattus norvegicus (Berkenhout, 1769)

For a full synonymy see Ellerman and Morrison-Scott (1951: 581-587).

1769. *Rattus norvegicus* Berkenhout: *Outl. Nat. Hist. Gt. Britain & Ireland*, **1**:5.

Holotype – No information on the holotype is available. Names were coined prior to the convention of assigning type specimens (K. Aplin *pers. comm*.).

Geographic distribution – *Rattus norvegicus* originated from the colder regions of Palaearctic Asia but now has a cosmopolitan geographic distribution as an invasive and commensal species (Skinner & Smithers 1990; Musser & Carleton 2005). The species is more widespread in colder areas, but is also found in human-modified environments in

warmer regions (Musser & Carleton 2005; Chapter 1; Fig. 1.3). In South Africa, *R. norvegicus* has traditionally been considered to be confined to coastal towns and harbours (Skinner & Smithers 1990; Apps 2000). Recently however, it has been recorded as far inland as Gauteng Province (Bastos *et al.* unpublished. data; Chapter 2; Fig. 2.2). For worldwide distribution see Musser & Carleton (2005).

Diagnosis – *Rattus norvegicus* has a relatively larger and robust skull, a relatively wide braincase, and a relatively shorter tail than the length of head and body that is also relatively thicker at its base than *R. rattus*/*R. tanezumi.* The supraorbital ridge is more curved in *R. norvegicus* than in *R. rattus*/*R. tanezumi* where it is more angular (Fig. 3.2a). The jugal bone of *R. norvegicus* is more robust, relatively thicker, and less fragile than that of *R. rattus*/*R. tanezumi* (Fig. 3.2b). The posterior margin of the palatal foramen of *R. novergicus* does not reach the anterior margin of the first set of maxillary molars (M^1), while that of *R. rattus*/*R. tanezumi* is level with or exceeds the anterior margin of the first set of maxillary molars (M^1) (Fig. 3.4). The antero-external cusp on M^1 of *R. norvegicus* is reduced while that of *R. rattus*/*R. tanezumi* is not (Fig. 3.4). *Rattus novergicus* has a relatively shorter and thicker front and hind toes, and relatively larger feet (Figs. 3.5 & 3.6), and relatively smaller ears than *R. rattus*/*tanezumi*. There are no discernible consistent differences in pelage colouration with all three species of *Rattus* known to occur in South Africa showing high degrees of pelage colour variation both within and between species that dorsally varies from brownish and blackish grey, brownish, to orange-yellowish, and ventrally from yellowish to completely white.

Etymology – The species name is derived from Latin for Norway where it is considered to have originated from (De Graaff 1981). The species is commonly referred to as either the brown rat, the Norway rat or the brown house rat (Meester & Setzer 1971; De Graaf 1981; Meester *et al.* 1986; Musser & Carleton 2005).

Ecology – The species is an invasive commensal that inhabits marshes, fields, basements and storage areas (Davis 1986) and wherever there are abundant food sources (Skinner & Smithers 1990). *Rattus norvegicus* is more dependent on water than *R. rattus* (Skinner & Smithers 1990), and it mainly prefers terrestrial habitats, particularly coastal and waterside environments (Amori & Clout 2003). Unlike *R. rattus*, the species holds its tail above the ground when running (Lawrence & Brown 1967). This, together

with the relatively shorter and thicker front and hind toes, and relatively larger feet may be attributed to the more terrestrial life style of the species than the more arboreal life styles of *R. rattus* and *R. tanezumi* that have a relatively longer tail that is also thinner at its base, longer and thinner front and hind toes, and relatively smaller feet.

Remarks – Rattus norvegicus has a chromosome diploid number of *2n* = 42 (Yosida 1980). The two haplotypes recovered in South Africa based on D-loop sequence data (Chapter 2) may be indicative of two separate introductions, but the centre of origin is difficult to determine because of the cosmopolitan distribution of the species. There is a critical need for extensive sampling in South Africa and beyond in order to determine the systematics (including population genetics), the centre of origin of the species in South Africa, and the extent of its geographic distribution. The recent discovery of *R. norvegicus* in Gauteng Province, South Africa indicates that the geographic distribution of the species is more widespread than previously considered. The species has been shown to be sympatric with *R. tanezumi* at one locality (Chapter 2; Figs. 2.2 & 2.3). There is also a critical need to investigate the ecology (including the population dynamics) of the three species of *Rattus* known to occur in Africa and how they interact with each other as well as with indigenous fauna in order to assess the nature and extent of zoonotic disease potential and damage to agricultural products. In South Africa, *R. norvegicus* is associated with various zoonotic diseases including *Bartonella elizabethae* which causes infective endocarditis in humans (Chapter 3) and with *Helicobacter muridarum,* a bacterial species with no known zoonotic potential. The prevalence of both bacterial genera was higher in *R. norvegicus* than in *R. rattus* and *R. tanezumi* (Chapter 4).

3.8 REFERENCES

ABDEL-RAHMAN, E. H., *et al.* 2008. Geometric craniometric analysis of sexual dimorphism and ontogenetic variation: A case study based on two geographically disparate species, *Aethomys ineptus* from southern Africa and *Arvicanthis niloticus* from Sudan (Rodentia: Muridae). *Mammalian Biology*, **74**: 361-373.

AMORI, G. & CLOUT, M. 2003. Rodents on islands: a conservation challenge. *In*: Rats, mice and people: rodent biology and management (Singleton, G. R., Hinds, L. A., Krebs, C. J. & Spratt, D. M.), Australian Centre for International Agricultural Research, Canberra. Pp. 63-68.

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.

APLIN, K. P., CHESSER, T., & TEN HAVE, J. 2003. Evolutionary biology of the genus *Rattus*: profile of an archetypal rodent pest. *In*: Rats, mice and people: rodent biology and management (*Singleton, G. R., Hinds, L. A., Krebs, C. J. & Spratt, D. M. eds*), Australian Centre for International Agricultural Research, Canberra. Pp. 487-498.

APPS, P. 2000. Smithers's mammals of Southern Africa. A field guide. Struik Publishers, Cape Town. p. 74

BAI, K. R. M. 2007. Rodent exclusion. *In*: Encyclopedia of Pest Management, Volume II (*Pimentel, D. eds*.), CRC Press, Taylor & Francis Group, USA. 562 pp.

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. *South African Society for Veterinary Epidemiology and Preventive Medicine*. Conference Proceedings, Pretoria, 11-12 August 2005. Pp. 78-83.

BAVERSTORCK, P. R. 1981. Book review. *Cytogenetics of the Black Rat* (Yosida T. H. eds), *Journal of Mammalogy* **62**: 866-867.

BAVERSTOCK, P. R., ADAMS, M., MAXSON, L. R. & YOSIDA, T. H. 1983. Genetic differentiation among karyotypic forms of the black rat, *Rattus rattus*. *Genetics* **105**: 969-983.

CARLETON, M. D. 1980. Phylogenetic relationships in neotomine–peromyscine rodents (Muroidea) and a reappraisal of the dichotomy within New World Cricetinae. Miscellaneous Publications, Museum of Zoology, University of Michigan **157**:1–146.

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

CHIMIMBA, C. T., DIPPENAAR, N. J. & 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.

CHINEN, A. A., SUZUKI, H., APLIN, K. P., TSUCHIYA, K. & SUZUKI, S. 2004. Preliminary genetic characterization of two lineages of black rats (*Rattus rattus* sensu lato) in Japan, with evidence for introgression at several localities. *Genes Genetic Systematics* **80**: 367–375.

DAVIS, E. D. 1986. The scarcity of rats and the black death: An ecological history. *Journal of Interdisciplinary History* **16**: 455-470.

DE GRAAFF, G. 1981. The rodents of southern Africa: notes on their identification, distribution, ecology and taxonomy. Butterworth, Durban. Pp. 218-228.

DIPPENAAR, N. J. & RAUTENBACH, I. L. 1986. Morphometrics and karyology of the southern African species of the genus *Acomys* I. Geoffroy Saint Hillaire, 1838 (Rodentia: Muridae). *Annals of the Transvaal Museum* **34**: 129-183.

DUPLANTIER, J-M., CATALAN, J., ORTH, A., GROLLEAU, B. & BRITTON-DAVIDIAN, J. 2003. Systematics of the black rat in Madagascar: consequences for the transmission and distribution of plague. *Biological Journal of the Linnean Society*, **78**: 335-341.

HARRISON, D. L. & BATES, P. J. J. 1991. The mammals of Arabia. Harrison Zoological Museum, Cornell University. 354pp.

HEANEY, L. & MOLUR, S. 2008. *Rattus tanezumi*. In: IUCN 2009. IUCN Red List of Threatened Species. Version 2009.1. <www.iucnredlist.org>. Downloaded on 15 July 2009.

HIRANO, M., DING, X., LI, T-C., TAKEDA, N., KAWABATA, H., KOIZUMI, N., KADOSAKA, T., GOTO, I., MASUZAWA, T., NAKAMURA, M., TAIRA, K., KUROKI, T., TANIKAWA, T., WATANABE, H. & ABE, K. 2003. Evidence for widespread infection of hepatitis E virus among wild rats in Japan. *Hepatology Research* **27**: 1-5.

HUGH-JONES, M. E., HUBBERT, W. T. & HAGSTAD, H. V. 1995. Zoonoses: recognition, control, and prevention. Iowa State University Press, Ames. 369 pp.

LAZARUS, A. B. 1989. Progress in rodent control and strategies for the future. *In*: Mammals as pests (*Putman, R. J. eds*), Chapman and Hall Ltd., London. Pp. 53-64.

LODAL, J. & LUND, M. 1989. Prevalence of *Pneumocystis carinii* and *Leptospira icterohaemorrhagiae* in Danish rodents. *In*: Mammals as pests (*Putman, R. J. eds*), Chapman and Hall Ltd., London. Pp. 51-52.

MATHIAS, M. D. & MIRA, A. 1992. On the origin and colonization of house mice in the Madeira Islands. *Biological Journal of the Linnean Society* **46**: 13-24.

MATISOO-SMITH, E. & ROBINS, J. H. 2004. Origins and dispersal of Pacific peoples: Evidence from mtDNA phylogenies of the Pacific rat. *Proceedings of the National Academy of Sciences of the USA* **101**: 9167-9172.

MATISOO-SMITH, E. & ROBINS, J. 2009. Mitochondrial DNA evidence for the spread of Pacific rats through Oceania. *Biological Invasions* **11**: 1521-1527.

MEESTER, J. & SETZER, H. W. 1971. The mammals of Africa: an identification manual. Smithsonian Institution Press, Washington DC. 505 pp.

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

MILJUTIN, A. & LEHTONEN, J. T. 2008. Probablility of competition between introduced and native rodents in Madagascar: An estimation based on morphological traits. *Estonian Journal of Ecology* **57**: 133-152.

MILLS, J. N. 1999. The role of rodents in emerging human disease: examples from the Hantaviruses and Arenaviruses. *In:* Ecologically-based Rodent Management (Singleton, G. R., Hinds, H. A., Leirs, H. & Zhang, Z. eds), Australian Centre for International Agricultural Research, Canberra. Pp. 134-160.

MOSTERT, M. E., CHIMIMBA, C. T. & BASTOS, A. D. S. 2007. *Helicobacter* and *Bartonella* prevalence in invasive rodents of the *Rattus* species complex (Rodentia: Muridae) in South Africa. Proceedings of the 7th South African Society for Veterinary Epidemiology and Preventive Medicine Conference, Somerset-West, South Africa, pp. 167.

MUSSER, G. G., & CARLETON, M. D. 2005. Family Muridae. *In*: Mammal Species of the World, 3rd Edition (*Wilson, D. E. & Reeder, D. M. eds*). The Johns Hopkins Univeristy Press, Baltimore. Pp. 894–1531.

MYERS, P., LUNDRIGAN, B. L., GILLESPIE, B. W. & ZELDITCH, M. L. 1996. Phenotypic plasticity in skull and dental morphology in the prairie deer mouse (*Peromyscus maniculatus bairdii*). *Journal of Morphology* **229**: 229-237.

PIMENTEL, D. 2007. Encyclopedia of pest management, Volume II. CRC Press, Taylor & Francis Group, Boca Raton. 784 pp.

PUTMAN, R. J. 1989. Mammals as pests. Chapman & Hall Ltd, London. 288 pp.

ROBINS, J. H., HINGSTON, M., MATISOO-SMITH, E. & ROSS, H. A. 2007. Identifying *Rattus* species using mitochondrial DNA. Molecular Ecology Notes **7**: 717-729.

SCHLICK, N. E., JENSEN-SEAMAN, M. I., ORLEBEKE, K., KWITEK, A. E., JACOB, H. J. & LAZAR, J. 2006. Sequence analysis of the complete mitochondrial DNA in 10 commonly used inbred rat strains. *American Journal of Physiology and Cell Physiology*, **291**, 1183-1192.

SKINNER, J. D. & SMITHERS, R. H. N. 1990. *The Mammals of the Southern African Subregion*. CTP Book Printers, Cape Town. 736 pp.

STEFEN, C. & RUDOLF, M. 2007. Contribution to the taxonomic status of the Chinese rats *Niviventer confucianus* and *N. fulvescens*. *Mammalian Biology* **72**: 213-223.

TAYLOR, P. J., ARNTZEN, L., HAYTER, M., ILES, M., FREAN, J. & 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 DEN BRANDT, J., KOVÁCS, P. & KLÖTING, I. 2000. Wild rats (*Rattus norvegicus*) for mapping of disease-resistant genes. *Journal of Experimental Animal Science* **41**: 57-60.

YABE, T., BOONSONG, P. & HONGNARK, S. 1998. The structure of the pawpad lamellae of four *Rattus* species. *Mammal study* **23**: 129-132.

YIĞIT, N., ÇOLAK, E. & SÖZEN, M. 1998. The taxonomy and karyology of *Rattus norvegicus* (Berkenhout, 1769) and *Rattus rattus* (Linnaeus, 1758) (Rodentia: Muridae) in Turkey. *Turkish Journal of Zoology* **22**: 203-212.

YOSIDA, H. T. 1980. Cytogenetics of the black rat: karyotype evolution and species differentiation. University of Tokyo Press, Tokyo. 256 pp.

YOSIDA, H. T. 1973. Similarity of giemsa banding patterns of chromosomes in several species of the genus *Rattus*. *Chromosoma* **41**: 93-101.

3.9 FIGURE LEGENDS

Fig. 3.1 Map of South Africa showing the sampling localities of *Rattus* in the present study. The geographic coordinates of these localities and the sample sizes are indicated in Table 3.1.

Fig. 3.2 The dorsal views of the cranium of *Rattus rattus* (*rr*), *R. tanezumi* (*rt*) and *R. norvegicus* (*rn*) from South Africa showing differences in supraorbital ridges (a) and the thickness of the jugal bone (b).

Fig. 3.3 The ventral views of the cranium of *Rattus rattus* (*rr*), *R. tanezumi* (*rt*) and *R. norvegicus* (*rn*) from South Africa showing the distance between the posterior margin of the palatal foramen and the anterior margin of the first set of molars (M^1).

Fig. 3.4 The ventral views of the cranium of *Rattus rattus* (*rr*), *R. tanezumi* (*rt*) and *R. norvegicus* (*rn*) from South Africa showing the degree of reduction of the antero-external cusp on the first maxillary molar (M^1) (d).

Fig. 3.5 The hind feet of *Rattus rattus* (*rr*), *R. tanezumi* (*rt*) and *R. norvegicus* (*rn*) from South Africa showing the differences in thickness of the toes (e).

Fig. 3.6 The front feet of *Rattus rattus* (*rr*), *R. tanezumi* (*rt*) and *R. norvegicus* (*rn*) from South Africa showing the difference in the length and thickness of the toe (f).


Hammanskraal
Hillcrest, Pretoria
Tembisa
Waterkloof, Pretoria
OR Tambo Int. Airport

Fig. 3.1

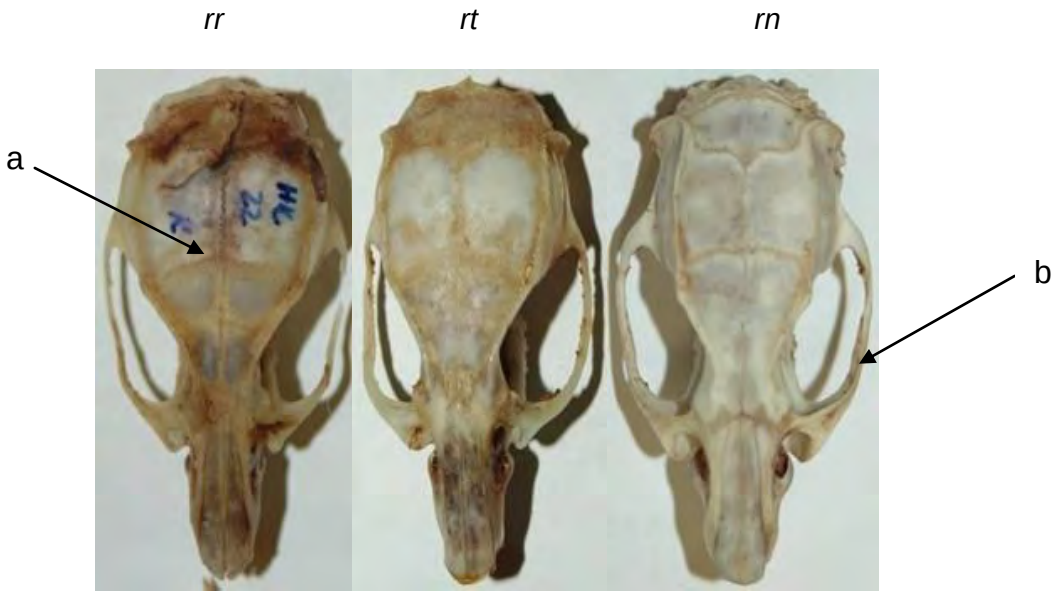

Fig. 3.2

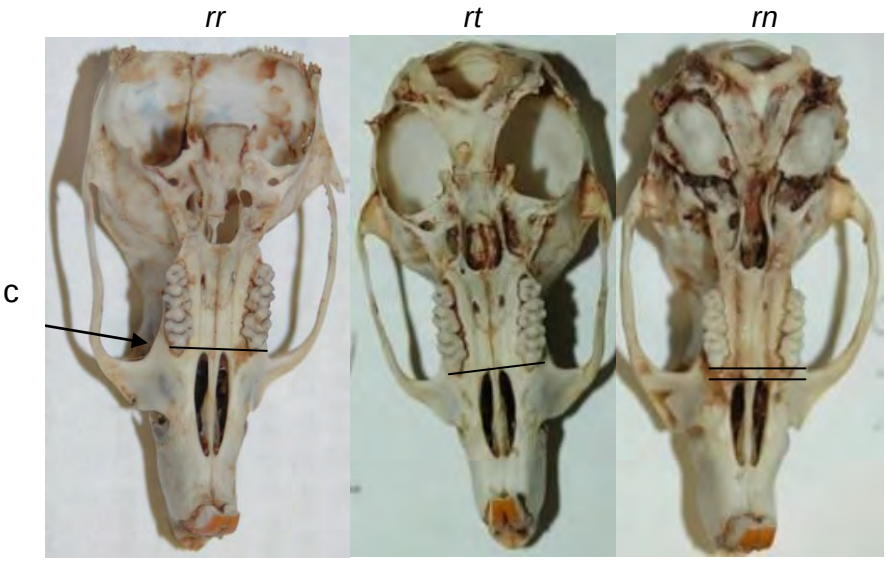

Fig. 3.3

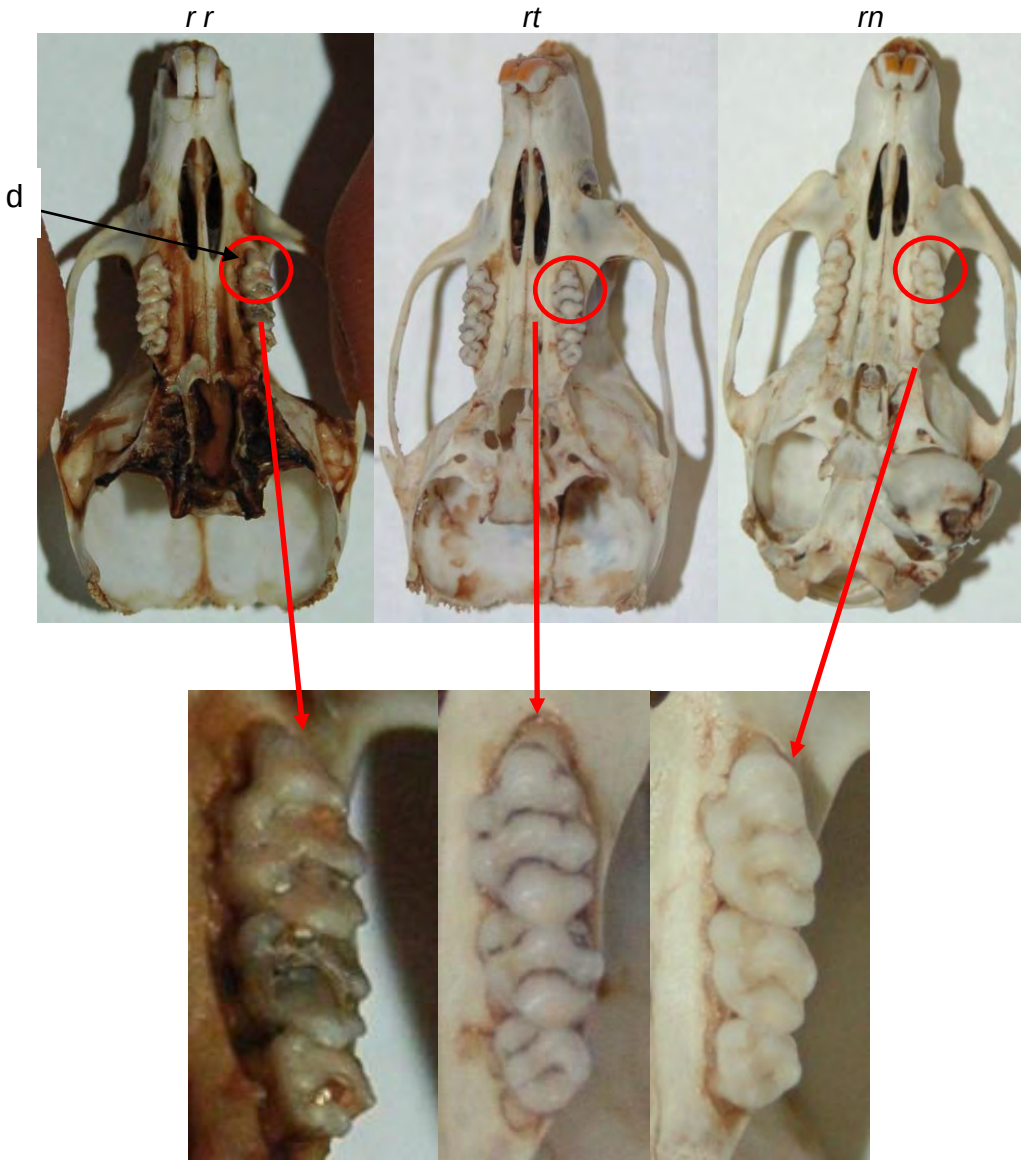

Fig. 3.4

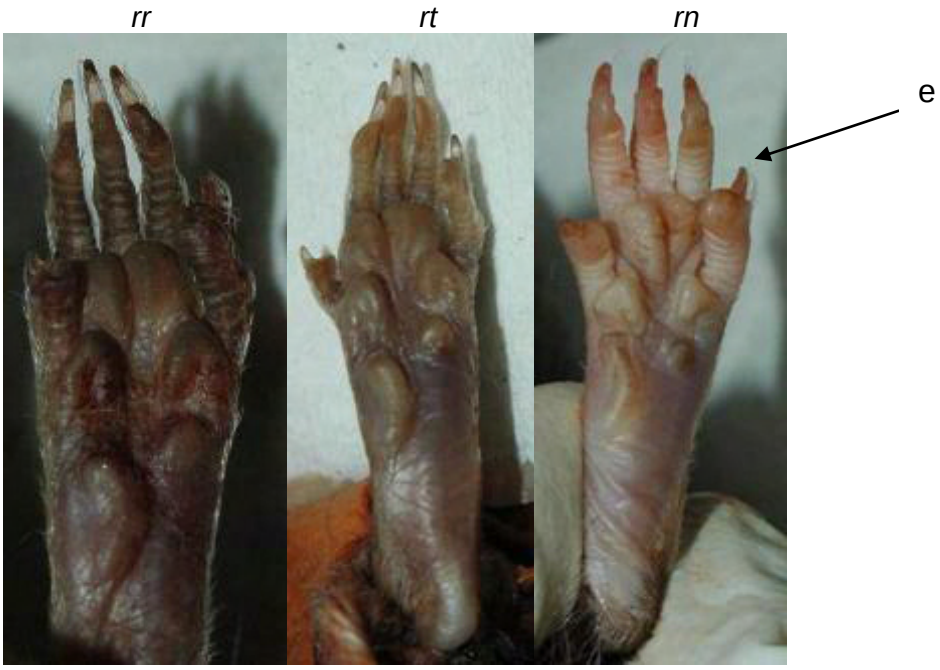

Fig. 3.5

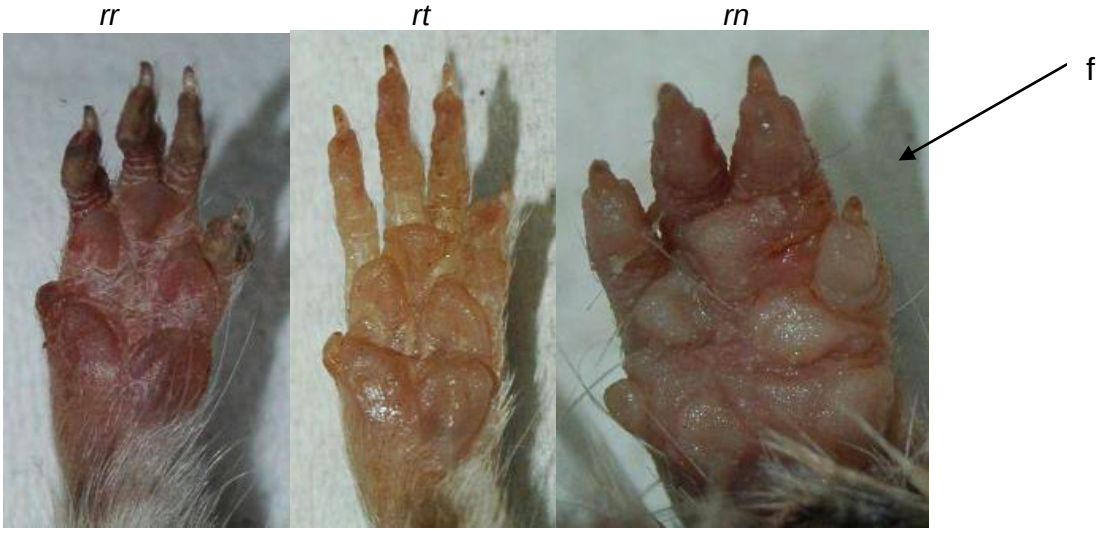

Fig. 3.6

Table 3.1 Geographic coordinates of the Gauteng Province sampling localities of *R. rattus* (*rr*), *R. tanezumi* (*rt*) and *R. norvegicus* (*rn*) from South Africa, and sample sizes used in the present chapter. A map showing these localites is shown in Fig. 3.2.

	Sampled locality	Sample size and species	Geographic coordinates
1	Hammanskraal	18 (8 *rr*; 10 *rt*)	25°22.27 S; 28°11.26 E
2	O.R. Tambo International Airport	23 (*rn*)	26°08.68 S; 28°13.57 E
3	Tembisa	4 (1 *rt;* 3 *rn*)	26°00.11 S; 28°12.78 E
4	Waterkloof, Pretoria	4 (*rt*)	25°49.13 S; 28°15.30 E
5	Hillcrest, Pretoria	1 (*rt*)	25°45.05 S; 30°59.28 E

CHAPTER 4

BARTONELLA PREVALENCE IN THREE INVASIVE AND COMMENSAL *RATTUS* SPECIES (RODENTIA: MURIDAE) FROM SOUTH AFRICA

4.1 ABSTRACT

Bartonella infection in animals is increasingly being reported, especially in members of the Order Rodentia. Currently, there are 19 recognised species of *Bartonella* Strong *et al.*, 1915 reported in the literature, of which many display zoonotic potential. *Bartonella elizabethae* (Daly *et al.,* 1993) Brenner *et al.,* 1993; a species known to cause severe cases of infective endocarditis in humans, has been recovered from members of the genus *Rattus* Fischer, 1803. This represents a potential threat to humans, especially in informal human settlements where rodent densities are high, but where *Bartonella* prevalence in *Rattus* is undetermined. In this study we assessed the prevalence of *Bartonella* in three members of the genus *Rattus*, namely *R. rattus* Linnaeus, 1758, *R. norvegicus* Berkenhout, 1769 and *R. tanezumi* Temminck, 1844, that occur commensally with humans in South Africa. One hundred and sixty two *Rattus* individuals were screened for bacterial presence by targeting the *gltA* gene region. Of these, 25 samples tested positive, corresponding to an overall prevalence of 15.4% which is markedly lower than that observed for endemic murid rodents (56.0%). There was however, a notable difference in the infection rate between the three *Rattus* congenerics evaluated. *Rattus rattus* and *R. tanezumi* had relatively low infection rates (4.7% and 6.8%, respectively) compared to *R. norvegicus* which had a prevalence of 39.0%. Of significant concern is the recovery of *B. elizabethae* from 24 of the 25 individuals that tested positive for *Bartonella*, as this bacterial species has been linked to infective endocarditis in humans. The recovery of *B. coopersplainensis* is also significant as it has only recently been documented for the first time in rats from Australia.

Key words: *Bartonella*; *Rattus*; invasive commensals; rodents; South Africa; zoonotic diseases

4.2 INTRODUCTION

The number of species in the genus *Bartonella* Strong *et al.,* 1915 has increased rapidly since 1992 when the genus comprised only a single species (Eremeeva *et al.* 2007), namely *B. bacilliformis* (Jacomo *et al.* 2002). Since then, 27 articles have been published on the recovery of novel species and currently the genus contains 33 named species in the National Centre for Biotechnology Information (NCBI) database. Of these, 19 species are officially recognised in the literature (Zeaiter *et al.* 2003; Fenollar & Raoult 2004; Eremeeva *et al.* 2007) with many having been linked to human diseases such as Carrion's disease, Oroya fever, endocarditis, trench fever and cat scratch disease (Anderson 1991; Fenollar & Raoult 2004; Birtles 2005). It has been confirmed that *Bartonella* has zoonotic potential (La Scola *et al.* 2003).

Members of the genus *Bartonella* are fastidious, gram-negative (Houpikian & Raoult 2001; La Scola *et al.* 2003) cocco-bacilli that infect the erythrocytes of their vertebrate hosts (La Scola *et al.* 2003). The bacterium is generally transmitted by haematophagous arthropod vectors (Birtles *et al.* 2001; La Scola *et al.* 2003; Birtles 2005), although vertical transmission is another mode by which it can be spread (Kosoy *et al.* 1998). *Bartonella* seem to be highly successful parasites as they have been confirmed in a wide variety of mammalian species studied to date (Birtles 2005), including bats (Concannon *et al.* 2005), carnivores (Breitschwerdt & Kordick 2000; Chomel *et al.* 2003; Engbæk & Lawson 2004; Boulouis *et al.* 2005), primates (Breitschwerdt & Kordick 2000; Chomel *et al.* 2003; Birtles 2005; Eremeeva *et al.* 2007), rabbits (Heller *et al.* 1999) and ungulates (Kelly *et al.* 2005). Bartonella is also present in a considerable number of rodent species in general and *Rattus* in particular (Table 4.1; Kosoy *et al.* 1997; Heller *et al.* 1998; Kosoy *et al.* 1998; Breitschwerdt & Kordick 2000; Birtles *et al.* 2001; Ying *et al.* 2002; Chomel *et al.* 2003; Kosoy *et al.* 2003; Engbæk & Lawson 2004; Pretorius *et al.* 2004; Boulouis *et al.* 2005; Mediannikov *et al.* 2005; Jardine *et al.* 2006; Bai *et al.* 2007; Bastos 2007; Knap *et al.* 2007).

4.2.1 Bartonella species identification

It has been suggested that DNA sequencing should be the tool for *Bartonella* species identifications because many species within the genus do not differ phenotypically (La Scola *et al.* 2003). To date a number of *Bartonella* species have been isolated from rodents, including *Rattus* (Table 4.1). Ellis *et al.* (1999) demonstrated that

members of the genus *Rattus* showed varying levels of infection in the United States of America. *Bartonella* prevalence for *R. norvegicus* ranged from 0.0% to 56.0%, depending on location, with an overall prevalence of 19.0% (Ellis *et al.* 1999). *Rattus rattus* had an overall prevalence of 12.0%, ranging from 9.0% to 60.0% depending on locality (Ellis *et al.* 1999). Species isolated were most similar to *B. elizabethae* (Ellis *et al*. 1999). Castle *et al.* (2004) showed that *Bartonella* isolated from *R. rattus* individuals from Thailand were most closely related to *B. elizabethae* and *B. grahamii*, both of which have zoonotic potential.

Consequently, it is possible that members of *Rattus* from South Africa may also be commonly infected based on the data from the USA and Thailand, particularly as infection rates of *Bartonella* in indigenous rodent species in South Africa are high (Pretorius *et al.* 2004; Bastos 2007). Given the regular contact between commensal rodent host species and humans, it is also possible that all *Bartonella* species are potentially pathogenic to humans (Birtles *et al.* 2001). *Bartonella* infections can demonstrate unusual clinical signs when unintentionally introduced into individuals that are immune compromised or into an incorrect host (Birtles 2005). Examples of these symptoms include neurological complications such as brainstem encephalopathy caused by cat scratch disease (CSD; Genizi *et al.* 2007), lepromatous leprosy or bacillary peliosis (BAP; Johnson 2000), and bacillary angiomatosis (BA; Gasquet *et al.* 1998).

4.2.2 The status of Rattus in South Africa

Traditionally, two species of *Rattus* were known to occur in South Africa as invasive commensals, *R. rattus* and *R. norvegicus* (Skinner & Smithers 1990). The first record of *R. tanezumi*, an otherwise Asian endemic, in South Africa (and Africa) increases the number of invasive commensal species from two to three (Bastos *et al.* 2005; Taylor *et al.* 2008). All three species are associated with humans and are known pests of agriculture, food crops and stored grain (Singleton *et al.* 1999). Diseases of medical importance have also been linked to members of *Rattus* that occur commensally with humans (Grantz 1997). Members of the genus have for example been associated with viral haemorrhagic fever caused by the hanta- and arenaviruses (Mills 1999), hepatitis E virus (Hirano *et al.* 2003) and rat bite fever (Elliot 2007). Taylor *et al.* (2008) also recovered antibodies for leptospirosis and toxoplasmosis in individuals of *Rattus* from Durban, KwaZulu-Natal Province, South Africa. However, the overall prevalence of

Bartonella in *Rattus* has to date not been determined for members of this genus in South Africa.

4.2.3 Genes used for the delineation of phylogenetic species of Bartonella

Various genes are used for the phylogenetic analysis of *Bartonella*, namely the heat-shock protein (*groEL*), citrate synthase (*gltA*), cell division protein (*ftsZ*), riboflavin sythetase (*ribC*), and the 17 kDa antigen, all of which are protein-encoding (Houpikian & Raoult 2001). In addition, two non-coding regions, the 16S/23S intergenic spacer region (*ITS*) and *16S rRNA* genes (Houpikian & Raoult 2001) are also often targeted. There has been some uncertainty on the dependability of ITS sequences when assessing molecular evolution (Birtles *et al.* 2000). Although the *16S rDNA* gene is informative in revealing deeper evolutionary relationships, it is considered to be insensitive for phylogenetic studies within the genus *Bartonella* (Houpikian & Raoult 2001).

The protein-encoding genes seem to be conserved when assessing intra-specific variation but are highly variable inter-specifically and have therefore received increasing attention (Birtles & Raoult 1996). For species delimitation and the elucidation of evolutionary relationships, *gltA* is considered to be one of the most reliable gene regions (Houpikian & Raoult 2001) as it is capable of differentiating between closely related species (Ellis *et al.* 1999). Consequently, the *gltA* gene was targeted in the present study, with positive PCRs being used to obtain bacterial prevalence estimates, and nucleotide sequencing and phylogenetic analysis of the amplicons being used to identify the *Bartonella* species present within members of the invasive commensal genus *Rattus* in South Africa.

4.3 MATERIALS AND METHODS

4.3.1 Genetic analysis

4.3.1.1 Sampling

A total of 162 individuals of *Rattus* were sampled (43 *R. rattus*, 46 *R. norvegicus*, 73 *R. tanezumi*) from 12 localities in three South African provinces, namely Limpopo, Gauteng and KwaZulu Natal Provinces (Fig. 4.1; Table 4.2). These collecting localities ranged from rural villages, informal settlements, to metropolitan areas within the cities of Pretoria and Johannesburg (Gauteng Province). Sample sizes and geographic coordinates of the localities are shown in Table 4.2. The samples used in this study represent the same

specimens used in the mitochondrial DNA (Chapter 2) and qualitative cranial and external morphological (Chapter 3) analyses for the multidisciplinary characterization of members of the genus *Rattus* from South Africa, as well as for the assessment of the prevalence of *Helicobacter* (Chapter 5). These specimens are also being used in an on-going parallel geometric morphometric study of *Rattus* from South Africa (Chimimba *et al.* unpublished).

Animals were obtained through a number of approaches as follows: 1) Live-trapping using Sherman traps (H.B. Sherman Traps Inc. Florida, U.S.A.) baited with a mixture of peanut butter, syrup, oatmeal and fish oil; 2) Samples obtained through a European Commission/DFID-funded community-participatory research project on rodents in southern Africa where 10 snap traps were placed in 10 community households in a number of villages and inspected daily; 3) Samples obtained opportunistically from pest control companies during their routine extermination programmes at facilities such as the O.R. Tambo International Airport; and 4) Samples provided by the general public.

After capture, during transportation and in the laboratory, live-trapped animals were kept in polyurethane cages with wood shavings provided as bedding and mouse pellets and water provided *ad libitum* as per the guidelines of the American Society of Mammalogists (ASM; http://www.mammalogy.org/committees/index.asp. Halothane inhalation was used to euthanize live animals. Animals were dissected and the livers were removed and stored in either absolute ethanol or frozen at -20° C for subsequent molecular analysis. Voucher specimens were prepared using standard natural history museum procedures for mammal specimens and will be deposited in the mammal reference collection of the Transvaal Museum (TM) of the Northern Flagship Institute (NFI), Pretoria, South Africa and the Durban Natural Science Museum, South Africa.

4.3.1.2 Laboratory procedures

Total genomic DNA was extracted from liver samples using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minnesota, U.S.A.), or alternatively with the Roche High Pure PCR Template Preparation Kit (Roche Applied Systems, Germany). Supplier specified protocols for the extraction of DNA from animal tissue protocols were followed. Overnight digestion using proteinase K at 55° C was done irrespective of the kit that was

used. DNA was eluted in a final volume of 50 µl. Host species were genetically identified by amplifying and sequencing either the 1.2 kbp mitochondrial region of the cytochrome *b* gene (cyt *b*), or the 501 bp mitochondrial D-loop region, as described in Chapter 2. In order to confirm the presence of *Bartonella*, a 509 bp region of the citrate synthase *(gltA*) gene of the bacterial genome was targeted using the primers designed by Bastos (2007). Polymerase chain reaction (PCR; Mullis & Faloona 1987) amplification was performed using a touchdown PCR reaction in which denaturation was always performed at 96° C for 12 s and extension at 72° C for 40 s. Annealing temperatures were 53° C for the first 3 cycles, 51° C for 5 cycles and 50° C for the remaining 32 cycles. These cycles were preceded by a 20 s denaturation step at 96° C and followed by a final extension at 72° C for 60 s.

Negative controls were included to control for reagent contamination. A positive control was included to exclude the possibility of false negatives, and to provide a reference band for molecular sizing. Products were electrophoresed on a 1.5% low-melting point agarose gel stained with Goldview and visualized under UV light. Bacterial genome presence was confirmed by PCR amplification of the 509 bp band and scored as positive. To ensure repeatability of results, all samples were screened three times and all PCR reactions were run on the same machine to mitigate variations introduced through the use of different machines. Positive PCR products were purified using the Roche Purification Kit and then cycle-sequenced with Big Dye version 3.1 terminator chemistry (Applied Biosystems [ABI]). Sample DNA was then precipitated for sequencing analysis.

4.3.1.3 Phylogenetic analysis

Sequence chromatograms were viewed and edited in Chromas (McCarthy 1996-1997) and then exported to DAPSA (Harley 1994) for multi-sequence alignment. Sequence data of reference strains homologous to that generated for positive samples, were downloaded from the Genbank database (www.ncbi.nlm.nih.gov) and used in all subsequent analyses.

Phenetic (distance methods) and molecular evolutionary analyses were performed using three programs. Neighbor-joining (NJ) clustering analysis was performed in MEGA version 4.0 (Tamura *et al.* 2007) and trees were midpoint rooted.

Model selection was guided by the best-fit model for the *Bartonella* nucleotide dataset determined in Modeltest 3.06b (Posada and Crandall 1998). Maximum Parsimony (MP) analyses were conducted using PAUP*4.0b10 (Swofford 2002) using stepwise addition with 1000 random addition replicates and tree-bisection-reconnection (TBR) branch-swapping. Bootstrap support (Felsenstein 1985) based on 10000 and 1000 replicates were used to assess confidence intervals for the NJ and Maximum Parsimony (MP) analyses, respectively. MrBayes version 3.1 (Huelsenbeck and Ronquist 2001) was used to perform Bayesian Inference (BI) analyses with the Markov Chain Monte Carlo (MCMC; Hastings 1970) method being used to determine posterior probabilities. Four chains were run simultaneously (three heated and one cold) in order to increase the chances of finding peaks in tree/parameter space. Priors were those identified in Modeltest under the best-fit model (K81uf+I+G) selected under the Akaike Information Criterion (AIC; Akaike 1974). A burn-in of 25% of the run length was discarded after runs of 10 000, 100 000 and 1 000 000 generations were performed.

4. 4 RESULTS

4.4.1 Bartonella sequence data

A homologous *Bartonella gltA* gene dataset, 403 nucleotides (nt) in length was generated for all *Rattus* samples that were positive and was complemented with homologous sequences recovered from the Genbank database, resulting in a final dataset containing 69 taxa, and inclusive of 15 *Bartonella* species (Table 4.3). The dataset contained 227/403 (56.3%) conserved sites and 176/403 (43.7%) variable sites, of which 137 (34.0%) were parsimony-informative and 39 were singletons (9.7%). The average nucleotide composition estimated under the K81uf+I+G model selected under the AIC in Modeltest was 30.7%, 19.4%, 16.0% and 33.9% for A, C, G and T, respectively, indicating a strong AT-bias (64.6%). Under this model, the proportion of invariable sites (I) and the gamma distribution shape parameter were 0.3705 and 0.6442, respectively.

4.4.2 Neighbor-joining (NJ), Maximum parsimony (MP) and Bayesian Inference (BI) analyses

The MP analysis of the *gltA* sequences yielded 216 possible trees (L = 581, CI = 0.382, RI = 0.846, RCI = 0.296, HI = 0.618) and from which a strict consensus tree was drawn. In addition, characters were reweighted using the rescaled consistency index

(RCI) values which resulted in the same number of trees being recovered (L = 167.5, CI = 0.539, RI = 0.846, RCI = 0.456 and HI = 0.461) and which was followed by drawing a strict consensus tree. No topological changes were noted between the consensus tree obtained with the equally weighted characters and that obtained following reweighting of characters with the RCI.

Tree topologies were similar for the MP and BI analyses with polytomies resulting with both methods, for the same species. The NJ tree had similar groupings to that of the MP and BI trees but the polytomies observed in the MP and BI trees at the shallower nodes, were resolved in the NJ tree. The phenetic tree with bootstrap support values from 1000 replications is therefore presented in Fig. 4.2 together with the support values > 50% obtained from the MP and BI analyses.

Samples used in this study were screened three times and if a sample was positive two out of three times, the individual was recorded as positive. By so doing, *Bartonella* was found to be present in 25 out of the 162 individuals screened (15.4%; Table 4.4). *Bartonella* prevalence differed markedly between the different host species within the genus *Rattus*. The *R. rattus* species complex, which consists of *R. rattus* Linnaeus, 1758 and *R. tanezumi* Temminck, 1844 (Musser & Carleton 2005) in South Africa, had an overall prevalence of 6 % (4.7% *R. rattus*, 6.8% *R. tanezumi*), whilst prevalence in *R. norvegicus* was substantially higher, with an overall infection rate of 39.0% being recorded for this introduced species (Table 4.4).

Phylogenetic analyses of the *gltA* gene region of *Bartonella* revealed the presence of five *Bartonella* haplotypes for the three invasive *Rattus* species screened in this study. Haplotype I was found in a single *R. rattus* individual from Nkomo-B in Limpopo Province, and is identical to a sequence generated for *Mastomys coucha* also sampled from Limpopo Province (73.0% bootstrap support - NJ) and characterized previously (Bastos 2007). Haplotype II is represented by three *R. norvegicus* individuals from O.R. Tambo International Airport (Johannesburg, Gauteng Province) and groups with *Bartonella* isolated from a *R. tanezumi* individual from Indonesia (66.0% bootstrap support - NJ). Haplotype III was recovered from two *Rattus* species, namely *R. norvegicus* (three individuals from Tembisa and 11 from O.R. Tambo International

Airport), and *R. tanezumi* (one individual from Ophuzane, three from Tholakele in northern Kwa-Zulu Natal and one individual from Hammanskraal in Gauteng Province).

Haplotype III grouped together with haplotype IV (78% bootstrap support - NJ; 63% bootstrap support - MP; 100% confidence interval - BI) which was recovered from a single *R. norvegicus* individual from Tembisa, Gauteng Province. These two haplotypes were most closely related to *Bartonella* identified in a *Rattus* (*Bartonella elizabethae*) individual from Peru (56% bootstrap support - NJ). All four haplotypes clustered within a *Bartonella elizabethae* lineage with strong support (90% bootstrap support - NJ; 99% bootstrap support - MP; 100% confidence interval - BI). Haplotype V, identified in a *R. rattus* individual from Nkomo-B in Limpopo Province, is identical to *Bartonella coopersplainensis* (100% bootstrap support - NJ; 100% bootstrap support - MP; 100% confidence interval - BI), a novel species recently isolated from Australian rats (Gundi *et al.* in press).

4.5 DISCUSSION

Bartonella infects various rodent species worldwide (eg. Birtles *et al*. 2001; Pretorius *et al.* 2004; Mediannikov *et al*. 2005; Bastos 2007; Bai *et al*. 2007; Knap *et al*. 2007). Bai *et al.* (2007) found a range of rodents in the grasslands of the United States infected with *Bartonella*, and recovered an overall prevalence of 52.4%. Similar results were recorded in rodents from Russia (60.0% to 83.0% prevalence; Mediannikov *et al.* 2005), Slovenia (40.4%; Knap *et al.* 2007) and the United Kingdom (64.2%; Birtles *et al.* 2001). In South Africa, small mammals from the Free State Province had a *Bartonella* infection rate of 44.0% (Pretorius *et al.* 2004) whereas endemic murid rodents living commensally with humans in Limpopo Province had a prevalence of 56.0% (Bastos 2007).

The 15.4% overall prevalence obtained for *Rattus* in this study contrasts markedly with the prevalence estimates recorded in the two previous South African studies on endemic species (Pretorius *et al.* 2004; Bastos 2007). In both studies, overall prevalence levels above 40.0% were recovered for a range of rodent species, which was consistent with results obtained following screening of rodent populations sampled in other parts of the world. The low prevalence recovered in this study is especially surprising as the individuals of *Rattus* from Limpopo Province in this study were captured

at two of the localities sampled in the study by Bastos (2007), in which endemic rodents were shown to have high levels of infection.

Results in the present study are however, consistent with those of Ellis *et al.* (1999) where overall prevalence of *Bartonella* in *R. rattus* and *R. norvegicus* was found to be 18.0%. These results indicate a relatively lower percentage of infection of individuals of *Rattus* with *Bartonella* than that observed in other small mammals screened to date (Birtles *et al.* 2001; Pretorius *et al.* 2004; Mediannikov *et al.* 2005; Bastos 2007; Knap *et al.* 2007). This suggests that in South Africa, endemic commensal small mammal species (Bastos 2007) may pose a greater threat to humans than the three *Rattus* species evaluated in this study. It is however, clear that of the three *Rattus* species screened, *R. norvegicus* poses the greatest zoonotic potential for *Bartonella* infection.

The difference between prevalence rates of endemic rodents (Pretorius *et al.* 2004; Bastos 2007) and invasive rodents (this study) in South Africa might indicate that indigenous species are more susceptible to *Bartonella* infection and hence have higher prevalence rates. It is possible that transmission of *Bartonella* between different rodent hosts may occur from the indigenous species to the invasive rodents where they occur sympatrically. Ellis *et al.* (1999) showed that native rodent species from the United States of America may be responsible for the occurrence of *Bartonella* in invasive *R. rattus* species. Members of the genus *Rattus* originated from the Old World whereas native United States of America rodents are classified as New World species. Ellis *et al.* (1999) showed that *Bartonella* isolates from *R. rattus* matched *Bartonella* associated with New World rodents and they therefore proposed that the occurrence of New World species of *Bartonella* in Old World *R. rattus* might be the result of native species infecting *Rattus* in areas where they come into contact with each other.

However, Ellis *et al.* (1999) also showed that *R. norvegicus* supposedly carried Old World *Bartonella* into the United States of America (Ellis *et al.* 1999) suggesting that *R. norvegicus* may also be a suitable host for *Bartonella* infection. This is supported by the high rates of *Bartonella* prevalence observed in *R. norvegicus* in the present study (39.0%; Table 4.4). The differences in prevalence rates in *R. rattus*, *R. tanezumi* and *R. norvegicus* in the present study and the results of Ellis *et al.* (1999) suggest that

Bartonella prevalence in members of *Rattus*, bacterial origins, host specificity and their transmission potential may not be consistent across congenerics, and therefore highlights the need for further investigation of these parameters and on the biology of the different host species.

The grouping of sequences from this study with those recovered from individuals occurring in other parts of the world may provide preliminary clues of the possible origin of the *Bartonella* species. The limited data and cosmopolitan distribution of members of *Rattus* should however, be taken into consideration. For example, the grouping of *Bartonella* isolated from a *R. rattus* individual with *B. coopersplainensis* may provide the first tenuous link between *Rattus* from Australia and South Africa, but would need additional confirmatory data than that provided by a single sample. Similarly, the *Bartonella* species isolated from *R. norvegicus,* groups together with a *Bartonella* species isolated from a *R. tanezumi* individual from Indonesia. The remaining *R. norvegicus* individuals as well as the *R. tanezumi* individuals from South Africa carry *Bartonella* that is most closely related to that isolated from a *Rattus* individual from Peru. The *B. coopersplainensis* link is especially interesting as it is the first time that this bacterial species has been documented in South Africa.

The recovery of *B. elizabethae* in the present study is significant as it is one of the species that has a confirmed zoonotic potential, having been recovered from cases of infective human endocarditis (Fenollar & Raoult 2004; Jacomo *et al.* 2006). Bacteria such as *B. elizabethae* that are capable of spreading zoonotically are particularly important especially since their hosts (e.g., *R rattus*, *R. norvegicus* and *R. tanezumi*) are commensal with humans (Amori & Clout 2003; Aplin & Singleton 2003; Chapter 2). In South Africa (this study; Chapter 2), animals were captured in urban (Hillcrest; Moreleta Park; Rietondale; Waterkloof), peri-urban (Tembisa; O.R. Tambo International Airport) and rural (Hammanskraal; Bloublommetjieskloof; Nkomo-B; Tholakele; Mvuzini; Ophuzane) settings in close association with humans (Chapter 2; Fig. 4.1; Table 4.2).

Approximately 5.27 million people in South Africa are living with HIV infection, and although HIV prevalence has remained relatively stable in the Gauteng Province, rural areas of the KwaZulu Natal and Limpopo Provinces have shown increased infection rates (National Department of Health Report 2008). The greater susceptibility of HIV-

positive individuals to infectious diseases (Stillwaggon 2001), highlights the need to evaluate lesser-known diseases, such as Bartonellosis. A wider variety of infections are connected to *Bartonella* in individuals with HIV/AIDS (Burgess *et al.* 1999), as immune compromised individuals are at greater risk of systemic problems when bitten by infected animals, causing endocarditis in severe cases (Brook 2003). These problems are exacerbated further as *Bartonella* infections, although treatable, are often mis-diagnosed or go undiagnosed in immune compromised individuals, mainly because these infections are diverse and symptoms are unclear (Spach & Koehler 1998). The recovery of *B. elizabethae* in *Rattus* individuals as well as endemic murid rodents (Bastos 2007) living commensally with humans is therefore highly significant and suggests the need for further studies on *Bartonella* infection in murid rodent commensals.

4.6 REFERENCES

AKAIKE, H. 1974. A new look at statistical model identification. *IEEE Transactions on Automatic Control* **19**: 716–722.

ANDERSON, B. E. & NEUMAN, M. A. 1997. Bartonella spp. as emerging human pathogens. *Clinical Microbiology Reviews* **10**: 203-219.

AMORI, G. & CLOUT, M. 2003. Rodents on islands: a conservation challenge. *In*: Rats, mice and people: rodent biology and management (Singleton, G. R., Hinds, L. A., Krebs, C. J. & Spratt, D. M.), Australian Centre for International Agricultural Research, Canberra. Pp. 63-68.

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.

APLIN, K. P. & SINGLETON, G. R. 2003. Balancing rodent management and small mammal conservation in agricultural landscapes: challenges for the present and the future. *In*: Rats, mice and people: rodent biology and management (Singleton, G. R., Hinds, L. A., Krebs, C. J. & Spratt, D. M.), Australian Centre for International Agricultural Research, Canberra. Pp. 80-88.

BAI, Y., KOSOY, M. Y., CULLY, J. F., BALA, T., RAY, C. & COLLINGE, S. K. 2007. Acquisition of non-specific *Bartonella* strains by the northern grasshopper mouse (*Onychomys leucogaster*). *FEMS Microbiology Ecology* **61**: 438-448.

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. *South African Society for Veterinary Epidemiology and Preventive Medicine*. Proceedings Pretoria, 11-12 August 2005. 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.

BIRTLES R. J. & 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., HAZEL, S. M., BENNETT, M., BOWN, K., RAOULT, D. and BEGON, M. 2001. Longitudinal monitoring of the dynamics of infections due to *Bartonella* species in UK woodland rodents. *Epidemiology and Infection* **126**: 323-329.

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

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

BROOK, I. 2003. Microbiology and management of human and animal bite wound infections. *Primary Care: Clinics in Office Practice* **30**: 25–39.

BOULOUIS, H-J., CHANG, C-C., HENN, J. B., KASTEN, R. W. & CHOMEL, B. B. 2005. Factors associated with the rapid emergence of zoonotic *Bartonella* infections. *Veterinary Research* **36**: 383-410.

BURGESS, A. W. O., JOHNSON, D. & ANDERSON, B. E. 1999. *In*: Opportunistic Intracellular Bacteria and Immunity, (*eds*) L. J. Paradise, M. Bendinelli & H. Friedman, Kluwer Academic / Plenum Publishers, Pp. 201-212.

CASTLE, K. T., KOSOY, M., LERDTHUSNEE, K., PHELAN, L., BAI, Y., GAGE, K. L., LEEPITAKRAT, W., MONKANNA, T., KHLAIMANEE, N., CHANDRANOI, K., JONES, J. W. & COLEMAN, R. E. 2004. Prevalence and diversity of *Bartonella* in rodents of northern Thailand: A comparison with *Bartonella* in rodent from

southern China. *American Journal of Tropical Medicine and Hygiene* **4**: 429-433.

CHOMEL, B. B., KASTEN, R. W., SYKES, J. E., BOULOUIS, H-J. & BREITSCHWERDT, E. B. 2003. Clinical impact of persistent *Bartonella* bacteremia in humans and animals. *Annals of the New York Academy of Sciences* **990**: 267-278.

CONCANNON, R., WYNN-OWEN, K., SIMPSON, V. R. & BIRTLES, R. J. 2005. Molecular characterization of haemoparasites infecting bats (Microchiroptera) in Cornwall, UK. *Parasitology* **131**: 489-496.

ELLIOT, S. P. 2007. Rat bit fever and *Streptobacillus moniliformis*. *Clinical Microbiology Reviews* **20**: 13-22.

ELLIS, B. A., REGNERY, R. L., BEATI, L., BACELLAR, F., ROOD, M., GLASS, G. G., MARSTON, E., KSIAZEK, T. G., JONES, D. & CHILDS, J. E. 1999. Rats of 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.

ENGBÆK, K. & LAWSON, P. A. 2004. Identification of *Bartonella* species in rodents, shrews and cats in Denmark: detection of two *B. henselae* variants, one in cats and the other in the long-tailed field mouse. *APMIS* **112**: 336-341.

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. & KOEHLER, J. E. 2007. Bacteremia, fever, and splenomegaly caused by a newly recognised *Bartonella* species. *The New England Journal of Medicine* **356**: 2381-2387.

FELSENSTEIN, J., 1985. Confidence limits of phylogenies: an approach using the bootstrap. *Evolution* **39**: 783-791.

FENOLLAR, F. & RAOULT, D. 2004. Molecular genetic methods for the diagnosis of fastidious microorganisms. *APMIS* **112**: 785-807.

GASQUET S., MAURIN, M., BROUQUI, P., LEPIDI H. & RAOULT, D. 1998. Bacillary angiomatosis in immunocompromised patients. *AIDS* **12**: 1793-1803.

GENIZI, J., KASIS, I., SCHIF, A. & SHAHAR, E. 2007. Effect of high-dose methyl-prednisolone on brainstem encephalopathy and basal ganglia impairment complicating cat scratch disease. *Brain & Development* **29**: 377-379.

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

GUNDI, V. A. K. B., TAYLOR, C., RAOULT, D., & LA SCOLA, B. *Bartonella* species in Australian rats: Description of *Bartonella rattaustraliani* sp. nov, *Bartonella queenslandensis* sp. nov., and *Bartonella coopersplainensis* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* (in press).

HARLEY, E. H. 1994. DAPSA, DNA and Protein Sequence Analysis, version 2.9. Department of Chemical Pathology, University of Cape Town.

HASTINGS, W. K. 1970. Monte Carlo sampling methods using Markov chains and their applications. *Biometrika* **57**: 97–109.

HELLER, R., RIEGEL, P., HANSMANN, Y., DELACOUR, G., BERMOND, D., DEHIO, C., LAMARQUE, F., MONTEIL, H., CHOMEL, B. & 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. & 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.

HIRANO, M., DING, X., LI, T-C., TAKEDA, N., KAWABATA, H., KOIZUMI, N., KADOSAKA, T., GOTO, I., MASUZAWA, T., NAKAMURA, M., TAIRA, K., KUROKI, T., TANIKAWA, T., WATANABE, H. & ABE, K. 2003. Evidence for widespread infection of hepatitis E virus among wild rats in Japan. *Hepatology Research* **27**: 1-5.

HOUPIKIAN, P. & RAOULT, D. 2001. Molecular phylogeny of the genus *Bartonella*: what is the current knowledge? *FEMS Microbiology Letters* **200**: 1-7.

HUELSENBECK, J. P. & RONQUIST, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Oxford University Press **17**: 754-755.

JACOMO, V. KELLY, P. J. & RAOULT, D. 2002. Natural history of *Bartonella* infections (an exception to Koch's Postulate). *Clinical and Diagnostic Immunology* **9**: 8-18.

JARDINE, C., McCOLL, D., WOBESER, G. & LEIGHTON, F. A. 2006. Diversity of *Bartonella* genotypes in Richardson's ground squirrel populations. *Vector-borne and Zoonotic Diseases* **6**: 395-403.

JOHNSON, R. A. 2000. The immune compromised host in the twenty-first century: Management of mucocutaneous infections. *Seminars in Cutaneous Medicine and Surgery* **19**; 19-61.

KELLY, P. J., DAVOUST, B., GOMEZ, J., RAOULT, D. & LA SCOLA, B. *Bartonella bovis* in cattle in Africa. *Veterinary Microbiology* **105**: 155-156.

KNAP, N., DUH, D., BIRTLES, R., TRILAR, T., PETROVEC, M. & AVŠIČ-ŽUPANC, T. 2007. Molecular detection of *Bartonella* species infecting rodents in Slovenia. *FEMS Immunology and Medical Microbiology* **50**: 45-50.

KOSOY, M. Y., REGNERY, R. L., TZIANABOS, T., MARSTON, E. L., JONES, D. C., GREEN, D., MAUPIN, G. O., OLSON, J. G. & CHILDS, J. E. 1997. Distribution, diversity, and host specificity of *Bartonella* in rodents from the Southeastern United States. *American Journal of Tropical Medicine and Hygiene* **57**: 578-588.

KOSOY, M. Y., REGNERY, R. L., KOSAYA, O. I., JONES, D. C., MARSTON, E. L. & 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., MURRAY, M., GILMORE, JR., R. D., BAI, Y. & GAGE, K. L. 2003. *Bartonella* strains from ground squirrels are identical to *Bartonella washoensis* isolated form a human patient. *Journal of Clinical Microbiology* **41**: 645-650.

LA SCOLA, B., ZEAITER, Z., KHAMIS, A. & RAOULT, D. 2003. Gene-sequence-based criteria for species definition in bacteriology: the *Bartonella* paradigm. *TRENDS in Microbiology* **11**: 318-321.

McCARTHY, C. 1996-1997. Chromas, version 1.43. School for Biomolecular and Biomedical Science. Griffith University, Australia.

MEDIANNIKOV, O., IVANOV, L., ZDANOVSKAYA, N., VYSOCHINA, N., FOURNIER, P-E, TARASEVICH, I. & RAOULT, D. 2005. Molecular screening of *Bartonella* species in rodents from the Russian Far East. *Ann. New York Academy of Science* **1063**: 308-311.

MILLS, J. N. 1999. The role of rodents in emerging human disease: examples from the Hantaviruses and Arenaviruses. *In*: Ecologically-based Rodent Management (*Singleton, G. R., Hinds, L. A., Leirs H. & Zhang, Z. eds*), Australian Centre for International Agricultural Research, Canberra. Pp. 134-160.

MULLIS, K. B. & FALOONA, F. A. 1987. Specific synthesis of DNA in vitro *via* a polymerase catalyzed chain reaction. *Methods in Enzymology* **155**: 335-350.

NATIONAL DEPARTMENT OF HEALTH, SOUTH AFRICA. 2008. The National HIV and syphilis prevalence survey South Africa 2007. http://us-cdn.creamermedia.co.za/assets/articles/attachments/16274_antenatal_report.pdf . Accessed 21 August 2009.

POSADA, D. & CRANDALL, K. A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817-818.

PRETORIUS, A-M., BEATI, L. & 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.

SINGLETON, G., HINDS, L., LEIRS, H. & ZHANG, Z. (*Eds*). 1999. *Ecologically based rodent management.* Australian Centre for International Agricultural Research, Canberra. 494 pp.

SKINNER, J. D. & SMITHERS, R. H. N. 1990. *The Mammals of the Southern African Subregion*. CTP Book Printers, Cape Town. 736 pp.

SPACH, D. H. & KOEHLER, J. E. 1998. *Bartonella*-associated infections. *Infectious Disease Clinics of North America* **12**: 137-155.

STILLWAGGON, E. 2002. HIV/AIDS in Africa: Fertile terrain. *Journal of Development Studies* **38**: 1-22.

SWOFFORD, D. L. 2002. PAUP*: Phylogenetic Analysis using Parsimony (* and other methods), Version 4.0b10. Sinauer Associates, Sutherland, MA.

TAMURA, K., DUDLEY, J., NEI, M. & KUMAR, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**: 1596-1599.

TAYLOR, P. J., ARNTZEN, L., HAYTER, M., ILES, M., FREAN, J. & 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.

YING, B., KOSOY, M. Y., MAUPIN, G. O., TSUCHIYA, K. R. & GAGE, K. L. 2002. Genetic and ecologic characteristics of *Bartonella* communities in rodents in

Southern China. *American Journal of Tropical Medicine and Hygiene* **66**: 622-627.

ZEAITER, Z., FOURNIER, P. E., GREUB, G. and RAOULT, D. 2003. Diagnosis of *Bartonella* endocarditis by a Real-time Nested PCR assay using serum. *Journal of clinical microbiology* **41**: 919-925.

4.7 FIGURE LEGENDS

Fig. 4.1 Map of South Africa showing the sampling localities of species of *Rattus* from South Africa examined in the present study. Collecting localities and their geographic coordinates are indicated in Table 3.1.

Fig. 4.2 *GltA* gene tree depicting genetic relatedness of members of *Bartonella* isolated from members of the genus *Rattus* from South Africa inferred using the Neighbor-joining (NJ) algorithm. Maximum parsimony (MP) bootstrap support as well as Bayesian Inference (BI) posterior probability values are indicated next to the relevant nodes. MP bootstrap values are indicated in black (above the line), BI values are indicated in blue (above the line) while NJ bootstrap values are indicated in red (below the line). Only bootstrap values > 50 are indicated. *Bartonella* from individuals of *R. rattus* are indicated in red, those from *R. norvegicus* are indicated in green and *Bartonella* haplotypes that are present in more than one *Rattus* host species are indicated in pink. Sequences obtained from the Genbank database are preceded by their Genbank accession number.

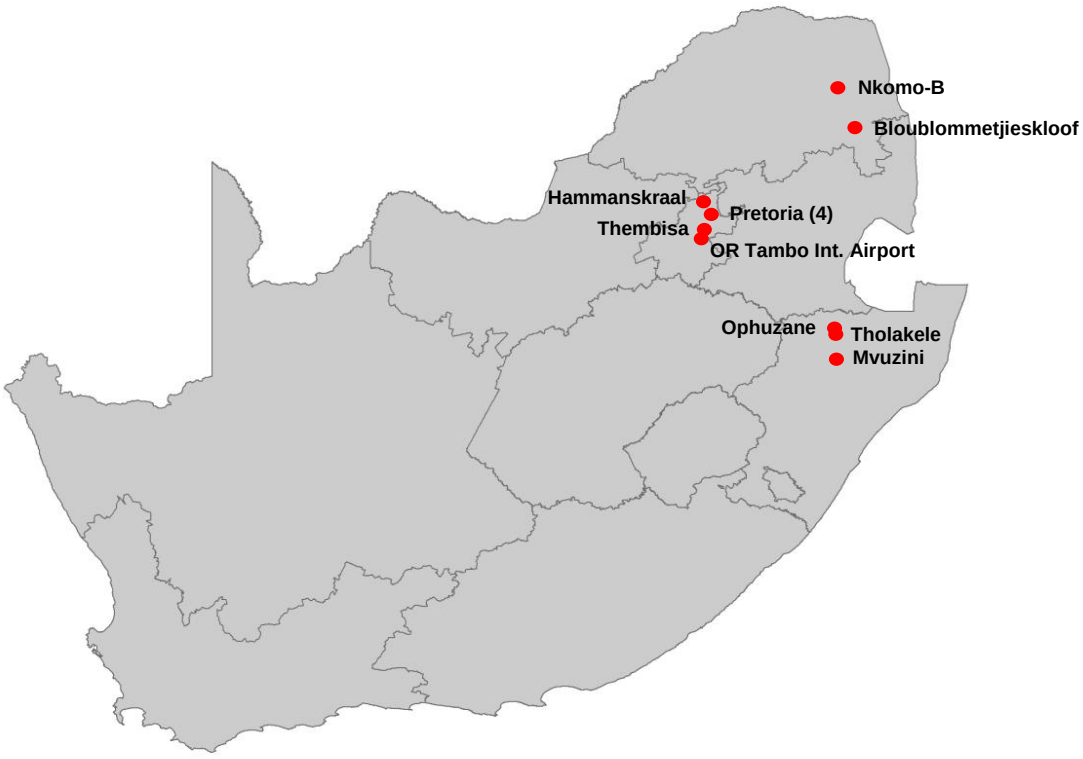
Nkomo-B
Bloublommetjieskloof
Hammanskraal
Pretoria (4)
Thembisa
OR Tambo Int. Airport
Ophuzane
Tholakele
Mvuzini

Fig. 4.1

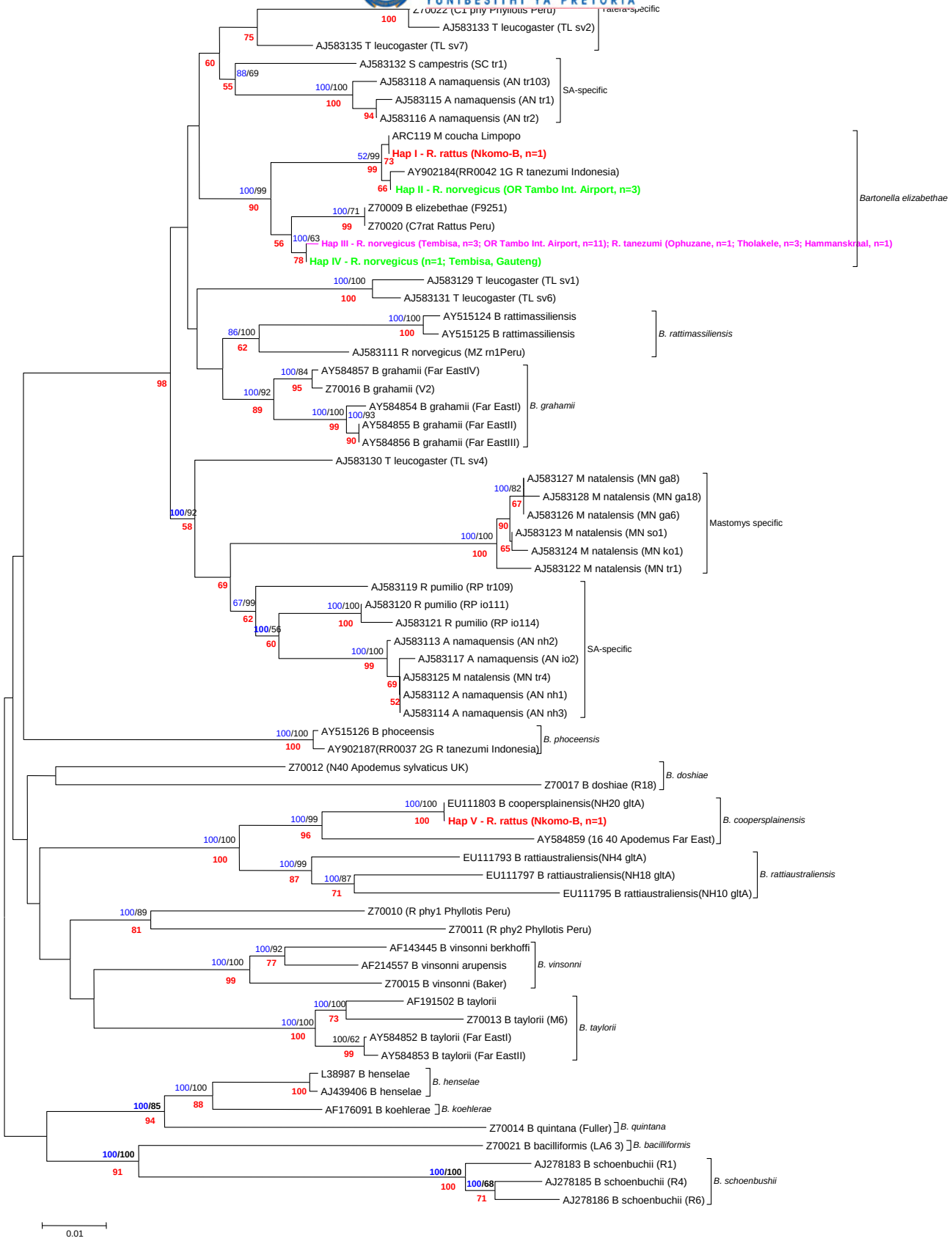
AJ583133 T leucogaster (TL sv2)
AJ583135 T leucogaster (TL sv7)
AJ583132 S campestris (SC tr1)
AJ583118 A namaquensis (AN tr103)
AJ583115 A namaquensis (AN tr1)
AJ583116 A namaquensis (AN tr2)
SA-specific
ARC119 M coucha Limpopo
Hap I - R. rattus (Nkomo-B, n=1)
AY902184(RR0042 1G R tanezumi Indonesia)
Hap II - R. norvegicus (OR Tambo Int. Airport, n=3)
Z70009 B elizebethae (F9251)
Z70020 (C7rat Rattus Peru)
Hap III - R. norvegicus (Tembisa, n=3; OR Tambo Int. Airport, n=11); R. tanezumi (Ophuzane, n=1; Tholakele, n=3; Hammanskraal, n=1)
Hap IV - R. norvegicus (n=1; Tembisa, Gauteng)
Bartonella elizabethae
AJ583129 T leucogaster (TL sv1)
AJ583131 T leucogaster (TL sv6)
AY515124 B rattimassiliensis
AY515125 B rattimassiliensis
AJ583111 R norvegicus (MZ rn1Peru)
B. rattimassiliensis
AY584857 B grahamii (Far EastIV)
Z70016 B grahamii (V2)
AY584854 B grahamii (Far EastI)
AY584855 B grahamii (Far EastII)
AY584856 B grahamii (Far EastIII)
B. grahamii
AJ583130 T leucogaster (TL sv4)
AJ583127 M natalensis (MN ga8)
AJ583128 M natalensis (MN ga18)
AJ583126 M natalensis (MN ga6)
AJ583123 M natalensis (MN so1)
AJ583124 M natalensis (MN ko1)
AJ583122 M natalensis (MN tr1)
Mastomys specific
AJ583119 R pumilio (RP tr109)
AJ583120 R pumilio (RP io111)
AJ583121 R pumilio (RP io114)
AJ583113 A namaquensis (AN nh2)
AJ583117 A namaquensis (AN io2)
AJ583125 M natalensis (MN tr4)
AJ583112 A namaquensis (AN nh1)
AJ583114 A namaquensis (AN nh3)
SA-specific
AY515126 B phoceensis
AY902187(RR0037 2G R tanezumi Indonesia)
B. phoceensis
Z70012 (N40 Apodemus sylvaticus UK)
Z70017 B doshiae (R18)
B. doshiae
EU111803 B coopersplainensis(NH20 gltA)
Hap V - R. rattus (Nkomo-B, n=1)
AY584859 (16 40 Apodemus Far East)
B. coopersplainensis
EU111793 B rattiaustraliensis(NH4 gltA)
EU111797 B rattiaustraliensis(NH18 gltA)
EU111795 B rattiaustraliensis(NH10 gltA)
B. rattiaustraliensis
Z70010 (R phy1 Phyllotis Peru)
Z70011 (R phy2 Phyllotis Peru)
AF143445 B vinsonni berkhoffi
AF214557 B vinsonni arupensis
Z70015 B vinsonni (Baker)
B. vinsonni
AF191502 B taylorii
Z70013 B taylorii (M6)
AY584852 B taylorii (Far EastI)
AY584853 B taylorii (Far EastII)
B. taylorii
L38987 B henselae
AJ439406 B henselae
B. henselae
AF176091 B koehlerae
B. koehlerae
Z70014 B quintana (Fuller)
B. quintana
Z70021 B bacilliformis (LA6 3)
B. bacilliformis
AJ278183 B schoenbuchii (R1)
AJ278185 B schoenbuchii (R4)
AJ278186 B schoenbuchii (R6)
B. schoenbushii
0.01

Fig. 4.2

Table 4.1 Summary of *Bartonella* species that have been isolated from rodents worldwide to date.

***Bartonella* spp.**	**Host (Genus)**	**References**
B. elizabethae	Rats (*Rattus; Aethomys; Bandicota*); Gerbil (*Tatera*); Mice (*Saccostomus*)	Ellis *et al.* (1999); Ying *et al.* (2002); Castle *et al.* (2004); Pretorius *et al.* (2004)
B. tribocorum	Rats (*Rattus*); Mice (*Apodemus*)	Heller *et al.* (1998); Engbæk & Lawson (2004)
B. grahamii	Voles (*Clethrionomys; Microtus*); Rats (*Rattus; Bandicota*); Mice (*Mus; Apodemus*); Gerbil (*Tatera*)	Ellis *et al.* (1999); Birtles *et al.* (2001); Ying *et al.* (2002); Castle *et al.* (2004); Engbæk & Lawson (2004); Pretorius *et al.* (2004); Meddianikov *et al.* (2005); Knap *et al.* (2007)
B. vinsonii	Rats (*Rattus*); Voles (*Microtus*); Mice (*Apodemus*)	Engbæk & Lawson (2004)
B. taylorii	Voles (*Clethrionomys*; *Microtus*); Mice (*Apodemus*)	Birtles *et al.* (2001); Engbæk & Lawson (2004); Meddianikov *et al.* (2005); Knap *et al.* (2007)
B. birtlesii	Shrews (*Sorex*); Voles (*Clethrionomys*); Mice (*Apodemus*)	Engbæk & Lawson (2004); Knap *et al.* (2007)
B. doshiae	Voles (*Clethrionomys; Microtus*); Mice (*Apodemus*)	Birtles *et al.* (2001); Knap *et al.* (2007)
B. alsatica	Rabbits (*Oryctolagus)*	Heller *et al.* (1999)
B. henselae	Mice (*Apodemus*)	Engbæk & Lawson (2004)
B. washoensis	Squirrel (*Spermophilus*)	Kosoy *et al.* (2003)
B. rattimassiliensis	Rats (*Rattus*)	Gundi *et al.* (2004)
B. phoceensis	Rats (*Rattus*)	Gundi *et al.* (2004)

Table 4.2 Geographic coordinates of sampled localities of *Rattus rattus*, *R. tanezumi* and *R. norvegicus* from South Africa.

	Locality	Province	Sample size	Geographic coordinates
1	Bloublommetjieskloof	Limpopo	2	24°18.66 S; 29°46.17 E
2	Hammanskraal	Gauteng	32	25°22.27 S; 28°11.26 E
3	Moreleta Park, Pretoria	Gauteng	2	25°49.67 S; 28°17.30 E
4	Mvuzini	Kwa-Zulu Natal	17	28°00.50 S; 30°40.52 E
5	Nkomo-B	Limpopo	37	23°24.96 S; 30°47.13 E
6	Ophuzane	Kwa-Zulu Natal	5	27°29.16 S; 30°56.03 E
7	O.R. Tambo International Airport	Gauteng	42	26°08.68 S; 28°13.57 E
8	Rietondale, Pretoria	Gauteng	1	25°26.10 S; 28°07.85 E
9	Tembisa	Gauteng	5	26°00.11 S; 28°12.78 E
10	Tholakele	Kwa-Zulu Natal	12	27°26.05 S; 30°59.28 E
11	Hillcrest, Pretoria	Gauteng	1	25°45.05 S; 30°59.28 E
12	Waterkloof, Pretoria	Gauteng	5	25°49.13 S; 28°15.30 E

Table 4.3 Sample identity and collecting localities that were positive for *Bartonella* infection isolated from members of the genus *Rattus* from South Africa. ORT Int. Airport denotes O.R. Tambo International Airport.

Sample Name	Location	*Bartonella* species isolated	Species isolated from
ARC89	Nkomo-B, Limpopo	*B. coopersplainensis*	*R. rattus*
ARC100	Nkomo-B, Limpopo	*B. elizabethae*	*R. rattus*
ARC128	Tholakele, Kwa-Zulu Natal	*B. elizabethae*	*R. tanezumi*
ARC131	Tholakele, Kwa-Zulu Natal	*B. elizabethae*	*R. tanezumi*
ARC148	Tholakele, Kwa-Zulu Natal	*B. elizabethae*	*R. tanezumi*
ARC165	Ophuzane, Kwa-Zulu Natal	*B. elizabethae*	*R. tanezumi*
OT7	ORT Int. Airport (Johannesburg), Gauteng	*B. elizabethae*	*R. norvegicus*
OT9	ORT Int. Airport (Johannesburg), Gauteng	*B. elizabethae*	*R. norvegicus*
OT10	ORT Int. Airport (Johannesburg), Gauteng	*B. elizabethae*	*R. norvegicus*
OT12	ORT Int. Airport (Johannesburg), Gauteng	*B. elizabethae*	*R. norvegicus*
OT20	ORT Int. Airport (Johannesburg), Gauteng	*B. elizabethae*	*R. norvegicus*
OT22	ORT Int. Airport (Johannesburg), Gauteng	*B. elizabethae*	*R. norvegicus*
OT24	ORT Int. Airport (Johannesburg), Gauteng	*B. elizabethae*	*R. norvegicus*
OT26	ORT Int. Airport (Johannesburg), Gauteng	*B. elizabethae*	*R. norvegicus*
OT29	ORT Int. Airport (Johannesburg), Gauteng	*B. elizabethae*	*R. norvegicus*
OT31	ORT Int. Airport (Johannesburg), Gauteng	*B. elizabethae*	*R. norvegicus*
OT33	ORT Int. Airport (Johannesburg), Gauteng	*B. elizabethae*	*R. norvegicus*
OT34	ORT Int. Airport (Johannesburg), Gauteng	*B. elizabethae*	*R. norvegicus*
OT35	ORT Int. Airport (Johannesburg), Gauteng	*B. elizabethae*	*R. norvegicus*
OT41	ORT Int. Airport (Johannesburg), Gauteng	*B. elizabethae*	*R. norvegicus*
HK13	Hammanskraal, Gauteng	*B. elizabethae*	*R. tanezumi*
ER1	Tembisa, Gauteng	*B. elizabethae*	*R. norvegicus*
ER2	Tembisa, Gauteng	*B. elizabethae*	*R. norvegicus*
ER3	Tembisa, Gauteng	*B. elizabethae*	*R. norvegicus*
ER4	Tembisa, Gauteng	*B. elizabethae*	*R. norvegicus*

Table 4.4 A summary of *Bartonella* prevalence in 162 samples of members of the genus *Rattus* from South Africa screened in this study, on a per-species basis

Species	Number tested	Number positive	Prevalence (%)
R. rattus	43	2	4.7
R. tanezumi	73	5	6.8
R. rattus complex	116	7	6.0
R. norvegicus	46	18	39.0
Total	162	25	15.4

CHAPTER 5

HELICOBACTER PREVALENCE IN THREE INVASIVE AND COMMENSAL *RATTUS* SPECIES (RODENTIA: MURIDAE) FROM SOUTH AFRICA

5.1 ABSTRACT

Helicobacter (Goodwin *et al.*, 1989 emend. Vandamme *et al.*, 1991) infections are increasingly being reported in a wide variety of animal species and in wild and laboratory rodents, in particular. To date, there are no less than 30 formally named species of *Helicobacter* with more than 46 species represented in the Genbank database. Members of the genus *Helicobacter* are known to infect either the stomach (gastric *Helicobacters*) or the liver and intestines (enterohepatic *Helicobacters*) of various animals and cause diseases such as peptic ulcers, gastric adenocarcinoma, mucosa-associated lymphoid tissue lymphoma and chronic gastritis. Some species such as *H. canis* (Stanley *et al.*, 1994), *H. rappini* (Dewhirst *et al.*, 2000) and *H. cinaedi* Totten *et al.*, 1988 (Vandamme *et al.*, 1991) are also known to have zoonotic potential. In this study, the prevalence of *Helicobacter* in three medically and agriculturally important invasive and commensal species of *Rattus* (Fischer, 1803) occurring in three provinces in South Africa, namely *Rattus rattus* (Linnaeus, 1758), *Rattus tanezumi* (Temminck, 1844) and *Rattus norvegicus* (Berkenhout, 1769) was assessed. The gastrointestinal tract (GIT) as well as the liver was evaluated by PCR for *Helicobacter* bacterial genome presence using a lineage-specific PCR approach developed specifically for this study. By so doing, an overall infection rate of 16.3% for the GIT and 0.6% for the liver was recovered for the South African *Rattus* congenerics. *Helicobacter rodentium* Shen *et al*., 1997 was identified from the liver of a single *R. rattus* individual (2.2% prevalence), whereas *Helicobacter muridarum* Lee *et al*., 1992 was found in the GIT of 13 individuals of *R. norvegicus* (28.3%). No *Helicobacter* infection was recorded in *R. tanezumi*. Currently, the two *Helicobacter* species identified by 16S gene sequencing have no known zoonotic potential and members of the genus *Rattus* infected with *Helicobacter* appear to pose no known threat to humans. However, the molecular approach used in this study holds potential for evaluating the prevalence in other murid rodents occurring commensally with humans and which may harbour *Helicobacter* species with known zoonotic potential.

Key words: *Helicobacter*, zoonotic disease; gastrointestinal tract; *Rattus*; enterohepatic species

5.2 INTRODUCTION

Bacteria belonging to the genus *Helicobacter* are gram-negative curved rods (Nester *et al.* 2001). *Helicobacter pylori* is probably the best known species in this genus causing diseases such as peptic ulcers, gastritis, mucosa-associated lymphoid tissue lymphoma and gastric adenocarcinoma in humans (Alm *et al.* 2000). *Helicobacter* infections are quite common among laboratory rodents (Whary & Fox 2006) such as mice (Shen *et al.* 1997; Goto *et al.* 2000; Shen *et al.* 2005; Bohr *et al.* 2006; Taylor *et al.* 2007), rats (Mendes *et al.* 1996; Goto *et al.* 2000), gerbils and shrews (Goto *et al.* 2000). *Helicobacter* has also been detected in wild rodent populations from Brazil (Comunian *et al.* 2006), rats from Italy (Giusti *et al.* 1998) and China (Goto *et al.* 2004) and in mice from Korea (Won *et al.* 2002). The potential significance of these flourishing micro-organisms cannot be ignored (Solnick & Schauer 2001). It is therefore, important to assess the reservoir host status of species that occur commensally with humans as they may harbour *Helicobacter* species with zoonotic potential (Solnick & Schauer 2001; Solnick 2003).

Currently, the genus *Helicobacter* consists of approximately 30 formally recognised species (Moyaert *et al.* 2008) which fall into two main groups based on tissue tropism, namely, gastric *Helicobacter* species (N≈9) and enterohepatic *Helicobacter* species (N≈21). Gastric species are able to survive the acidity of the stomach because of their strong urease activity (Ceelen *et al.* 2007). The enterohepatic species (EHS) are usually found in the liver and/or intestines (Fox 1997; Solnick & Schauer 2001) and cause gastroenteritis and hepatitis (Solnick & Schauer 2001). They have also been linked to adenocarcinoma of the colon and typhlocolitis in immune compromised mice (Fox *et al.* 1996). EHS are very common in laboratory rodents and can cause disease in immunocompetent rodents (Solnick & Schauer 2001). Various *Helicobacter* species have been associated with rodents (Table 5.1), and whilst a great deal is known about *Helicobacter* in captive/laboratory rodents, prevalence estimates are less well-studied in wild, commensal species. No study has been conducted to date on *Helicobacter* infection of *Rattus* in South Africa.

5.2.1 Rattus in South Africa and disease association

Currently there are three species of *Rattus* occurring in South Africa, namely *R. rattus* and *R. norvegicus* (Skinner & Smithers 1990) and the recently discovered *R. tanezumi* (Bastos *et al.* 2005; Taylor *et al.* 2008; Bastos *et al.* unpublished). All three species have been found in association with humans in rural, peri-urban and urban settings (Chapter 2). In addition to the damage they cause on crops and stored food,

their close association with humans increases the possibility of *Rattus* acting as a reservoir of disease for humans. Many zoonotic diseases of medical importance have been associated with *Rattus* species occurring commensally with humans (Grantz 1997). More specifically, *Rattus* has been linked to diseases such as the Seoul virus (Mills 1999), haemorrhagic fever (Lee *et al.* 1980), murine typhus and Lyme disease (Grantz 1997). In South Africa in particular, members of the genus *Rattus* have been associated with diseases such as leptospirosis and toxoplasmosis (Taylor *et al.* 2008).

Zoonotic diseases are important in areas where their hosts occur commensally with humans, such as in rural villages. People living in these settings are usually poor with limited access to healthcare. A 2008 report by the South African National Department of Health (2008), reported that approximately 5.27 million people are living with HIV/AIDS infections. Stillwaggon (2002) reported that individuals infected with HIV/AIDS are more vulnerable to infectious diseases, irrespective of how they are transmitted. Various diseases, such as diarrhoea are also prevalent with higher fatalities in poorer communities (Stillwaggon 2002). The aim of the present study is therefore to determine whether members of South African *Rattus* are carriers of *Helicobacter* species with zoonotic potential, in light of their close association with humans.

5.2.3 Helicobacter identification methods

Various methods are used for the detection of *Helicobacter* in their host species and include serological tests, cellular fatty acid profiles, culturing, pyrolysis mass spectrometry, restriction, protein profiling, nuleic acid probes, phenotypic tests, lectin agglutination, PCR detection and enzyme analysis (On 1996; Riley *et al.* 1996; Zenner 1999; Whary & Fox 2006). Discrepancies between the different methods are known to occur (Linton *et al.* 1997; Dewhirst *et al.* 2005), but PCR amplification is regarded as being the most precise and insightful means of *Helicobacter* detection (Zenner 1999; Whary & Fox 2006) and 16S RNA or 23S RNA gene regions are usually targeted (Zenner 1999; Dewhirst *et al.* 2005; Whary & Fox 2006) with genus-specific primers and species-specific primers (Whary & Fox 2006).

5.3 MATERIALS AND METHODS

5.3.1 Genetic analysis

5.3.1.1 Sampling

Liver and gastrointestinal tract (GIT) samples were tested for *Helicobacter* presence. Most studies use GIT samples as most Helicobacters are gastric species. However, since there are also enterohepatic Helicobacters (Taylor *et al.* 2007), liver tissue of 162 *Rattus* individuals (*n* = 162; 43 *R. rattus*, 46 *R. norvegicus* and 73 *R. tanezumi*) from 12 localities in Limpopo, Gauteng and KwaZulu Natal Provinces, South Africa (Fig. 5.1; Table 5.2), was also screened. For the GIT, fewer individuals (N=80) were screened, due to unavailability of this tissue type for the Limpopo Province *Rattus*. The sampling localities included rural villages, informal settlements and metropolitan areas within the cities of Pretoria and Johannesburg (Gauteng Province). Sample sizes and geographic coordinates of the sampling localities are shown in Table 5.2.

Animals were obtained through a number of approaches as follows: 1) Live-trapping using Sherman traps (H.B. Sherman Traps Inc. Florida, U.S.A.) baited with a mixture of peanut butter, syrup, oatmeal and fish oil; 2) Samples obtained through a European Commission/DFID-funded community-participatory research project on rodents in southern Africa where 10 snap traps were placed in 10 community households in a number of villages and inspected daily; 3) Samples obtained opportunistically from pest control companies during their routine extermination programmes at facilities such as the O.R. Tambo International Airport; and 4) Samples provided by the general public.

After capture, during transportation and in the laboratory, live-trapped animals were kept in polyurethane cages with wood shavings provided as bedding and mouse pellets and water provided *ad libitum* in accordance with the guidelines of the American Society of Mammalogists (ASM; http://www.mammalogy.org/committees/index.asp; Animal Care and Use Committee 1998). Halothane inhalation was used to euthanize live animals. Animals were dissected and the livers were removed and stored in either absolute ethanol or frozen at -20° C for subsequent molecular analysis. GIT samples were collected from 80 of the total of 162 animals used in this study and subsequently frozen at -20 ° C. Voucher specimens were prepared using standard natural history museum procedures for mammal specimens and will be deposited in the mammal reference collection of the Transvaal Museum (TM) of the Northern Flagship Institute (NFI), Pretoria, South Africa and the Durban Natural Science Museum, South Africa.

For direct comparison with the genetic data, the specimens from this study represent the same specimens that were genetically identified in Chapter 2 using

mtDNA (D-loop) in the multidisciplinary characterization of *Rattus* from South Africa. The same genetically-identified specimens were also used in the classical qualitative assessment (Chapter 5) and are also being used in an on-going parallel geometric morphometric study (Chimimba *et al.* unpublished).

5.3.1.2 Laboratory procedures

Total genomic DNA was isolated from liver and GIT samples using the Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minnesota) as well as Roche High Pure PCR Template Preparation Kit (Roche Applied Systems, Germany). Supplier specified protocols for animal tissue were used and overnight digestion was performed with proteinase K at 55° C, for both kits. DNA was eluted in a final volume of 50 µl. Host species identification was determined genetically by amplification and sequencing of either the 501 bp hypervariable region I (HVR I) of the mitochondrial D-loop region, or the 1.2 kbp mitochondrial region of the cytochrome *b* (cyt *b*) gene as detailed in Chapter 2. A 906 bp region of the 16S gene of the bacterial genome was targeted to determine pathogen presence using Polymerase Chain Reaction (PCR; Mullis & Faloona 1987) amplification. This was done using a touch-down PCR reaction at the following thermal cycling conditions: initial denaturation at 96° C for 20 s, followed by 3 cycles of 96° C, 12 s; 58° C, 20 s and 72° C, 55 s; 5 cycles of 96° C, 12 s, 57° C, 18 s, 72° C, 50 s and finally 32 cycles of 96° C, 20 s; 56° C, 15 s, 72° C, 45 s. Final extension was at 72° C for 60 s.

A positive control was always included as a molecular marker and to preclude false negatives, whilst a negative control was included to control for false positives arising from reagent contamination. Gel electrophoresis was done on a 1.5% low-melting point agarose gel (stained with Goldview) and then visualized under ultra-violet (UV) light to determine if any product was present. Positive PCRs indicated bacterial presence in the sample. All PCR reactions were run on the same machine to control for variables such as temperature and machine efficiency. Positive PCR products were purified from the tube using the Roche Purification Kit and then cycle-sequenced with Big Dye terminator chemistry (Applied Biosystems [ABI]). DNA was precipitated for sequencing analysis using sodium acetate.

5.3.1.3 Lineage-specific primer design

Primers were designed specifically for this study by aligning Genbank sequences of approximately 35 different *Helicobacter* species (Table 5.3) in order to ensure that all of these species would be detectable when using this approach. Sequences were

aligned and a phylogeny was inferred which indicated the presence of two discrete lineages (Fig. 5.2). This guided the separation of sequences, according to lineage and directed the lineage-specific primer design. A universal forward primer that was conserved across both lineages termed Heli-F and two lineage-specific reverse primers termed Heli-R1 and Heli-R2 were designed with the latter two primers amplifying lineage 1 and lineage 2 species, respectively in separate reactions, when combined with the Heli-F primer.

5.3.1.4 Phylogenetic analysis

Chromas (McCarthy 1996-1997) was used to view and edit sequence chromatograms which were then exported to DAPSA (Harley 1994) for multi-sequence alignment. Nucleotide sequences generated from positive *Helicobacter* samples (Table 5.4) were aligned to homologous sequences downloaded from the Genbank (www.ncbi.nlm.nih.gov) database, and to those used for the initial primer design (Table 5.3).

A gene phylogeny was inferred using distance and discrete methods of analysis. Neighbor-joining (NJ) clustering analysis with trees being midpoint rooted, was performed in MEGA version 4.0 (Tamura *et al.* 2007) using the TrN+I+G model, which distinguishes between transitional substitution rates between purines and transversional substitution rates between pyrimidines. Modeltest version 3.06b (Posada and Crandall 1998) was used to determine the best-fit model for the *Helicobacter* data. PAUP* version 4.0b10 (Swofford 2002) was used to conduct Maximum parsimony (MP) analyses. Nodal support was estimated by nonparametric bootstrap support (Felsenstein 1985). Stepwise addition with 1000 random addition replicates and tree-bisection-reconnection (TBR) branch-swapping was performed.

Bayesian Inference (BI) analyses were performed using MrBayes version 3.1 (Huelsenbeck and Ronquist 2001) and posterior probabilities were determined by a Markov Chain Monte Carlo (MCMC; Hastings 1970) method in which four chains were run simultaneously (three heated and one cold) at default temperatures. Priors were identified under the best-fit model (TrN+I+G) which was selected under the Akaike Information Criterion (AIC; Akaike 1974) in Modeltest. A burn-in of 25% of the run length was discarded after a run of 5 000 000 generations was performed.

5. 4 RESULTS

5.4.1 *Helicobacter sequence data*

All sequences (844 bp) generated from *Rattus* samples that tested positive for the presence of *Helicobacter* were aligned with homologous sequences from the Genbank database. The final dataset contained 56 taxa which represented unique haplotypes and corresponded to 35 different *Helicobacter* species. The dataset comprised 138 (16.4%) parsimony-informative sites, with 677/844 (80.2%) being conserved and 163/844 (19.3%) being variable (Fig. 5.3). Only 25 sites (3.0%) were singletons. The average nucleotide composition estimated in Modeltest under the best-fit model selected under the Akaike Information Criterion (AIC) was 28.9%, 19.8%, 26.5% and 24.8% for A, C, G and T, respectively.

5.4.2 *Neighbor-joining (NJ), Maximum parsimony (MP) and Bayesian Inference (BI) analyses*

The TrN+I+G model was selected under the AIC when run in Modeltest version 3.06 (Posada 2000) as the model of evolution that best fitted the dataset. The gamma distribution shape parameter was $\gamma = 0.6368$ and the proportion of invariable sites (I) was 0.7245.

In the MP analysis, a consensus tree was drawn from the four possible trees that had the following tree statistics: L = 573, CI = 0.382, RI = 0.834, RCI = 0.319 and HI = 0.618. The rescaled consistency index (RCI) was used to reweight the characters, which resulted in the same number of trees being recovered (L = 180, CI = 0.567, RI = 0.890, RC = 0.505 and HI = 0.618). No further changes were noted in the topology when RCI weighting was performed for a third time.

Tree topologies were similar for the MP and BI analyses. However, BI gave better overall node resolution, whereas the MP analysis resulted in numerous polytomies. The NJ analysis (SBL = 0.58) gave better overall node resolution than MP or BI. A phylogram is presented in Fig. 5.3 with bootstrap values from the NJ and MP analyses and posterior probability values from the BI analysis.

Three different *Helicobacter* haplotypes were recovered from the 14 sequences that represented the positive *Helicobacter* samples (GIT and liver; Table 5.5). Thirteen samples tested positive for *Helicobacter* infection in the GIT corresponding to an overall prevalence of 16.3% for *Rattus* (Table 5.5). These samples are represented by two haplotypes, Haplotypes I and II, which group with *H.*

muridarum. Both haplotypes were recovered from individuals of *R. norvegicus*, with Haplotype I being identified in one individual from O.R. Tambo International Airport, Gauteng Province and one individual from the nearby Tembisa, Gauteng Province (see locality data in Fig. 5.1 and Table 5.2). Haplotype II was found in nine individuals from O.R. Tambo International Airport and two individuals from Tembisa. The prevalence of *H. muridarum* on a per-species basis was thus highest in individuals of *R. norvegicus* with the prevalence of 28.3% (13/46). No *Helicobacter* infections were recorded for either *R. rattus* or *R. tanezumi* in the GIT.

The third haplotype (Haplotype III) was only recovered in one individual of *R. rattus* from Nkomo-B, Limpopo Province (see locality data in Fig. 5.1 and Table 5.2) and this bacterial sequence which was detected in the liver, groups together with *H. rodentium*. This corresponds to a prevalence of 2.2% (1/45; Table 5.5) for *Helicobacter* in wild *R. rattus* and 0.0% for *R. tanezumi*. Overall *Helicobacter* prevalence recorded in the liver was thus 0.6%.

5.5 DISCUSSION

Since the 1982 description of *Helicobacter pylori*, the type species of the genus, the number of *Helicobacter* species has increased rapidly with many more almost certainly waiting discovery (Franklin *et al.* 2001). Elucidation not only of *Helicobacter* species but also their zoonotic potential is needed as possible zoonoses of *Helicobacter* species are probably under accounted (Whary & Fox 2004). The genus *Helicobacter* is pathogenic in a variety of species including rodents (Fox 1997; De Groote *et al.* 2000). At least one species found in rodents, *H. bilis* might be zoonotically important (Shomer *et al.* 1998).

The screening of samples in order to determine *Helicobacter* presence can be a lengthy process as reported by Goto *et al.* (2000) who screened for *Helicobacter* infection using PCR amplification. Samples are first run using genus-specific primer sets so as to determine the presence of *Helicobacter* bacteria within the samples. Positive samples are then screened with species-specific primer sets in order to establish which species are present. If positive samples do not amplify with species-specific primers, they are sequenced (Goto *et al.* 2000). Other methods include the use of restriction enzymes in restriction fragment length polymorphisms (Riley *et al.* 1996; Shen *et al.* 2000) and fluorogenic polymerase chain reactions (Ge *et al.* 2001). However, PCR amplification methods are still the most sensitive and specific for

uncovering the presence of *Helicobacter* species in a sample (Whary & Fox 2006). The newly designed primer set used in the current study saves time in terms of PCR analysis by screening for the presence of various species of *Helicobacter* simultaneously instead of screening a sample multiple times when primer sets are species-specific. Whilst the primer sets hold clear research value, they are of limited use in a diagnostic setting due to the dependence on nucleotide sequencing for a definitive *Helicobacter* species assignment.

The two haplotypes (Haplotypes I & II) recovered from the GIT samples screened grouped with *H. muridarum,* a *Helicobacter* species that is common in the intestines of mice and rats (Whary & Fox 2006; Ceelen *et al.* 2007). Jiang *et al.* (2002) found that *scid* mice infected with *H. muridarum* developed inflammatory bowel disease. *Helicobacter muridarum* is also capable of colonizing the stomachs of mice (Fox 1997; Zenner 1999; Whary & Fox 2006). In aging mice, the occurrence of this *Helicobacter* species in the stomach contributes to the development of chronic gastritis (Fox 1997; Zenner 1999). *Helicobacter muridarum* has been isolated from rat intestines, but no disease association has yet been recorded (Zenner 1999; Whary & Fox 2006), and there is also no evidence that it spreads zoonotically.

The single positive *Helicobacter* liver sample was identified as *H. rodentium*, a bacterial species found in diverse species of rodents and which is believed to have a restricted tissue tropism, namely the intestine (Whary & Fox 2006). Confirmation of this species in the liver therefore indicates that tissue tropism for this species is broader than previously documented. *Helicobacter rodentium* has been linked to severe epidemics of diarrhoeal diseases in immunodeficient mice (De Groote *et al.* 2000). This bacterium can also be spread through faecal-oral contact as it colonises the lower bowel of the hosts (Whary & Fox 2006). *Helicobacter bilis* can, in conjunction with *H. rodentium*, induce diarrhoea and proliferative typhlocolitis in *scid* mice (Shomer *et al.* 1998).

Significantly higher *Helicobacter* infection rates were found in the house rat (*Rattus rattus*) in a study by Comunian *et al.* 2006 (30.0%), and Goto *et al.* 2000 recorded a *H. rodentium* prevalence of 29.4% in rats and 38.3% in mice. However, these results are based on *H. rodentium* cultured from the GIT and not the liver, as was the case in our study. Of significance is that no *H. rodentium* was recovered from the GIT in this study.

A *Helicobacter* prevalence of 28.3% was obtained for *R. norvegicus* in this study, a level of prevalence that is similar (23.0%) to that recorded by Guisti *et al.* (1998) in Italy in which the bacterial species detected in wild-caught *R. norvegicus* was *H. heilmannii*. Ricci *et al.* (2006) reported that this species is zoonotic and is usually derived from cats and dogs.

Currently, *Helicobacter* infection from *Rattus* is not of immediate concern to human health in South Africa as the two species identified in this study have to date, not been shown to have zoonotic potential. However, other *Helicobacter* species with known zoonotic potential have been isolated from rodents such as *H. heilmannii* (Guisti *et al.* 1998) and *H. bilis* (Shomer *et al.* 1998; Whary & Fox 2006). Although the latter two *Helicobacter* species were not identified from any of the samples examined in this study, the possibility of their presence in other murid rodents living commensally with humans cannot be ruled out and requires further investigation. Similarly, the possibility that *Helicobacter* species identified in the present study may be able to cause disease in immune compromised individuals can also not be ruled out.

The marked difference between the infection rates of *R. norvegicus* compared to that of the two members of the *Rattus rattus* species complex (*R. rattus* and *R. tanezumi*) may indicate differences in host specificity of *Helicobacter*. Behavioural characteristics of the host species could influence rodent-to-rodent spread of *Helicobacter* (Comunian *et al.* 2006) and warrants further investigation in South Africa.

5.6 REFERENCES

AKAIKE, H. 1974. A new look at statistical model identification. *IEEE Transactions on Automatic Control* **19**: 716–722.

ALM, R. A., BINA, J., ANDREWS, B. M., DOIG, P. & HANCOCK, R. E. W. 2000. Comparative genomics of *Helicobacter pylori*: analysis of the outer membrane protein families. *Infection and Immunology* **68:** 4155-4168.

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.

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. *South African Society for Veterinary Epidemiology and Preventive Medicine*. Proceedings Pretoria, 11-12 August 2005. Pp. 78-83.

BOHR, U. R. M., SELGRAD, M., OCHMANN, C., BACKERT, S., KÖNIG, W., FENSKE, A., WEX, T. & MALFERTHEINER, P. 2006. Prevalence and spread of enterohepatic *Helicobacter* species in mice reared in a specific-pathogen-free animal facility. *Journal of Clinical Microbiology* **44**: 738-742.

CEELEN, L. M., HAESEBROUCK, F., DUCATELLE, R. & DECOSTERE, A. 2007. The occurrence and clinical significance of enterohepatic *Helicobacter* species in laboratory rodents. *Vlaams Diergeneeskundig Tijdschrift* **76**: 103-116.

COMUNIAN, L. B., MOURA, S. B., PAGLIA, A. P., NICOLI, J. R., GUERRA, J. B., ROCHA, G. A. & QUEIROZ, D. M. M. 2006. Detection of *Helicobacter* species in the gastrointestinal tract of wild rodents from Brazil. *Current Microbiology* **53**: 370-373.

DE GROOTE, D., DUCATELLE, R. & HAESEBROUCK, F. 2000. *Helicobacters* of possible zoonotic origin: a review. *Acta Gastro-Enterologica Belgica* **63**: 380-387.

DEWHIRST, F. E., SHEN, Z., SCIMECA, M. S., STOKES, L. N., BOUMENNA, T., CHEN, T., PASTER, B. J. & FOX, J. G. 2005. Discordant 16S and 23S rRNA gene phylognies for the genus Helicobacter: Implications for phylogenetic inference and systematic. Journal of Bacteriology **187**: 6106-6118.

FELSENSTEIN, J., 1985. Confidence limits of phylogenies: an approach using the bootstrap. *Evolution* **39**: 783-791.

FOX, J. G., CABOT, E. B., TAYLOR, N. S. & LARAWAY, R. 1988. Gastric colonization by *Campylobacter pylori* subsp. *mustelae* in ferrets. *Infection and Immunity* **56**: 2994-2996.

FOX, J. G., YAN, L., SHAMES, B., CAMPBELL, J., MURPHY, J. C. & LI, X. 1996. Persistent hepatitis and enterocolitis in germfree mice infected with *Helicobacter hepaticus*. *Infection and Immunology* **64**: 3673-3681.

FOX, J. G. 1997. The expanding genus of *Helicobacter*: pathogenic and zoonotic potential. *Seminars in Gastrointestinal Disease* **8**: 124-141.

FOX, J. G. SHEN, Z., XU, S., FENG, Y., DANGLER, C. A., DEWHIRST, F. E., PASTER, B. J. & CULLEN, J. M. 2002. *Helicobacter marmotae* sp. nov. isolated from livers of woodchucks and intestines of cats. *Journal of Clinical Microbiology* **40**: 2513-259.

FRANKLIN, C. L., BECKWITH, C. S., LIVINGSTON, R. S., RILEY, L. K., GIBSON, S. V., BESCH-WILLIFORD & HOOK JR., R. R. 1996. Isolation of novel *Helicobacter* species, *Helicobacter cholecystus* sp. nov., from the gallbladders of Syrian hamsters with cholangiofibrosis and centrilobular pancreatitis. *Journal of Clinical Microbiology* **34**: 2952-2958.

FRANKLIN, C. L., GORELICK, P. L., RILEY, L. K., DEWHIRST, F. E., LIVINGSTON, R. S., WARD, J. M., BECKWITH, C. S. & FOX, J G. 2001. *Helicobacter typhlonius* sp. nov., a novel murine urease-negative *Helicobacter* species. *Journal of Clinical Microbiology* **39**: 3920-3926.

GE, Z., WHITE, D. A., WHARY, M. T. & FOX, J. G. 2001. Fluorogenic PCR-based quantitative detection of a murine pathogen, *Helicobacter hepaticus*. *Journal of Clinical Microbiology* **39**: 2598-2602.

GEBHART, C. J., FENNELL, C. L., MURTAUGH, M. P. & STAMM, W. E. 1989. *Campylobacter cinaedi* is normal intestinal flora in hamsters. *Journal of Clinical Microbiology* **27**: 1692-1694.

GIUSTI, A. M., CRIPPA, L., BELLINI, O., LUINI, M. & SCANZIANI, E. 1998. Gastric spiral bacteria in wild rats from Italy. *Journal of Wildlife Diseases* **34**: 168-172.

GOTO, K., OHASHI, H., TAKAKURA, A. & ITOH, T. 2000. Current status of *Helicobacter* contamination of laboratory mice, rats, gerbils and house musk shrews in Japan. *Current Microbiology* **41**: 161-166.

GOTO, K., JINAG, W., ZHENG, Q., OKU, Y., KAMIYA, H., ITOH, T. & ITO, M. 2004. Epidemiology of *Helicobacter* infection in wild rodents in the Xinjiang-Uygur autonomous region of China. *Current Microbiology* **49**: 221-223.

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

HASTINGS, W. K. 1970. Monte Carlo sampling methods using Markov chains and their applications. *Biometrika* **57**: 97–109.

HARLEY, E. H. 1994. DAPSA, DNA and Protein Sequence Analysis, version 2.9. Department of Chemical Pathology, University of Cape Town.

HUELSENBECK, J. P. & RONQUIST, F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Oxford University Press **17**: 754-755.

JALAVA K., KAARTINEN, M., UTRIAINEN, M., HAPPONEN, I. & HÄNNINEN, M-L. 1997. *Helicobacter salomonis* sp. nov., a canine gastric *Helicobacter* sp. related to *Helicobacter felis* and *Helicobacter bizzozeronii. International Journal of Systematic Bacteriology* **47**: 975-982.

JEON, W. J., YUN, Y. M., ROH, K. J., LEE, Y. C., CHOI, Y. K., HYUN, B. H., KIM, C. K., CHO, J. S. & SEONG, J. K. 2001. *Helicobacter apodemus* sp. nov.,

isolated from intestine of the striped field mouse (*Apodemus agrarius*). *Experimental Animals* (Tokyo): **48**, 228.

JIANG, H. Q., KUSHNIR, N., THURNHEER, M. C., BOS, N. A. & CEBRA, J. J. 2002. Monoassociation of *scid* mice with *Helicobacter muridarum*, but not four other enterics, provokes IBD upon receipt of T cells. *Gastroenterology* **122**: 1346-1354.

LEE, H. W., PARK, D. H., BAEK, L. J., CHOI, K. S., WHANG, Y. N. & WOO, M. S. 1980. Korean hemorrhagic fever patients in urban areas of Seoul. *Korean Journal of Virology* **10**: 16.

LEE, A., PHILLIPS, M. W., O'ROURKE, J. L., PASTER, B. J., DEWHIRST, F. E., FRASER, G. J., FOX, J. G., SLY, L. I., ROMANIUK, P. J., TRUST, T. J. & KOUPRACH, S. 1992. *Helicobacter muridarum* sp. nov., a microaerophilic helical bacterium with a novel ultrastructure isolated from the intestinal mucosa of rodents. *International Journal of Systematic Bacteriology* **42**: 27-36.

LINTON, D., LAWSON, A. J., OWEN, R. J. & STANLEY, J. 1997. PCR detection, identification to species level, and fingerprinting of *Campylobacter jejuni* and *Campylobacter coli* direct from diarrheic samples. *Journal of Clinical Microbiology* **35**: 2568-2572.

McCARTHY, C. 1996-1997. Chromas, version 1.43. School for Biomolecular and Biomedical Science. Griffith University, Australia.

MENDES, E. N., QUEIROZ, D. M. M., DEWHIRST, F. E., PASTER, B. J., BOURA, S. B. & FOX, J. G. 1996. *Helicobacter trogontum* sp. nov., Isolated from the rat intestine. *International Journal of Systematic Bacteriology* **46**: 916-921.

MILLS, J. N. 1999. The role of rodents in emerging human disease: examples from the Hantaviruses and Arenaviruses. *In*: Ecologically-based Rodent Management (*Singleton, G. R., Hinds, L. A., Leirs H. & Zhang, Z. eds*), Australian Centre for International Agricultural Research, Canberra. Pp. 134-160.

MOYAERT, H., PASMANS, R., DUCATELLE, R., HAESEBROUCK, F. & BAELE, M. 2008. Evaluation of 16S rRNA gene-based PCR assays for genus-level identification of *Helicobacter* species. *Journal of Clinical Microbiology* **46**: 1867-1869.

MULLIS, K. B. & FALOONA, F. A. 1987. Specific synthesis of DNA in vitro *via* a polymerase catalyzed chain reaction. *Methods in Enzymology* **155**: 335-350.

NATIONAL DEPARTMENT OF HEALTH, SOUTH AFRICA. 2008. The National HIV and syphilis prevalence survey South Africa 2007. http://us-cdn.creamermedia.co.za/assets/articles/attachments/16274_antenatal_report.pdf. Accessed 21 August 2009.

NESTER E. W., ANDERSON, D. G., ROBERTS JR., C. E., PEARSALL, N. N. & NESTER, M. T. 2001. Microbiology: A human perspective. McGraw-Hill Companies, Inc. Pp. 291.

ON, S. L. W. 1996. Identification methods for Campylobacters, Helicobacters, and related organisms. *Clinical Microbiology Reviews* **9**: 405-422.

PATTERSON, M. M., SCHRENZEL, M. D., FENG, Y., XU, S., DEWHIRST, F. E., PASTER, B. J., THIBODEAU, S. A., VERSALOVIC, J. & FOX, J. G. 2000. *Helicobacter aurati* sp. nov., a urease-positive *Helicobacter* species cultured from gastrointestinal tissues of Syrian hamsters. *Journal of Clinical Microbiology* **38**: 3722-3728.

POSADA, D. & CRANDALL, K. A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817-818.

POSADA, D. 2000. Testing models of evolution – Modeltest version 3.06. Brigham Young University, USA.

RICCI, C., HOLTON, J. & VAIRA, D. 2006. Diagnosis of *Helicobacter pylori*: Invasive and non-invasive tests. *Best Practice & Research Clinical Gastroenterology* **21**: 299-313.

RILEY, L. K., FRANKLIN, C. L., HOOK JR., R. R. & BESCH-WILLIFORD, C. 1996. Identification of murine *Helicobacters* by PCR and restriction enzyme analyses. *Journal of Clinical Microbiology* **34**: 942-946.

ROBERTSON, B. R., O'ROURKE, J. L., VANDAMME, P., ON, S. L. W. & LEE, A. 2001. *Helicobacter ganmani* sp. nov., a urease-negative anaerobe isolated from the intestines of laboratory mice. *International Journal of Systematic and Evolutionary Microbiology* **51**: 1881-1889.

SCHAUER, D. B., GHORI, N. & FALKOW, S. 1993. Isolation and characterization of "*Flexispira rappini*" from laboratory mice. *Journal of Clinical Microbiology* **31**: 2709-2714.

SHEN, Z., FOX, J. G., DEWHIRST, F. E., PASTER, B. J., FOLTZ, C. J., YAN, L., SHAMES, B. & PERRY, L. 1997. *Helicobacter rodentium* sp. nov., a urease-negative *Helicobacter* species isolated form laboratory mice. *International Journal of Systematic Bacteriology* **47**: 627-634.

SHEN, Z., FENG, Y. & FOX, J. G. 2000. Identification of enterohepatic *Helicobacter* species by restriction fragment-length polymorphism analysis of the 16S rRNA gene. *Helicobacter* **5**: 121-128.

SHEN, Z., XU, S., DEWHIRST, F. E., PASTER, B. J., PENA, J. A., MODLIN, I. M., KIDD, M. & FOX, J. G. 2005. A novel enterohepatic *Helicobacter* species '*Helicobacter mastomyrinus*' isolated from the liver and intestine of rodents. *Helicobacter* **10**: 59-70.

SHOMER, N. H., DANGLER, C. A., MARINI, R. P. & FOX, J. G. 1998. *Helicobacter bilis/Helicobacter rodentium* co-infection associated with diarrhea in a colony of *scid* mice. *Laboratory Animal Science* **48**: 455-459.

SIMMONS, J. H., RILEY, L. K., BESCH-WILLIFORD, C. L. & FRANKLIN, C. L. 2000. *Helicobacter mesocricetorum* sp. Nov., a novel *Helicobacter* isolated from the feces of Syrian hamsters. *Journal of Clinical Microbiology* **38**: 1811-1817.

SKINNER, J. D. & SMITHERS, R. H. N. 1990. *The Mammals of the Southern African Subregion*. CTP Book Printers, Cape Town. 736 pp.

SOLNICK, J. V. 2003. Clinical significance of *Helicobacter* species other than *Helicobacter pylori*. *Clinical Practice* **36**: 349-354.

SOLNICK, J. V. & SCHAUER, D. B. 2001. Emergence of diverse *Helicobacter* species in the pathogenesis of gastric and enterohepatic diseases. *Clinical Microbiological Review* **14**: 59-97.

STILLWAGGON, E. 2002. HIV/AIDS in Africa: Fertile terrain. *Journal of Development Studies* **38**: 1-22.

SWOFFORD, D. L. 2002. PAUP*: Phylogenetic Analysis using Parsimony (* and other methods), Version 4.0b10. Sinauer Associates, Sutherland, MA.

TAMURA, K., DUDLEY, J., NEI, M. & KUMAR, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**: 1596-1599.

TAYLOR, N. S., XU, S., NAMBIAR, P., DEWHIRST, F. E. & FOX, J. G. 2007. Enterohepatic *Helicobacter* species are prevalent in mice from commercial and academic institutions in Asian, Europe, and North America. *Journal of Clinical Microbiology* **45**: 2166-2172.

TAYLOR, P. J., ARNTZEN, L., HAYTER, M., ILES, M., FREAN, J. & 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.

WHARY, M. T. & FOX, J. G. 2004. Natural and experimental *Helicobacter* infections. *Comparative Medicine* **54**: 128-158.

WHARY, M. T. & FOX, J. G. 2006. Detection, eradication, and research implications of *Helicobacter* infections in laboratory rodents. *Lab Animal* **35**: 25-36.

WON, Y. S., VANDAMME, P., YOON, J. H., PARK, Y. H., HYUN, B. H., KIM, H. C., ITOH, T., TANIOKA, Y. & CHOI, Y. K. 2007. *Helicobacter callitrichis* sp. nov., a novel *Helicobacter* species isolated from the feces of the common marmoset (*Callithrix jacchus*). *FEMS Microbiology Letters* 271: 239-244.

ZENNER, L. 1999. Pathology, diagnosis and epidemiology of the rodent *Helicobacter* infection. *Comparative Immunology, Microbiology & Infectious Diseases* **22**: 41-61.

5.7 FIGURE LEGENDS

Fig. 5.1 A map of South Africa showing sampling localities of the *Rattus* species examined in this study. The locality data and sample sizes are indicated in Table 4.1. Pretoria (4) indicates four sampling localities in Pretoria namely Moreleta Park, Waterkloof, Rietondale and Hillcrest.

Fig. 5.2 The phylogeny of *Helicobacter* species used to design lineage specific primers for the current study. The sequences form two distinct lineages with the first lineage representing mostly enterohepatic *Helicobacter* species (although some have also been found in stomachs) and the second lineage representing only gastric species.

Fig. 5.3 The phylogeny of *Helicobacter* species based on Neighbor-joining (NJ) clustering analysis. Maximum parsimony (MP) data and Bayesian Inference (BI) values are indicated on the tree. MP bootstrap values are indicated in black (above the line), BI values are indicated in blue (above the line) while NJ bootstrap values are indicated in red (below the line). Only bootstrap values > 50 are shown. *Helicobacter* isolated from individuals of *Rattus rattus* are indicated in red and those isolated from *R. norvegicus* are indicated in green. Sequences obtained from the Genbank database is preceded by their Genbank accession number.

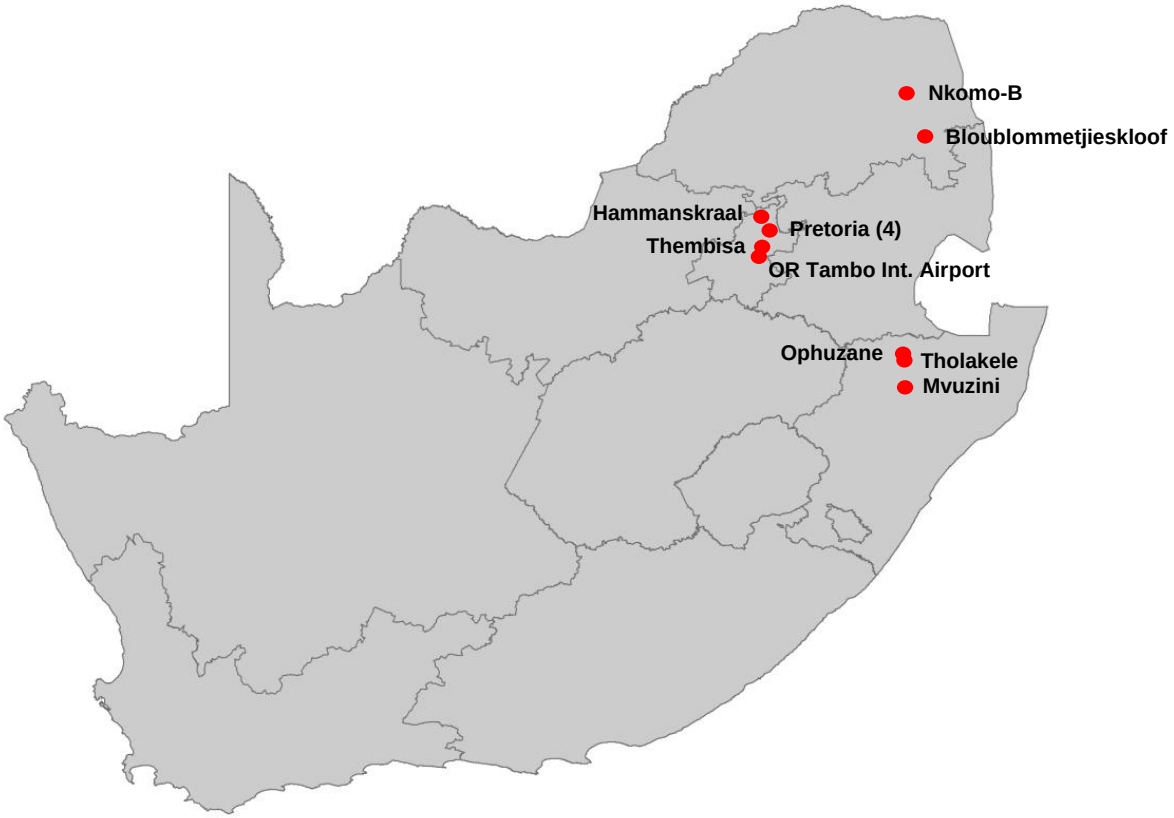
Nkomo-B
Bloublommetjieskloof
Hammanskraal
Pretoria (4)
Thembisa
OR Tambo Int. Airport
Ophuzane
Tholakele
Mvuzini

Fig. 5.1

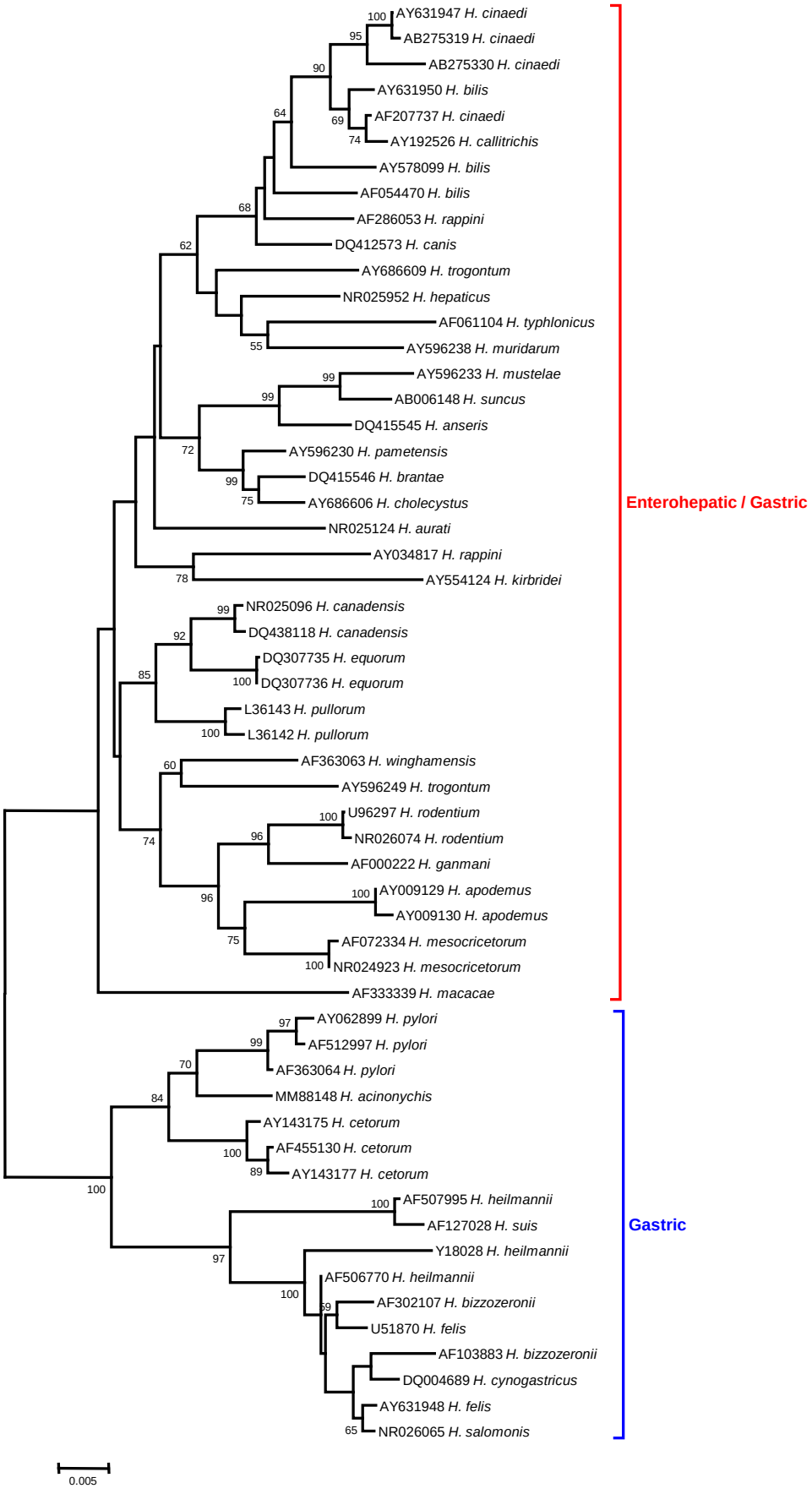
AY631947 H. cinaedi
AB275319 H. cinaedi
AB275330 H. cinaedi
AY631950 H. bilis
AF207737 H. cinaedi
AY192526 H. callitrichis
AY578099 H. bilis
AF054470 H. bilis
AF286053 H. rappini
DQ412573 H. canis
AY686609 H. trogontum
NR025952 H. hepaticus
AF061104 H. typhlonicus
AY596238 H. muridarum
AY596233 H. mustelae
AB006148 H. suncus
DQ415545 H. anseris
AY596230 H. pametensis
DQ415546 H. brantae
AY686606 H. cholecystus
NR025124 H. aurati
AY034817 H. rappini
AY554124 H. kirbridei
NR025096 H. canadensis
DQ438118 H. canadensis
DQ307735 H. equorum
DQ307736 H. equorum
L36143 H. pullorum
L36142 H. pullorum
AF363063 H. winghamensis
AY596249 H. trogontum
U96297 H. rodentium
NR026074 H. rodentium
AF000222 H. ganmani
AY009129 H. apodemus
AY009130 H. apodemus
AF072334 H. mesocricetorum
NR024923 H. mesocricetorum
AF333339 H. macacae
AY062899 H. pylori
AF512997 H. pylori
AF363064 H. pylori
MM88148 H. acinonychis
AY143175 H. cetorum
AF455130 H. cetorum
AY143177 H. cetorum
AF507995 H. heilmannii
AF127028 H. suis
Y18028 H. heilmannii
AF506770 H. heilmannii
AF302107 H. bizzozeronii
U51870 H. felis
AF103883 H. bizzozeronii
DQ004689 H. cynogastricus
AY631948 H. felis
NR026065 H. salomonis
Enterohepatic / Gastric
Gastric
0.005

Fig. 5.2

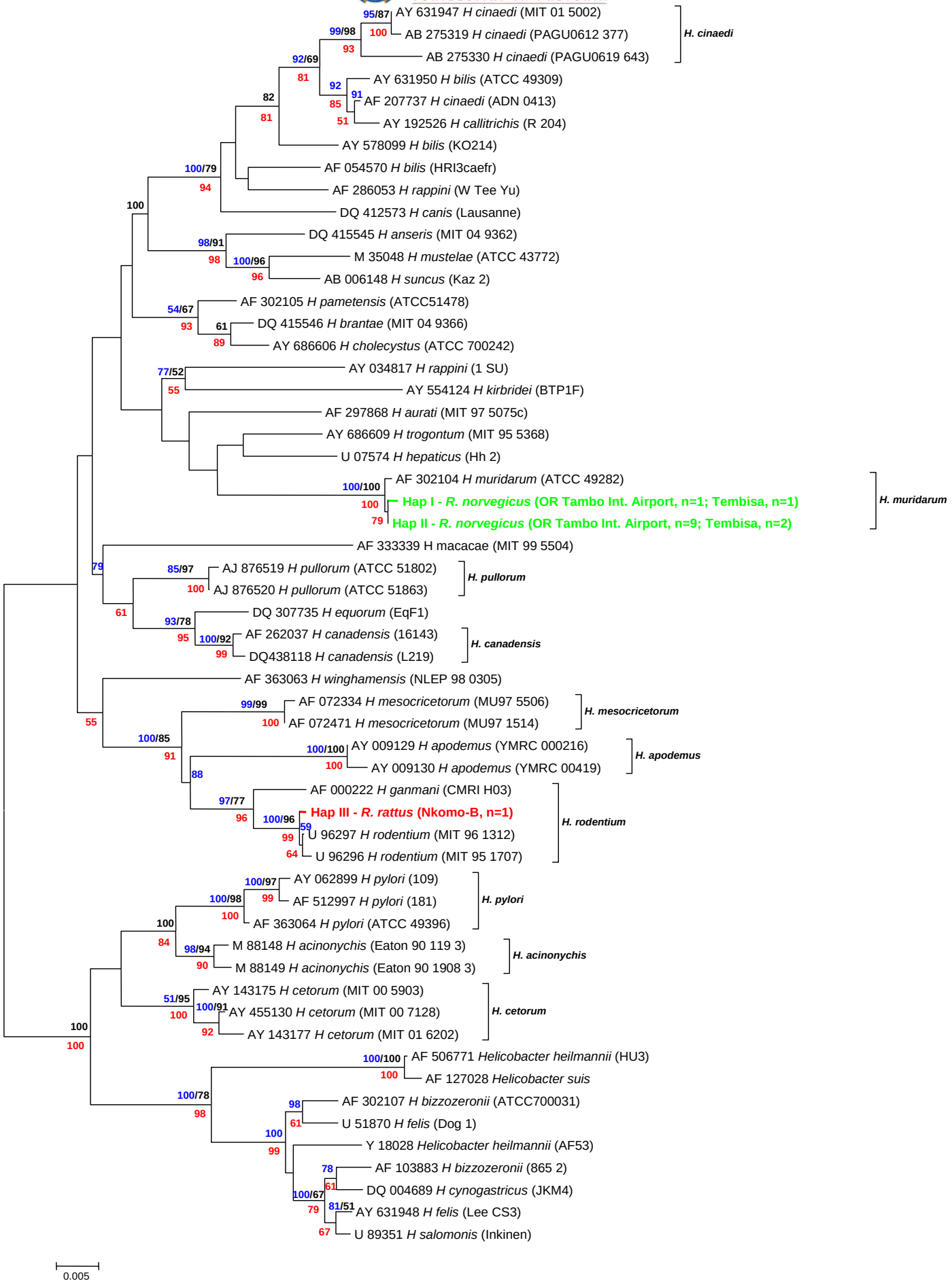
AY 631947 H cinaedi (MIT 01 5002)
AB 275319 H cinaedi (PAGU0612 377)
AB 275330 H cinaedi (PAGU0619 643)
H. cinaedi
AY 631950 H bilis (ATCC 49309)
AF 207737 H cinaedi (ADN 0413)
AY 192526 H callitrichis (R 204)
AY 578099 H bilis (KO214)
AF 054570 H bilis (HRI3caefr)
AF 286053 H rappini (W Tee Yu)
DQ 412573 H canis (Lausanne)
DQ 415545 H anseris (MIT 04 9362)
M 35048 H mustelae (ATCC 43772)
AB 006148 H suncus (Kaz 2)
AF 302105 H pametensis (ATCC51478)
DQ 415546 H brantae (MIT 04 9366)
AY 686606 H cholecystus (ATCC 700242)
AY 034817 H rappini (1 SU)
AY 554124 H kirbridei (BTP1F)
AF 297868 H aurati (MIT 97 5075c)
AY 686609 H trogontum (MIT 95 5368)
U 07574 H hepaticus (Hh 2)
AF 302104 H muridarum (ATCC 49282)
Hap I - R. norvegicus (OR Tambo Int. Airport, n=1; Tembisa, n=1)
Hap II - R. norvegicus (OR Tambo Int. Airport, n=9; Tembisa, n=2)
H. muridarum
AF 333339 H macacae (MIT 99 5504)
AJ 876519 H pullorum (ATCC 51802)
AJ 876520 H pullorum (ATCC 51863)
H. pullorum
DQ 307735 H equorum (EqF1)
AF 262037 H canadensis (16143)
DQ438118 H canadensis (L219)
H. canadensis
AF 363063 H winghamensis (NLEP 98 0305)
AF 072334 H mesocricetorum (MU97 5506)
AF 072471 H mesocricetorum (MU97 1514)
H. mesocricetorum
AY 009129 H apodemus (YMRC 000216)
AY 009130 H apodemus (YMRC 00419)
H. apodemus
AF 000222 H ganmani (CMRI H03)
Hap III - R. rattus (Nkomo-B, n=1)
U 96297 H rodentium (MIT 96 1312)
U 96296 H rodentium (MIT 95 1707)
H. rodentium
AY 062899 H pylori (109)
AF 512997 H pylori (181)
AF 363064 H pylori (ATCC 49396)
H. pylori
M 88148 H acinonychis (Eaton 90 119 3)
M 88149 H acinonychis (Eaton 90 1908 3)
H. acinonychis
AY 143175 H cetorum (MIT 00 5903)
AY 455130 H cetorum (MIT 00 7128)
AY 143177 H cetorum (MIT 01 6202)
H. cetorum
AF 506771 Helicobacter heilmannii (HU3)
AF 127028 Helicobacter suis
AF 302107 H bizzozeronii (ATCC700031)
U 51870 H felis (Dog 1)
Y 18028 Helicobacter heilmannii (AF53)
AF 103883 H bizzozeronii (865 2)
DQ 004689 H cynogastricus (JKM4)
AY 631948 H felis (Lee CS3)
U 89351 H salomonis (Inkinen)
0.005

Fig. 5.3

Table 5.1 *Helicobacter* species that have been isolated from ro[illegible]st species.

***Helicobacter* spp.**	**Host**	**References**
F. rappini	Mice (*Mus*)	Schauer *et al.* (1993); Zenner (1999); Whary & Fox (2006)
H. apodemus	Gerbils (*Meriones*); Jerboas (*Dipus; Euchoreutes*); Mice (Apodemus; Wiedomys); Rats (Nesokia; Oligoryzomys)	Jeon *et al.* (2001); Goto *et al.* (2004); Comunian *et al.* (2006)
H. aurati	Hamsters (*Mesocricetus*)	Patterson *et al.* (2000); Whary & Fox (2006)
H. bilis	Gerbils (lab gerbils e.g. *Meriones*); Mice (*Mus*); Rats (*Rattus*)	Zenner (1999); Goto *et al.* (2000); Whary & Fox (2006); Taylor *et al.* (2007)
H. bizzozeroni	Rats (*Rattus*)	Whary & Fox (2006)
H. Canadensis	Jerboas (*Dipus; Euchoreutes*)	Goto *et al.* (2004)
H. cholecystus	Hamsters (*Mesocricetus*)	Franklin *et al.* (1996); Zenner (1999); Whary & Fox (2006)
H. cineadi	Hamsters (*Mesocricetus*); Mice (*Calomys*); Rats (*Oryzomys*)	Gebhart *et al.* (1989); Zenner (1999); Comunian *et al.* (2006); Whary & Fox (2006)
H. ganmani	Gerbils (*Meriones*); Mice (*Mus; Bolomys*); Rats (*Oryzomys*)	Robertson *et al.* (2001); Goto *et al.* (2004); Comunian *et al.* (2006); Whary & Fox (2006)
H. heilmanni	Rats (*Rattus*)	Giusti *et al.* (1998)
H. hepaticus	Gerbils (*Brachiones; Meriones*); Mice (*Mus*); Rats (*Rattus*)	Zenner (1999); Goto *et al.* (2000); Goto *et al.* (2004); Comunian *et al.* (2006); Taylor *et al.* (2007)
H. marmotae	Rats (*Oryzomys*); Woodchuck (*Marmota*)	Fox *et al.* (2002); Comunian *et al.* (2006)
H. mastomyrinus	Mice (*Mastomys*)	Shen *et al.* (2005); Whary & Fox (2006)
H. mesocricetorum	Hamsters (*Mesocricetus*)	Simmons *et al.* (2000); Whary & Fox (2006)
H. muridarum	Mice (*Mus*), Rats (*Rattus*)	Lee *et al.* (1992); Zenner (1999); Whary & Fox (2006)
H. mustelae	Ferret (*Mustela*)	Fox *et al.* (1988); Zenner (1999)
H. pametensis	Rats (*Nesokia*)	Goto *et al.* (2004)
H. rodentium	Mice (*Mus*); Rats (*Oryzomys; Rattus*)	Shen *et al.* (1997); Zenner (1999); Goto *et al.* (2000); Comunian *et al.* (2006); Whary & Fox (2006); Taylor *et al.* (2007)
H. trogontum	Rats (*Rattus*)	Mendes *et al.* (1996); Zenner (1999)
H. typhlonius	Mice (*Mus*); Rats (*Rattus*)	Franklin *et al.* (2001); Whary & Fox (2006)
H. winghamensis	Gerbils (*Brachiones; Meriones*); Rats (*Nesokia*)	Goto *et al.* (2004)

Table 5.2 Geographic coordinates of the sampling localities of *Rattus rattus*, *R. tanezumi* and *R. norvegicus* from South Africa examined in the present study.

	Locality	Province	Geographic coordinates
1	Bloublommetjieskloof	Limpopo	24°18.66 S - 29°46.17 E
2	Hammanskraal	Gauteng	25°22.27 S - 28°11.26 E
3	Moreleta Park, Pretoria	Gauteng	25°49.67 S - 28°17.30 E
4	Mvuzini	Kwa-Zulu Natal	28°00.50 S - 30°40.52 E
5	Nkomo-B	Limpopo	23°24.96 S - 30°47.13 E
6	Ophuzane	Kwa-Zulu Natal	27°29.16 S - 30°56.03 E
7	O.R. Tambo International Airport	Gauteng	26°08.68 S - 28°13.57 E
8	Rietondale, Pretoria	Gauteng	25°26.10 S - 28°07.85 E
9	Tembisa	Gauteng	26°00.11 S - 28°12.78 E
10	Tholakele	Kwa-Zulu Natal	27°26.05 S - 30°59.28 E
11	Hillcrest, Pretoria	Gauteng	25°45.05 S - 30°59.28 E
12	Waterkloof, Pretoria	Gauteng	25°49.13 S - 28°15.30 E

Table 5.3 *Helicobacter* species, their Genbank accession numbers and the strain used in the design of the lineage specific primers in the present study.

Species	Genbank accession number	Strain	Reference
H. cinaedi	AY631947	MIT 01-5002	Dewhirst *et al.* (2005)
	AB275319	PAGU0612 (=377)	Kitamura *et al.* (2007)
	AB275330	PAGU0619 (=643)	Kitamura *et al.* (2007)
	AF207737	ADN 0413	Vandamme *et al.* (2000)
H. bilis	AY578099	KO214	Hanninen *et al.* (2005)
	AF054570	HRI3caefr	Robertson (unpubli.)
H. callitrichis	AY192526	R-2O4	Won *et al.* (2007)
H. rappini	AF286053	W. Tee-Yu	Tee *et al.* (2001)
	AY034817	1 SU	Hanninen *et al.* (2003)
H. canis	DQ412573	Lausanne	Leemann *et al.* (2006)
H. trogontum	AY686609	MIT 95-5368	Dewhirst *et al.* (2005)
	AY596249	ATCC 43968	Dewhirst *et al.* (2005)
H. hepaticus	NR025952	Hh-2	Fox *et al.* (1994)
H. typhlonicus	AF061104		Franklin *et al.* (1999)
H. muridarum	AY596238	ATCC 49282	Dewhirst *et al.* (2005)
H. aurati	NR025124	MIT 97-5075c	Patterson *et al.* (2000)
H. mustelae	AY596233	ATCC 43772	Dewhirst *et al.* (2005)
H. suncus	AB006148	Kaz-2	Goto *et al.* (1998)
H. anseris	DQ415545	MIT 04-9362	Fox *et al.* (2006)
H. pametensis	AY596230	ATCC 51478	Dewhirst *et al.* (2005)
H. brantae	DQ415545	MIT 04-9362	Fox *et al.* (2006)
H. cholecystus	AY686606	ATCC 700242	Dewhirst *et al.* (2005)
H. canadensis	NR025096	NLEP-16143	Fox *et al.* (2000)

Species	Genbank accession number	Strain	Reference
	DQ438118	L219	Inglis *et al.* (2006)
H. equorum	DQ307735	EqF1	Moyaert *et al.* (2007)
	DQ307736	EqF2	Moyaert *et al.* (2007)
H. pullorum	L36143	NCTC 12826	Stanley *et al.* (1994)
	L36142	NCTC 12825	Stanley *et al.* (1994)
H. macacae	AF333339	MIT 99-5504	Fox *et al.* (2001)
H. kirbridae	AY554124	BTP1F	Coldham *et al.* (unpubli.)
H. winghamensis	AF363063	NLEP 98-0305	Melito *et al.* (2001)
H. apodemus	AY009129	YMRC 000216	Jeon *et al.* (unpubli.)
	AY009130	YMRC 000419	Jeon *et al.* (unpubli.)
H. mesocricetorum	AF072334		Simmons *et al.* (2000)
	NR024923	MU97-1514	Simmons *et al.* (2000)
H. rodentium	U96297		Shen *et al.* (1997)
	NR026074	MIT 95-1707	Shen *et al.* (1997)
H. ganmani	AF000222	CMRI H03	Robertson *et al.* (2001)
H. cetorum	AF455130	MIT 00-7128	Harper *et al.* (2002)
	AY143177	MIT 01-6202	Harper *et al.* (2002)
	AY143175	MIT 00-5903	Harper *et al.* (2002)
H. acinonychis	MM88148		Eaton *et al.* (unpubli.)
H. pylori	AY062899	109	Trieber *et al.* (2002)
	AF512997	181	Gerrits *et al.* (2002)
	AF363064	ATCC 49396	Melito *et al.* (unpubli.)
H. heilmanni	AF507995	HU3	O'Rourke *et al.* (2004)
	AF506770	AD1	O'Rourke *et al.* (2004)
	Y18028		Andersen *et al.* (1999)
H. suis	AF127028		De Groote *et al.* (1999)
H. bizzozeronii	AF302107		Melito *et al.* (unpubli.)
	AF103883		Cattoli *et al.* (1999)
H. felis	U51870		Eaton *et al.* (1996)
	AY631948	Lee CS3	Dewhirst *et al.* (2005)
H. cynogastricus	DQ004689	JKM4	Van den Bulck *et al.* (2006)
H. salomonis	NR026065	Inkinen	Jalava *et al.* (1997)

Table 5.4 Sample names and localities of samples of *Rattus* from South Africa that tested positive for *Helicobacter* infection. O.R. Tambo Int. Airport denotes O.R. Tambo International Airport.

Sample name	Location	*Helicobacter* species isolated	Tissue isolated from	Species isolated from
ARC83	Nkomo-B, Limpopo	*H. rodentium*	Liver	*R. rattus*
HB01	O.R. Tambo Int. Airport, Gauteng	*H. muridarum*	GIT	*R. norvegicus*
HB02	O.R. Tambo Int. Airport, Gauteng	*H. muridarum*	GIT	*R. norvegicus*
HB03	O.R. Tambo Int. Airport, Gauteng	*H. muridarum*	GIT	*R. norvegicus*
HB05	O.R. Tambo Int. Airport, Gauteng	*H. muridarum*	GIT	*R. norvegicus*
HB06	O.R. Tambo Int. Airport, Gauteng	*H. muridarum*	GIT	*R. norvegicus*
HB07	O.R. Tambo Int. Airport, Gauteng	*H. muridarum*	GIT	*R. norvegicus*
HB10	O.R. Tambo Int. Airport, Gauteng	*H. muridarum*	GIT	*R. norvegicus*
HB13	O.R. Tambo Int. Airport, Gauteng	*H. muridarum*	GIT	*R. norvegicus*
HB20	O.R. Tambo Int. Airport, Gauteng	*H. muridarum*	GIT	*R. norvegicus*
HB26	O.R. Tambo Int. Airport, Gauteng	*H. muridarum*	GIT	*R. norvegicus*
HB71	Tembisa, Gauteng	*H. muridarum*	GIT	*R. norvegicus*
HB72	Tembisa, Gauteng	*H. muridarum*	GIT	*R. norvegicus*
HB74	Tembisa, Gauteng	*H. muridarum*	GIT	*R. norvegicus*

Table 5.5 Number of individuals from each *Rattus* species from South Africa tested for *Helicobacter* infection in the liver and the gastro intestinal tract (GIT), the number of samples that were positive in the liver and the GIT and the prevalence of *Helicobacter* infection in each species as well as tissue prevalence.

Species	No. tested Liver	No. positive liver	Prevalence liver (%)	No. tested GIT	No. Positive GIT	Prevalence GIT (%)
R. rattus	45	1	2.2	6	0	0
R. tanezumi	73	0	0.0	28	0	0
R. norvegicus	46	0	0.0	46	13	28.3
Total	164	1	0.6	80	13	16.3

CHAPTER 6

GENERAL DISCUSSION

The current study acknowledged the occurrence of three invasive *Rattus* species in South Africa. Traditionally, two species of invasive *Rattus*, *Rattus rattus* and *R. norvegicus* have been known to occur in South Africa (De Graaf 1981; Skinner & Smithers 1990) as pests in rural households and human-mediated areas. During the trapping of *Rattus* at some of these villages, *R. tanezumi* was discovered in 2005 and increasing the number of invasive species to three (Bastos *et al.* 2005; Taylor *et al.* 2008; Bastos *et al*. unpublished). *Rattus rattus* and *R. norvegicus* are two of the most familiar commensal species and occur worldwide (De Graaf 1981; Skinner & Smithers 1990; Aplin *et al.* 2003; Chinen *et al.* 2005; Musser & Carleton 2005) and the capture of *R. tanezumi* in and around human dwellings indicates that it most likely can also be considered a true commensal.

This study was aimed at evaluating the molecular and morphological differences observed in members of South African *Rattus* and their role in transmitting zoonotic diseases such as *Bartonella* and *Helicobacter* especially given their close association with humans. The study represents the most comprehensive inland *Rattus* dataset generated to date, and genetic analyses consisting of cytochrome *b* data (from a previous study as well as sequences generated in this study) and D-loop from this study represented the first genetic data for *Rattus* occurring in South Africa. The D-loop phylogeny recovered only four haplotypes for *R. rattus* which most likely represented three separate introductions that occurred relatively recently. Sequences were closely related to those from Indonesia, the Pacific Islands and New Zealand, but due to the relatively global distribution of *Rattus* (De Graaf 1981; Skinner & Smithers 1990; Aplin *et al*. 2003; Musser & Carleton 2005), it is difficult to establish with certainty exactly where these individuals originated from.

No genetic variation was observed within *R. tanezumi* which indicates a recent, single introduction into South Africa, especially if this lack of diversity can be observed throughout the distributional range of *R. tanezumi's* in South Africa. The closest link with South African *R. tanezumi* based on D-loop data is to sequences generated from *Rattus*

from Indonesia. The *Bartonella* data confirms the Indonesian link as *Bartonella* haplotypes recovered in South Africa are similar to those isolated from *R. tanezumi* from Indonesia. This may not be surprising since *R. tanezumi* has a south-east Asian origin.

Two *R. norvegicus* haplotypes were recovered from the D-loop data are indicative of two separate introductions. However, their origin was difficult to establish because samples grouped with individuals from countries as far apart as such as Great Britain and French Polynesia. Because of the relatively global distribution of *R. norvegicus* (De Graaf 1981; Skinner & Smithers 1990; Aplin *et al*. 2003; Musser & Carleton 2005), and limited available sequence data for wild-caught individuals (Bastos *et al.* unpublished), it is difficult to determine with certainty the origin of South African *R. norvegicus*. This species was unexpectedly captured during inland sampling for specimens of *R. rattus* and *R. tanezumi*. Prior to this study, *Rattus norvegicus* was believed to only occur along the coastal regions of South Africa and within harbour towns (De Graaf 1981; Skinner & Smithers 1990). It would be interesting to compare sequences of inland South African *R. norvegicus* with coastal individuals from their traditional distribution ranges (Bastos *et al.* unpublished) to determine if there is more variation within South African *R. norvegicus* especially between populations representing such diverse habitat types with significant differences in altitude and geography. With the growing number of entries of *Rattus* sequences in the Genbank database, it may become possible to more accutately determine the origin and migration patterns of members of the genus *Rattus* worldwide.

The external cranial morphology and external characters showed some differences between members of the *R. rattus* species complex and *R. norvegicus*. However, it was more difficult to distinguish between *R. rattus* and *R. tanezumi* as many observed differences were not consistent throughout the samples. There is therefore a need for further morphological studies that should also include both traditional and geometric morphometric studies of *R. rattus* and *R. tanezumi*. Similar studies should also be conducted on members of the *R. rattus* species complex from other parts of the world as it is possible that extralimital members of the *R. rattus* species complex may have been wrongly identified morphologically. However, the morphological component of this study was valuable as it was the first time that morphological comparisons were based on genetically identified samples.

Future studies should combine the current data of South African *Rattus* with samples from the rest of Africa to determine similarity and species distribution across the continent as well as to determine the extent of the distribution of *R. tanezumi* in Africa. It would also be very informative to incorporate all available data into computer modelling programs (e.g., Harper *et al*. 2005; Neerinckx *et al*. 2008; Bastos *et al.* unpublished), in order to predict possible areas that may be threatened by invasion based on the current distributional ranges in South Africa. These models may also provide insights into the number of possible introduction events which could be compared to a number of possible introductions determined through molecular analyses.

More extensive cytogenetic studies are needed on South African members of the *R. rattus* species complex as these individuals are known to have different chromosome numbers and karyotype diversity has not been extensively studied for local *Rattus*. Nuclear genes should be targeted in order to determine the introgression capabilities of these rats as this in conjunction with the cytogenetics would provide useful information on the breeding patterns of *R. rattus* and *R. tanezumi*. Preliminary data on South African *R. rattus* complex specimens in which the BRCA, GHR and IRBP gene regions (e.g., Adkins *et al.* 2001; Chinen *et al*. 2005; Lecompte *et al.* 2008) were targeted, indicated that GHR provided the best resolution capabilities and may therefore be a good region to use in future studies (Mostert *et al.*, unpublished).

The recovery of *Bartonella elizabethae* from members of *Rattus* living commensally with humans was especially important due to this bacterium's known zoonotic potential resulting in severe cases of endocarditis. It was, however, surprising that members of *Rattus* had lower prevalence rates than observed for endemic species (Bastos 2007). Future studies should, however, include the assessment of *Bartonella* prevalence in humans through health-authority assisted screening of blood samples, as well as serological studies on humans, especially in rural areas where people come into contact with wild animals and where healthcare is poor. More studies should also be considered on other diseases that can spread zoonotically through their *Rattus* hosts as many of these potentially threatening diseases could present symptoms that are not easily recognised. *Helicobacter* prevalence was markedly lower than *Bartonella* prevalence determined in Chapter 4 and the *Helicobacter* species recovered have no known zoonotically potential.

Of particular importance, however, is the higher number of infections observed within *R. norvegicus* compared to members of the *R. rattus* complex not only with regard to *Helicobacter*, but also *Bartonella* infection. *Rattus norvegicus* individuals also showed co-infection with *Bartonella* and *Helicobacter*. Out of the 46 *R. norvegicus* individuals screened for the disease aspect of the study, seven individuals (15.2%) demonstrated co-infection with *Helicobacter* and *Bartonella*. This difference in infection prevalence between species may be indicative of differences in the biology between *R. norvegicus* and the *R. rattus* species complex, or differences in species selection by the arthropod vectors which requires further investigation, not only on rodent biology, but also on host preference of the bacteria and arthropod vectors responsible for spreading the bacteria.

6.1 REFERENCES

ADKINS, R. M., GELKE, E. L., ROWE, D. & HONEYCUTT, R. L. 2001. Molecular phylogeny and divergence time estimates for major rodent groups: evidence form multiple genes. *Molecular Biology and Evolution* **18**: 777-791.

APLIN, K. P. & SINGLETON, G. R. 2003. Balancing rodent management and small mammal conservation in agricultural landscapes: challenges for the present and the future. *In*: Rats, mice and people: rodent biology and management (Singleton, G. R., Hinds, L. A., Krebs, C. J. & Spratt, D. M.), Australian Centre for International Agricultural Research, Canberra. Pp. 80-88.

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. *South African Society for Veterinary Epidemiology and Preventive Medicine*. Proceedings Pretoria, 11-12 August 2005. 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.

CHINEN, A. A., SUZUKI, H., APLIN, K. P., TSUCHIYA, K. & SUZUKI, S. 2004. Preliminary genetic characterization of two lineages of black rats (*Rattus rattus* sensu lato) in Japan, with evidence for introgression at several localities. *Genes Genetic Systematics* **80**: 367–375.

DE GRAAFF, G. 1981. The rodents of southern Africa: notes on their identification, distribution, ecology and taxonomy. Butterworth, Durban. Pp. 218-228.

HARPER, G. A., DICKINSON, K. J. M. & SEDDON, P. J. 2005. Habitat use by three rat species (*Rattus* spp.) on Stewart Island/Rakiura, New Zealand. *New Zealand Journal of Ecology* **29**: 251-260.

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**: 1-21.

MUSSER, G. G., & CARLETON, M. D. 2005. Family Muridae. *In*: Mammal Species of the World, 3rd Edition (*Wilson, D. E. & Reeder, D. M. eds*). The Johns Hopkins Univeristy Press, Baltimore. Pp. 894–1531.

NEERINCKX, S. B., PETERSON, A. T., GULINCK, H., DECKERS, J. & LEIRS, H. 2008. Geographic distribution and ecological niche of plague in sub-Saharan Africa. *International Journal of Health Geographics* **7**: 1-12.

SKINNER, J. D. & SMITHERS, R. H. N. 1990. *The Mammals of the Southern African Subregion*. CTP Book Printers, Cape Town. 736 pp.

TAYLOR, P. J., ARNTZEN, L., HAYTER, M., ILES, M., FREAN, J. & 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.