Molecular prevalence and diversity of zoonotic bacteria of invasive *Rattus* from South Africa, with emphasis on the genera *Rickettsia* and *Streptobacillus*

by

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GENERAL ABSTRACT

The study investigated the origin and diversity of the three matrilineally-defined invasive, commensal *Rattus* species, namely *R. norvegicus*, *R. rattus* and *R. tanezumi* known to occur in South Africa after routine identification of the species using molecular techniques. Subsequently, their role as potential zoonotic disease reservoirs in primarily urban environments with particular interest in their potential to transmit and spread zoonotic disease through direct contact as well as indirect contact through the urinary route were investigated. Bacterial prevalence and diversity were determined by PCR and nucleotide sequencing, respectively.

Genetic diversity of *Rattus* in southern Africa was previously explored and despite additional samples being characterised in the present study, the genetic diversity in the southern African *Rattus* population remained relatively low when compared to global genetic diversity of the three matrilineally-defined *Rattus* species. Evidently, the observed genetic diversity is probably a result of a combination of introduction events and subsequent diversification which is possibly limited through interspecific competition with indigenous rodents. However, samples from the west and south coast of South Africa are under-represented in the data set and this would need to be addressed for better insight into the genetic diversity of the South African *Rattus* population.

An overall bacterial prevalence of 49 % was observed in kidney samples using a 16S broad range PCR primer assay which when sequenced identified a diverse range of bacteria genera namely, *Acinetobacter*, *Bartonella*, *Brochothrix*, *Rickettsia* and *Streptococcus*. These bacteria are implicated in causing opportunistic infection and may contribute to food spoilage. *Streptobacillus moniliformis* is the etiological agent of rat-bite fever and Haverhill fever. A prevalence of 50.9 % was obtained in oral swabs while in kidney tissue this was only

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6.3 %. Nucleotide sequencing of *S. moniliformis* revealed two *S. moniliformis* strains specific to the three matrilineally-defined *Rattus* hosts present in South Africa. Evidence of host specificity exists but could not be linked to the ecological factors tested. Rickettsial DNA was detected in only one *R. tanezumi* sample, corresponding to a prevalence of 0.9 %. Subsequent nucleotide sequencing and phylogenetic analysis confirmed the presence of pathogenic, *Rickettsia felis*. Consequently, *Rattus* is likely to be only an incidental host and the infection is probably maintained by their associated ectoparasites.

The study has determined that introductions of some invasive, commensal *Rattus* have occurred relatively recently. Apart from the accompanying diseases introduced with them, the continuous influx of genetic diversity may influence rodent control efforts. In addition, the role of *Rattus* in the direct and indirect transmission and spread of zoonotic disease has been demonstrated, and their presence poses a public health and food security threat. Continuous disease surveillance and rodent control is crucial not only in urban areas where conditions are conducive to disease outbreaks but also at points of entry such as sea- and airports.

KEYWORDS: bacteria, commensal, invasive species, matrilineally-defined species, mitochondrial haplotypes, public health, *Rattus*, South Africa

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Die verhandeling word opgedra aan my ouma, wie geglo het dat God 'n spesiale plek het vir diegene wat werk met kinders en diere.

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DECLARATION

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

SIGNATURE…………………………

DATE…………11 July 2013………………………

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DISCLAIMER

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

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Chapter 1

GENERAL INTRODUCTION

A species of either plant or animal living in close association with another is known as a commensal species. Some members of the genus *Rattus* have become as such and habitually congregate with humans as they have learnt to exploit human habitation for food and shelter (Brooks & Jackson 1973). Rodents of the genus *Rattus* have been historically associated with human settlements since 1500 BC (Atkinson 1985). This long evolutionary relationship of rats and humans has had many pros and cons. In the past, rats played an important role in social and cultural activities and were hunted for sport, skin (used in textiles), whiskers (for use in paintbrushes) and meat (Barnett 2001). Some believe that the eating of rats by sailors may have unknowingly provided a source of vitamin C preventing scurvy (Barnett 2001). In modern medicine, *R. norvegicus* became specifically bred and used as biological model organisms in which to test experimental treatments (Richter 1968). Given optimal conditions, their similar physiology, enables them to transmit diseases to humans that otherwise have no effect on them and are therefore often undetected. Commensal *Rattus* species also exhibit extraordinary adaptive biology and behaviour. They become serious pests in agriculture when they destroy and consume food crops (Singleton 1999), and many species of the genus are successful invasive species in both anthropologically altered and pristine environments (Atkinson 1985). Here, they often reach high densities, making them difficult to control. This is exacerbated by the evasive behaviour of these rats, and it has been suggested that many years of hunting and trapping of rats by humans may have resulted in characteristic neophobia displayed by commensal rodents as a result of natural selection (Barnett 2001).

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Taxonomy

There are approximately 66 species within the genus *Rattus* (Musser & Carleton 2005) of which the black rat, *R. rattus* and the brown rat, *R. norvegicus*, are the most widespread invasive, commensal species of rats (Long 2003; Musser & Carleton 2005). This approximation of the number of species is needed as introgression between *R. tanezumi* and *R. rattus* genomes has recently been reported in areas where both species are invasive (Lack *et al.* 2012; Conroy *et al.* 2013). Conversely, recent genetic assessments indicate that the genus is far more species rich than suggested by morphology (Pagès *et al.* 2010; Aplin *et al.* 2011). Their worldwide distribution may be due to their adaptation to commensalism (Long 2003). *Rattus rattus* is native to southern and eastern Asia (Indian peninsula) and *R. norvegicus* is believed to have originated from northern China, Siberia and Japan (Musser & Carleton 2005) but both are now widely distributed in Africa. Individuals of some populations of *R. tanezumi* are morphologically indistinguishable from *R. rattus* (Musser & Carleton 2005; Mostert 2009) and the two species often occur in sympatry in areas to which they have been introduced. Along with a number of other genetically discrete lineages but morphologically uniform lineages, they constitute the *R. rattus* species complex (Taylor *et al.* 2008; Aplin *et al.* 2011; Bastos *et al.* 2011). *Rattus tanezumi* was believed to be restricted to southeast Asia (Musser & Carleton 2005) but may be more widespread than previously known as it may often be confused with *R. rattus* in most parts of the world (Plyusnina *et al.* 2009). *Rattus tanezumi* for example, was recorded for the first time in South Africa (and Africa) as recently as 2005 (Bastos *et al.* 2005).

Invasion biology

Invasive species present a global risk to indigenous species of all ecosystems and invasive rats significantly impact island ecosystems (Atkinson 1985). Three species in particular, *R. rattus*, *R. exulans* and *R. norvegicus* are believed to have colonised at least 82 % of the 123

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major island groups worldwide (Atkinson 1985). In South Africa, 16 out of 247 total mammalian species are exotics (Pimentel *et al.* 2001) although many more are expected to be discovered with the aid of molecular techniques. *Rattus tanezumi* was recorded in South Africa (and Africa) when molecular techniques were employed to genetically identify rodents (Bastos *et al.* 2005).

Historically, most rat introductions to islands and non-native continents occurred unintentionally as stowaways of trade and cargo ships (Atkinson 1985). In some instances, rats became introduced to islands when humans exploited resources close to island shorelines (Atkinson 1985). Most likely, rats are currently introduced and spread more extensively by additional modes of transportation such as aeroplanes, trains and other vessels (Bastos *et al.* 2011).

The success of rats as biological invaders is mostly attributed to their biology. They are cautious (neophobic) (Barnett 2001), have high reproduction rates (Davis 1948), are omnivorous and highly adaptable with the ability to exploit disturbed habitats (Brown 1989). In addition, they become introduced to ecosystems where no existing natural equivalent species or predators occur (Brown 1989; Courchamp *et al.* 2003) and may replace those endemic species with which they share an ecological niche, through competition (Atkinson 1985).

In New-Zealand, *R. exulans* is responsible for at least six avifaunal and several reptile species declines and local extinctions (Towns & Broome 2003), while *R. rattus* invasion has led to the extinction of several endemic rodent species on various islands (Amori & Clout 2003). Rats also affect indigenous flora when they consume plant seed and seedlings (Atkinson 1985; Amori & Clout 2003) and influence plant species compositions (Towns & Broome 2003).

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Improper control measures for rats have had catastrophic effects by contributing to native species declines (Atkinson 1985; Amori & Clout 2003). This has led to the conclusion that vulnerable environments especially those with rich endemic ground nesting avifauna and highly diverse rodent fauna should avoid rat invasion at all costs (Amori & Clout 2003; Towns & Broome 2003).

The role of *Rattus* in tracing human migrations

The invasion of rats has proved to be useful in tracing human migrations (Matisoo-Smith *et al.* 1998; Matisoo-Smith & Robins 2004). Phylogeographic analysis of rat populations has revealed evidence on the origin of Polynesian people and subsequent pattern of human settlement on the Pacific islands (Matisoo-Smith *et al.* 1998).

Rattus exulans, originally from island or peninsular south-eastern Asia, was intentionally introduced by the first human settlers to islands of the Pacific, possibly as food source. Although ancestral Polynesians introduced plant and animal species in common with those associated with European colonisation, the association of *R. exulans* and ancestral Polynesians was a unique phenomenon. It appears in the earliest geological layers of remote Pacific islands and throughout all archaeological sites associated with the ancestral Polynesian people (Matisoo-Smith *et al.* 1998; Matisoo-Smith & Robins 2004). Because *R. rattus* and *R. norvegicus*, which were introduced by Europeans, do not interbreed with *R. exulans* (Matisoo-Smith & Robins 2004), the study used mitochondrial DNA (mtDNA) sequences of *R. exulans* as genetic markers to trace human migration. This is possible because rats (as with other rodents) have rapid generation turnover and *R. exulans* have high genetic variation that reflects recent evolutionary events (Matisoo-Smith *et al.* 1998). This highlights the value of using *Rattus* as a proxy for other human migration studies.

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Economic importance

Invasive rodents, specifically rats, have the ability to cause mass destruction leading to major monetary loss. Their omnivorous nature and behaviour affords them the ability to gnaw on and consume a very broad range of materials (Putman 1989). Consequently, they often gnaw on electrical wiring, cardboard storage boxes, building structures and many other goods and materials. This behaviour has safety implications for humans as it may lead to fire, explosions and disintegration of buildings (Pimentel *et al.* 2000) potentially causing loss of life. Rats also infest, contaminate and consume stored grains and other stored food products (Putman 1989) and destroy agricultural crops, causing reduced food supply and income leading to famine in poor households. Biological invasion of rats on islands has resulted in severe ecosystem disruption due to localised native species declines.

Routine repair to damaged building structures and electrical wiring as a result of rat infestation is constantly needed and incur significant costs. In addition, costs escalate with on-going control measures such as trapping and baiting. Furthermore, efforts to reduce and reverse adverse ecological effects of rat invasions are associated with great economic costs.

It is thus not surprising that of all the invasive mammals, rats are responsible for causing the greatest economic loss in the USA (Pimentel *et al*. 2000). Economic losses from agricultural crops alone due to introduced vertebrate species have been estimated to US$ 2.4 billion per year (Pimentel *et al.* 2001) and the presumption that rats contribute mostly to these losses cannot be excluded. Elsewhere, Ahmad *et al.* (1995) reported a large annual loss of 740kg of rice per shop in Pakistan as result of a combination of spillage, contamination and consumption by rats. Clearly, this phenomenon is evident worldwide and amounts to an enormous global loss of foodstuffs due to rat infestations.

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Rats as food source

Rats, in especially Asia, form an important source of meat for human consumption (Barnett 2001; Khiem *et al.* 2003). In addition, rats are also used in traditional medicine (Mangoendihardjo & Wagiman 2003). In India consumption of rat meat is believed to increase semen production in men (Negi & Palyal 2007; Benarjee *et al.* 2010), while in Mexico it is believed to promote general health (Puente 1999). The rat meat market in the Mekong Delta, Vietnam although centralised, generates livelihoods to local farmers that act as rat catchers and to meat processers that set up businesses (Khiem *et al.* 2003). Rat meat is popular as there are few protein sources available with an average of 77-480 kg of rat meat processed daily (Khiem *et al.* 2003). Rat meat is typically part of the bushmeat trade (Karesh and Noble 2009) and exports of indigenous species such as the cane rat (*Thryonomys* sp.) are common to Europe and America (Schmadeke 2010). The extent of consumption of rodents of the genus *Rattus* however, is largely unknown but is believed to be reduced due to widespread knowledge of well-known diseases such as plague being associated with them (Deutsch & Murakhver 2012).

Rats in the pet industry

Rats have become popular in the pet trade (Elliot 2007) and have been described as affectionate, intelligent, social and clean animals that develop intense connections to their owner (Mann 2001). This pet had become so popular that it had an established club of over 200 members namely the Rat & Mouse Fanciers for Excellence (RMFE) which included an international rodent registry database (rodentfancy.com). Pet rats are usually traditional laboratory rats that are specifically bred as pet rats and not wild rats which were tamed. For this reason they are termed "disease-free" or known as specified pathogen free (SPF) rats, as a wide variety of known pathogens have been excluded initially through hysterectomy from donor animals (Gaastra *et al.* 2009) but later through breeding (Richter 1968). Unfortunately,

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diseases such as rat-bite fever are often overlooked because the pathogen is a commensal in oral cavities of rats and may consequently be transmitted to humans through bites (Wullenweber 1995). Nevertheless, qualities credited to the pet rat such as little space and low food requirements, low-cost grooming and housing needs still make rats very attractive pets and they will continue to be so in future.

Biomedical importance

Biomedical model organisms

The brown rat, *R. norvegicus*, was the first animal to be domesticated strictly for scientific purposes (Richter 1968). Rats are used as model organisms for experiments on which to simulate effects on humans, and have been used in nearly all fields of biological research. In fact, most knowledge of endocrinology and nutrition of humans have come from experimental observations made of rats (Richter 1968).

Rattus norvegicus, specifically, has many valuable properties making it a good model organism on which to base experiments and its effects on humans. Its high reproduction rate and relatively short lifespan (Richter 1968) allows investigations on growth and aging factors, and to test various pharmacological treatments. The rat's nutritional demands are much the same as humans and pioneering research on the nutritive effects of vitamins was done with experiments on rats (Richter 1968). These nutritional studies revealed an exceptional ability of the rat to assess the nutritive value of food-stuffs by taste and smell, which enables the rat to avoid harmful or non-nutritive substances (Richter 1968). It is this characteristic that makes it particularly difficult to use poison bait traps as a means to control rats. The anatomy, high resistance to infection and ample size of rats makes it a good subject for surgical manipulation (Richter 1968). A high resistance to infection, however, allows rats

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to have high parasitic loads and given their similar physiology enables them to transmit diseases to humans.

Disease carriers

Invasive rats are known to harbour a variety of parasites of veterinary and medical importance. Several hanta- and arena viruses known to cause haemorrhagic fevers (Gratz 1997; Mills & Childs 1998), disease-causing bacterial pathogens (Azad & Beard 1998; Easterbrook *et al.* 2007), helminths and protozoan parasites (Webster & MacDonald 1995; Claveria *et al.* 2005) have been isolated from rats. In addition, rats may also harbour several ectoparasites that act as vectors capable of transmitting disease-causing parasites to humans (Carter & Cordes 1980; Azad & Beard 1998).

Table 1 gives an indication of bacterial pathogens already isolated from *Rattus* spp. which are implicated in causing diseases in humans. The bacterial pathogens listed are those where rodents of the genus *Rattus* are, or have the potential to become, the reservoir hosts and may thus play a significant role in disease transmission to humans (zoonoses).

Table 1 Bacterial pathogens isolated from *Rattus* spp. and their associated diseases in humans.

Pathogen	**Transmission**	**Medical condition in humans**	**Reference**	**Prevalence in *Rattus***	**Reference**
Bartonella spp.	Vector-borne	Endocarditis	Smith *et al.* 2002	High	Ellis *et al.* 1999; Chomel *et al.* 2006; Easterbrook *et al.* 2007
Borrelia burgdorferi	Vector-borne	Lyme disease	Barbour & Fish 1993; Matuschka *et al.* 1996	High	Matuschka *et al.* 1996; Peavy *et al.* 1997; Richter *et al.*1999
Campylobacter spp.	Food-borne	Gastroenteritis	Meerburg *et al.* 2006	Low	Le Moine *et al.*1987; Meerburg *et al.*2006
Coxiella burnetii	Vector-borne	Q-Fever	Stanford *et al.* 1990; Domingo *et al.* 1999	High	Webster & MacDonald 1995; Comer *et al.* 2001
Francisella tularensis	Vector-borne/Food-borne	Tularaemia	Christova *et al.* 2004	High	Christova *et al.* 2004

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Helicobacter spp.	Environmental	Gastritis, peptic ulcers, lymphoma	Solnick 2003	High	Giusti *et al.* 1998; Comunian *et al.* 2006
Leptospira spp.	Environmental (excrement)	Leptospirosis	Binder & Mermel 1998; Taylor *et al.* 2008; Adler & De la Pen˜a Moctezuma 2010	Low to high	Carter & Cordes 1980; Webster *et al.* 1995; Taylor *et al.* 2008
Listeria monocytogenes	Food-borne	Listeriosis	Allerberger & Wagner 2010	Low	Webster & MacDonald 1995
Pasteurella pneumotropica	Direct/Environmental (excrement)	Endocarditis, septicemia, peritonitis	Campos *et al.* 2000; Nimri *et al.* 2001; Frebourg *et al.* 2002	Low to high	Carter & Cordes 1980; Shepherd *et al.* 1982; Le Moine *et al.*1987
Rickettsia spp.	Vector-borne	Typhus/spotted Fevers/tick bite fever	Comer *et al.* 2001; Kelly *et al.* 2004	High	Azad & Beard 1998; Comer *et al.* 2001
Salmonella spp.	Food-borne	Gastritis	Meerburg *et al.*2006; NICD 2008	Low	Le Moine *et al.*1987; Hilton *et al.* 2002
Spirillum minus	Direct	Spirillosis/rat-bite fever	Freels & Elliott 2004	Low to high	Gaastra *et al.* 2009
Staphylococcus aureus	Environmental (aerosol/excrement)	Staphylococcal bacteremia, sepsis	Lowy 1998; Van de Giessen *et al.* 2009	Low to high	Webster & MacDonald 1995; Wincewicz 2002; Van de Giessen *et al.* 2009
Streptobacillus moniliformis	Direct/Environmental (excrement)	Streptobacillosis /rat-bite fever	Van Nood & Peters 2005; Elliot 2007	High	Wullenweber 1995; Gaastra *et al.* 2009
Streptococcus pneumoniae	Direct/Environmental (aerosol)	Pneumococcal disease	Van der Linden *et al.* 2009	High	Webster & MacDonald 1995; Wincewicz 2002; Goodman 2004; Van der Linden *et al.* 2009
Yersinia enterocolitica	Food-borne	Yersiniosis	Hubbert 1972; Guven *et al.* 2010	Low	Iinuma *et al.* 1992; Webster & MacDonald 1995
Yersinia pestis	Vector-borne	Plague	Perry & Fetherston 1997; Neerinckx *et al.* 2010	Low to high	Perry & Fetherston 1997
Yersinia pseudotuberculosis	Food-borne	Yersiniosis	Hubbert 1972	Low	Kaneko *et al.*1979; Iinuma *et al.* 1992

In recent years, there have been numerous discoveries of novel diseases, while established diseases appeared to show increased incidence (McMichael 2004). A re-emerging disease, frequently associated with *Rattus*, is the well-known zoonotic disease, plague, caused by the *Yersinia pestis* bacterium (Perry & Fetherston 1997). This disease shaped history by weakening empires, altering socioeconomic structures and changing medical practice and public health regulation (Perry & Fetherston 1997; Barnett 2001). Plague is a vector-borne

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disease and typically transmitted to humans through bites from infected rat-fleas (Perry & Fetherston 1997). Sub Saharan Africa is most affected by plague with indigenous rodents acting as maintenance hosts of the bacteria (Gratz 1997; Neerinckx *et al.* 2010) and it is now formally recognized as being a re-emerging disease that is currently in a quiescent stage in South Africa (NICD 2008). Prone to sporadic outbreaks, it caused severe socio-economic implications when it reappeared in Botswana, Madagascar, Algeria and Tanzania, after being quiescent for over 30-60 years (Neerincx *et al.* 2010).

Diseases that are indirectly transmissible through environmental contamination usually have a widespread distribution and leptospirosis is no exception. A worldwide distribution with several pathogenic *Leptospira* species and serovars (Adler & De la Pen˜a Moctezuma 2010) makes leptospirosis and other food-borne pathogens an omnipresent public health threat. Furthermore, it is particularly of high incidence in urban areas where it is most commonly transmitted to humans through contact with faeces or urine from infected rats (Binder & Mermel 1998). The disease can range from mild to a very severe systemic infection with a mortality of 10 %. Fortunately, the mild "anicteric" form is most commonly presented (Binder & Mermel 1998).

Rat-bite fever presents an example of a zoonosis where (in most cases) direct contact through a bite or scratch is needed for disease transmission to occur. The risk of transmission may seem low, but with the high abundance of rats in both urban and rural areas in South Africa (Kirsten & von Maltitz 2005) and elsewhere (Childs *et al.* 1998), frequent contact between rats and humans occur. *Streptobacillus moniliformis* is one etiological agent of the disease which has non-specific presentation with a mortality rate estimated at 13 % if un-treated (Gaastra *et al.* 2009).

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Pest management

A pest species as defined by Putman (1989) is an animal species or population which by its activities conflicts with the interests of humans to such an extent that the damage caused becomes of economic significance. Animal species frequently become pests when their population densities become excessively high or when they occur in non-native or disturbed habitats (Putman 1989). Rodents of the genus *Rattus* in part owe their pest status to their successful reproductive biology, adaptive behaviour and their ability to invade and persist in non-native and disturbed habitats. In agriculture, action is often taken only when the amount of damage excessively exceeds costs of control (Richards 1989) yet, even at low densities, rats may still pose a public health threat for diseases such as salmonellosis and leptospirosis (Richards 1989). It is therefore apparent that reducing the damage and not necessarily the numbers of the pest is imperative for effective control. It is however, difficult to quantify damage in terms of aesthetics and public health concerns (Richards 1989), resulting in commensal rodents being considered as pest species regardless of population size.

Effective control of rats has been challenging and limited in the past by the use of single management strategies (Putman 1989). Management practices that increase mortality rates of rats through infrequent poison baiting have in fact exacerbated levels of population densities following control (Putman 1989). Regular use of rodenticides however, increases the risk of rodenticide resistance (Putman 1989; Singleton *et al.* 1999). A change to integrated pest management (Singleton *et al.* 1999) and adaptive management (Putman 1989) has been more successful with longer lasting effects, and management practises incorporating ecological principles and biology of the pest species have a better chance at success. The Malayan wood rat (*Rattus tiomanicus*) was successfully controlled by incorporating elements of its population dynamics into the baiting programme (Singleton *et al.* 1999). In addition, alternate or complementary use of poison types and live-trapping (Singleton *et al.* 1999) coupled with

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continuous trapping regimes (Putman 1989) can overcome problems of development of resistance and counter effects of population reduction.

Evidently, it is clear that as much information as possible regarding the pest species in question is needed for effective management of the species to take place. It is certain that research is lacking in terms of preventing the development and spread of rodenticide resistance, species specific behavioural and population ecology and rodent taxonomy and phylogeny (Singleton *et al.* 1999). This knowledge is imperative for the development of more ecological and economically sound pest management strategies.

JUSTIFICATION

The ever expanding human population enhances and promotes contact with wild rodent populations as competition for space and food sources increase (Singleton *et al.* 1999). In addition, urbanisation is on the increase particularly in Latin America, Africa and Asia (Gratz 1999). This leads to the formation of informal settlements (townships), where density of dwellings is high and adequate sanitation and rodent control measures are absent (Gratz 1999; Easterbrook *et al.* 2005; Wekesa *et al.* 2011). This creates a breeding ground for rodents as suitable habitat patches are created where food, water and shelter are freely available (Langton *et al.* 2001) enabling the population to attain high numbers. Consequently, humans are frequently exposed to rodents and their associated ectoparasites, and are therefore at risk to disease.

Moreover, the incidence of rat bites is also more frequent than expected according to a survey by Kirsten & Von Maltitz (2005). In South Africa, recent media reports such as "*Ma vort, laat kinders vol rotbytmerke* (mother gone, leaving children covered in rat-bite marks") , by Zwecker (2010) for the Beeld newspaper; *"Soweto baby bitten to death by rats"*, South African Press Association (SAPA) (2011); *"Helen Zille tweets about rat bite"*, (SAPA)

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(2013) and "*Rats, That's Not Funny - Khayelitsha Child Bitten By Rat*", Matsolo (2013) highlight the increased incidence of rat bites. Elsewhere, a two-year survey revealed that rodents accounted for 4.3 % of all animal bites recorded (Childs *et al.* 1998). Considering, the proportion of immune-compromised individuals such as people living with HIV/AIDS, those suffering from alcoholism or drug addiction as well as the very young and elderly, precaution is needed as many of these rodent-borne diseases develop as a result of opportunistic infections in such individuals (Comer *et al.* 2001).

Although a variety of parasites are associated with *Rattus*, bacterial diseases generally have a widespread occurrence, involve various modes of transmission (Gratz 1997), cause severe outbreaks and as mentioned earlier are involved in several zoonoses. In practice, bacteria are relatively easy to isolate and identify with the availability of several techniques (Pickup 1991). It is therefore surprising that only a few studies in southern Africa have investigated bacterial prevalence in *Rattus*. These studies are summarised in Table 2 and formed the baseline for the current study.

Table 2 Previous studies of bacterial zoonoses associated with *Rattus* from southern Africa

Bacteria	Output	Authors
Rickettsia spp.	Publication	Gear 1954
Pasteurella pneumotropica	Publication	Shepherd *et al.* 1982
Leptospira spp.	Publication	Taylor *et al.* 2008
Bartonella and *Helicobacter* spp.	MSc thesis	Mostert 2009
Bartonella spp.	PhD thesis	Brettschneider 2010

With *Rattus* closely associated with human habitation, they pose an enormous public health risk if disease transmission should occur. The discovery of a third invasive, commensal *Rattus* species in southern Africa during routine molecular monitoring of indigenous murid rodents (Bastos *et al.* 2011), emphasises the need for continuous surveillance of not only pathogens but also their hosts. The outcome of this study may

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provide information to be used in preventative measures against disease outbreaks as well as management of the *Rattus* hosts.

AIM

The study investigated the origin and diversity of the three matrilineally-defined *Rattus* species known to occur in South Africa. Subsequently, their role as potential zoonotic disease reservoirs in primarily urban environments with particular interest in their potential to transmit and spread zoonotic disease through direct contact as well as indirect contact through the urinary route were investigated.

RESEARCH QUESTIONS

The key research questions were as follows:

1. What is the identity and diversity of invasive, commensal *Rattus* in South Africa?;
2. What is the diversity, prevalence and zoonotic potential of bacteria potentially transmitted through environmental contamination by invasive, commensal *Rattus* in South Africa?;
3. What is the diversity and prevalence of zoonotic, *Streptobacillus moniliformis* in relation to invasive, commensal *Rattus* host specificity in South Africa? and
4. What is the diversity and prevalence of potentially zoonotic *Rickettsia* in invasive, commensal *Rattus* species in South Africa?

HYPOTHESES

Hypothesis 1

Null (H_0) hypothesis: There is no genetic diversity within members of invasive, commensal *Rattus* in South Africa.

Alternative (H_{A1}) hypothesis 1: There is extensive genetic diversity within members of invasive, commensal *Rattus* in South Africa.

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Alternative (H_{A2}) hypothesis 2: There is low genetic diversity within members of invasive, commensal *Rattus* in South Africa.

Hypothesis 2

Null (H_0) hypothesis: There is no diversity, prevalence or zoonotic potential of bacteria that are potentially transmitted through environmental contamination by invasive, commensal *Rattus* in South Africa.

Alternative (H_A) hypothesis: There is diversity and prevalence of bacteria that are of zoonotic potential which are potentially transmitted through environmental contamination by invasive, commensal *Rattus* in South Africa.

Hypothesis 3

Null (H_0) hypothesis: There is no diversity and prevalence of zoonotic, *Streptobacillus moniliformis* in relation to invasive, commensal *Rattus* host specificity in South Africa.

Alternative (H_A) hypothesis: There is diversity and prevalence of zoonotic, *Streptobacillus moniliformis* in relation to invasive, commensal *Rattus* host specificity in South Africa.

Hypothesis 4

Null (H_0) hypothesis: There is no prevalence and diversity of potentially zoonotic, *Rickettsia* in invasive, commensal *Rattus* species in South Africa.

Alternative (H_A) hypothesis: There is prevalence and diversity of potentially zoonotic, *Rickettsia* in invasive, commensal *Rattus* species in South Africa.

STUDY APPROACH

Site Selection

Rodent sampling took place in the Gauteng Province authorised by a valid collection permit (CPF6 0032) (issued by the Department of Nature Conservation) and with permission from

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the landowner. Site selection was initially chosen based on presence data obtained as part of a previous study (Mostert 2009; Bastos *et al.* 2011) and were representative of urban and semi-urban areas. These sites included residential properties, informal settlements, office buildings and small holdings.

Laboratory Procedures

Laboratory procedures were carried out in a biosafety level 2 (BCL 2) facility and research on infective material was done with permission from the Department of Agriculture, Fisheries and Forestry (Ref no. 12/11/1/1/8). Animals were sacrificed using halothane inhalation administered by a veterinarian as approved by the Animal Ethics Committee (formerly the Animal Use and Care Committee, AUCC) of the University of Pretoria, Pretoria, South Africa (Ethics clearance number EC025-10), after which the fur was sprayed with absolute alcohol and combed to collect ectoparasites. Standard external measurements were recorded and oral and rectal swabs were collected and stored in 1:1 PBS/glycerol solution while organ tissue were dissected and preserved at -20ºC for subsequent molecular analysis.

Kidney tissue extracts were used in chapter 3 to determine which bacteria are potentially transmitted through environmental contamination by urine/faeces. These same genomic extracts were then subsequently used in chapter 2 for *Rattus* host identification, in chapter 4 for detection of *Streptobacillis moniliformis* with regards to Haverhill fever and in chapter 5 for detection of *Rickettsia* which has previously been isolated from kidneys (Abramowicz *et al.* 2011). Oral swab extracts were used in chapter 4 for *S. moniliformis* detection as the organism is considered to be part of the commensal flora of the pharyngeal region in *Rattus*. Voucher specimens of all *Rattus* samples will be prepared and deposited in the small

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mammal reference collection of the Ditsong National Museum of Natural History (formerly Transvaal Museum), Pretoria, South Africa.

Methods of detection

Rattus *host identification*

Molecular techniques were used to accurately identify cryptic *Rattus* species and confirm identities of juvenile non-cryptic *Rattus* hosts. This was done to avoid misidentification of the source of infection which may impede control measures and limit future investigation (Mills & Child 1998; Leirs *et al.* 1999). *Rattus* species were identified using mitochondrial cytochrome *b* (cyt *b*) primer sets (Bastos *et al.* 2011) in conventional PCR and sequencing. The cytochrome *b* gene is a protein-coding gene and therefore, it is a highly conserved gene region and has been extensively used to resolve mammalian phylogenies (Irwin *et al.* 1991; Robins *et al.* 2007).

Bacterial detection

Traditionally, identification of bacteria relied on isolating pure cultures but culture methods have been found to be problematic where bacterial biochemical needs are quite complex and unknown (Pickup 1991; Amman *et al.* 1995). This renders certain bacteria "non-culturable" and makes culturing methods insensitive to target species (Pickup 1991; Amann *et al.* 1995). Additionally, culture methods also incorporate morphological characteristics for distinguishing bacterial populations (Pickup 1991), but many bacteria lack distinct characteristics and as such, phenotypic errors may lead to misidentification of the cause of an infection (Benslimani *et al.* 2005). For the reasons mentioned above, this study employed molecular techniques of PCR and genetic sequencing to accurately identify bacterial species.

Various gene regions were used in the study depending on the bacterial genus in question. In chapter 3 broad range universal primer sets (Hauben *et al.* 1997; Marchesi *et al.* 1998)

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were used which amplified a section of the 16S ribosomal subunit of the bacterial genome. The 16S ribosomal subunit or 16S r-RNA is a highly conserved gene in the bacterial domain with an average length of about 1,500 nucleotides (Amann *et al.* 1995). It is therefore extensively used as it contains enough information for reliable phylogenetic resolution up to the genus level within the domain (Amann *et al.* 1995).

Detection of *S. moniliformis* in chapter 4 also involved sequencing of a fragment characterising the 16S ribosomal subunit. Molecular techniques to detect *S. moniliformis* were originally developed based on broad range universal primer sets (Boot *et al.* 2002) and consequently, all available primer sets characterise the 16S gene region. However, non-specific amplification was reported for some available primer sets (Kimura *et al.* 2008) and selection of the primer set for use in this study was made accordingly.

Although several primer sets were available for detection of *Rickettsia* (see La Scola & Raoult 1997 for a review), primers most commonly used, amplify regions of the citrate synthase- (*gltA*) and outer membrane protein genes (*ompA* & *ompB*). The citrate synthase gene region is regarded as genus-specific while the outer membrane protein gene regions are regarded as species-specific (La Scola & Raoult 1997; Roux & Raoult 2000). Therefore, in chapter 5, two primer sets were chosen - one characterising the *glt A* and the other characterising the omp *B* gene region to facilitate generic and specific resolution. These primer sets were selected based on the target rickettsial species, the number of times the primers were cited as well as their reported specificity.

STUDY OUTLINE

The main objective of chapter 2 was to accurately identify *Rattus* host species and determine the proportional abundance of each species. Sequences generated for this purpose were then used to infer phylogenies among the commensal *Rattus* species in a global context.

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Subsequently, haplotype network analyses provided insights into colonisation and dispersal patterns of the three invasive, commensal matrilineal *Rattus* species currently known to occur in South Africa. The diversity of bacteria within *Rattus* hosts and their potential for spread as environmental contaminants were assessed in chapter 3 with subsequent analyses of between species differences in bacterial prevalence and diversity among *Rattus* species. This information was also used in subsequent chapters 4 and 5. Prevalence and diversity of *S. moniliformis* within and among *Rattus* host species was assessed in chapter 4. This provided a way to assess the transmission potential of rat-bite fever through direct contact with *Rattus*. Furthermore, the study also investigated potential transmission through environmental contamination associated with a type of rat-bite fever called Haverhill fever. Lastly, diversity in bacterial strains and subsequent host specificity of these strains were determined and assessed by phylogenetic analyses. Chapter 5 assessed the prevalence of vector-borne *Rickettsia* and diversity within and among *Rattus* host species, also exploring the role of the rodent host in transmission of the disease. In addition, the study determined phylogenetic relationships of *Rickettsia* present in invasive commensal *Rattus* in South Africa and their implication in disease epidemiology. Finally, chapter 6 provides a general discussion and concluding remarks on the findings in this study and highlights its limitations with recommendations for future studies.

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Chapter 2

Molecular identification and diversity of invasive, commensal *Rattus* species in southern Africa

ABSTRACT

Rats of the genus *Rattus* carry a wide range of pathogens and when disease outbreaks occur, accurate identification of the source of infection is important not only from an epidemiological perspective but also for effective rodent control measures. Invasive, commensal *Rattus* were sampled in coastal and inland southern Africa. Matrilineal origin of *R. rattus* complex species in southern Africa, (*viz. R. rattus* and *R. tanezumi*) was determined by nucleotide sequencing of a mitochondrial gene, whereas adult *R. norvegicus* were identified solely on morphology. It was established that a high global diversity exists for all three *Rattus* species, yet haplotype network analyses revealed a low genetic diversity of *Rattus* species in southern Africa, similar to that reported previously (Bastos *et al.* 2011), and in spite of the substantial increase in the sample size arising from the present study. The frequency of unique haplotypes and the relationships of the haplotypes suggest that this observed diversity is possibly a result of a combination of discrete introductions into southern Africa and subsequent diversification. The formation of distinct clades in the phylogenetic trees of all mtDNA *Rattus* lineages supports the introduction events as revealed by the haplotype networks. In addition, haplotype networks of *R. tanezumi* revealed that this species was recently introduced to southern Africa possibly directly from its native range, while phylogenetic analyses revealed that the *R. tanezumi* lineage present in southern Africa is recently diverged and more commensal than the wild endemic ancestral lineage. In areas where the two *R. rattus* complex species occur sympatrically in their invasive range in southern Africa, introgression is likely and has implications for rodent control measures and disease epidemiology.

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KEYWORDS: cytochrome *b*, invasive species, haplotype network analysis, phylogeny

INTRODUCTION

The first documented record of *Rattus* in South Africa was in 1945, when it was estimated that they destroyed grain amounting to at least R1 million annually (Lever 1985). Currently, three species of invasive, commensal *Rattus* namely *R. norvegicus*, *R. rattus* and *R. tanezumi* are known to occur in South Africa, the latter being only identified in South Africa in 2005 using a genetic approach (Bastos *et al.* 2005). The three species of *Rattus* originated from Eurasia (Musser & Carleton 2005) and are likely to have reached the African continent through the shipping trade (Atkinson 1985). These invasive rodents are known as commensal species (Atkinson 1985; Taylor *et al.* 2008; Bastos *et al.* 2011), as they habitually congregate with humans and exploit human habitation for food and shelter. However, not all *Rattus* species are commensal and live in and around human dwellings. Some species occupy natural forest but show preference to disturbed over pristine, undisturbed habitat (Aplin *et al.* 2011).

Rattus norvegicus is readily distinguished by its significantly larger body size and external morphological features. By contrast, *R. tanezumi* and *R. rattus* are cryptic species that form part of the *R. rattus* species complex (Taylor *et al.* 2008; Aplin *et al.* 2011; Bastos *et al.* 2011) and although often occurring in sympatry cannot be distinguished from each other morphologically (Musser & Carleton 2005; Mostert 2009). Various genetic techniques can be used to distinguish between the two cryptic species. *Rattus rattus* has a diploid number of $2n = 38\text{-}40$ and *R. tanezumi* a diploid number of $2n = 42$ (Aplin *et al.* 2011). In addition, the matrilineal origin of each species can be determined by sequencing of the cytochrome *b* (cyt *b*) gene (Bastos *et al.* 2011).

Rattus is known to carry a wide range of zoonotic pathogens (Gratz 1999). The lack of adequate sanitation, pest control and poor housing conditions that prevail in much of rural

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and urban South Africa (Taylor *et al.* 2008; Wekesa *et al.* 2011), results in thriving rodent populations, promoting contact with humans (Gratz 1999) and increased risk of zoonotic disease transmission. When disease outbreaks occur it is important to accurately identify the source of infection as misidentification of the host species may impede control measures and possibly confound future investigations (Mills & Childs 1998; Leirs *et al.* 1999).

In this study, a mitochondrial gene sequencing approach was used to: 1) determine the identity and genetic diversity of cryptic *Rattus* species in South Africa; 2) place this regional diversity in context of current knowledge of global diversity; 3) unravel the colonisation history of *Rattus* in southern Africa and 4) verify molecular identities of morphologically-identified *R. norvegicus* samples.

MATERIALS AND METHODS

Sample collection

Rats (*Rattus* spp.) were obtained by a combination of captures and donations from members of the public and pest control companies. Rats were trapped using Sherman traps (H.B. Sherman Inc. Florida, USA) as well as snap traps (Scientific Supa-Kill Kempton Park, South Africa).

The one hundred and forty nine samples from 29 localities in four provinces (Gauteng, Kwa-Zulu- Natal, Limpopo and Mpumalanga Provinces) in South Africa and neighbouring Swaziland (Figure 1) sampled between 2003-2008 have been reported previously (Bastos *et al.* 2005; Taylor *et al.* 2008; Mostert 2009; Bastos *et al.* 2011). Mitochondrial data generated in the course of these earlier studies was complemented with an additional 107 samples captured from 16 localities in the Gauteng Province during 2010-2011 (Figure 1) in the course of this study. This combined dataset of 256 samples will hereinafter collectively be referred to as the southern African samples.

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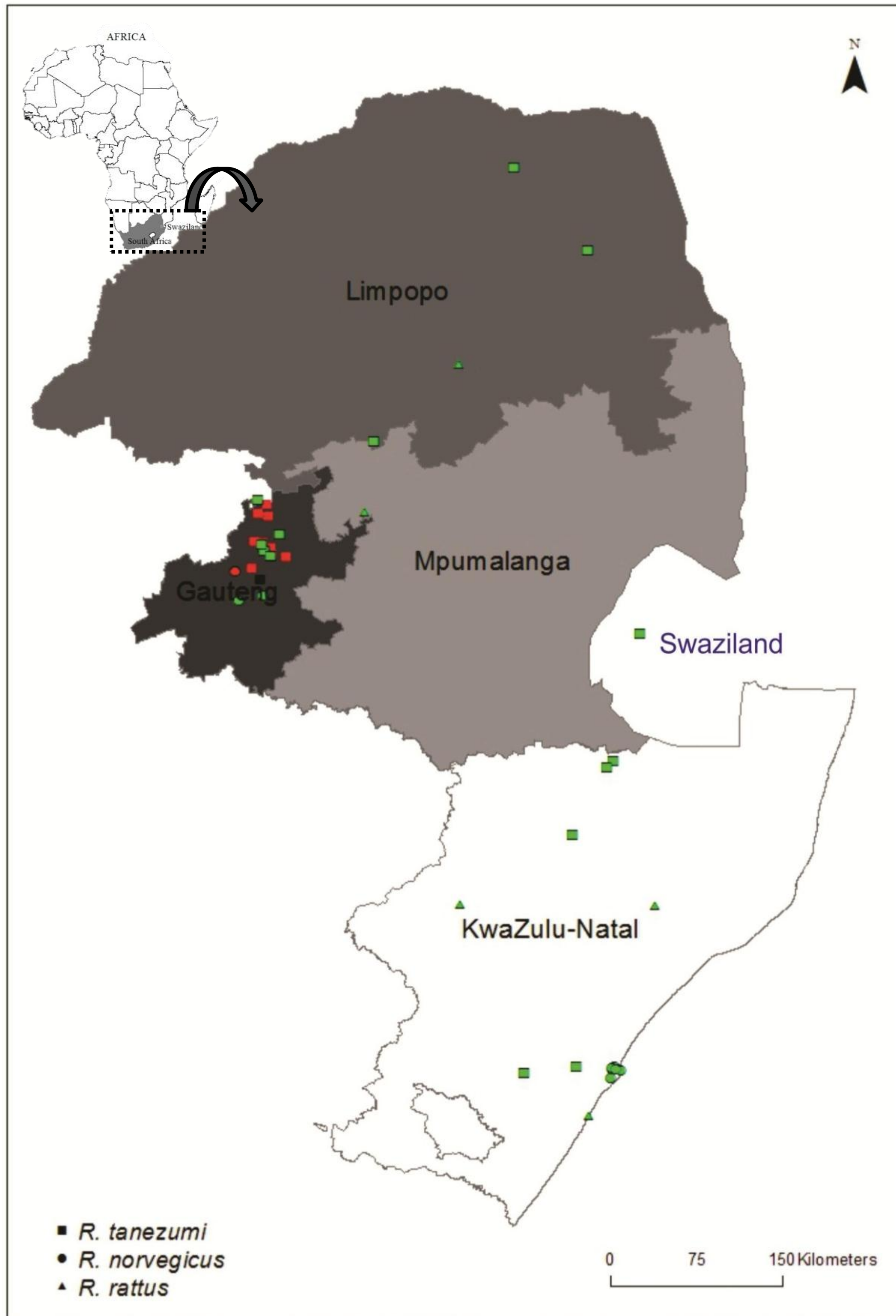

Figure 1 Map of the southern African region where *Rattus* species were sampled with sampling localities of the 2003-2008 sampling period indicated in green while those collected between 2010 and 2011 being denoted in red. Symbols denote different matrilineal *Rattus* species where squares denote *R. tanezumi*, circles denote *R. norvegicus* and triangles denote *R. rattus*.

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Table 1 GPS coordinates of localities where *Rattus* specimens were sampled from southern Africa (*n* = sample size).

Sampling locality	*N*	Province	Latitude	Longitude	Reference
Boschkop, Pretoria	6	Gauteng	-25.815	28.407	This study
Cato Crest IS, Durban	4	Kwa-Zulu Natal	-29.850	30.978	Bastos *et al.* (2011)
Cato Manor Road, Durban	1	Kwa-Zulu Natal	-29.858	30.978	Bastos *et al.* (2011)
Centurion, Pretoria	1	Gauteng	-25.905	28.138	This study
Diepsloot, Pretoria	4	Gauteng	-25.937	28.012	This study
Durban Central Business District	3	Kwa-Zulu Natal	-29.859	31.016	Bastos *et al.* (2011)
Durban Harbour	2	Kwa-Zulu Natal	-29.871	31.048	Bastos *et al.* (2011)
Garsfontein, Pretoria	2	Gauteng	-25.802	28.302	This study
Giyani	16	Limpopo	-23.416	30.786	Bastos *et al.* (2011)
Grobler Plot, Hammanskraal	3	Gauteng	-25.481	28.193	This study
Hammanskraal	18	Gauteng	-25.374	28.190	Bastos *et al.* (2011)
Hatfield, Pretoria	1	Gauteng	-25.749	28.238	This study
Industrial Area, Magriet Monamudi area, Hammanskraal	4	Gauteng	-25.498	28.269	This study
Johannesburg Zoological Gardens, Johannesburg	8	Gauteng	-26.168	28.037	Bastos *et al.* (2011)
Ladysmith	1	Kwa-Zulu Natal	-28.55	29.783	Bastos *et al.* (2011)
Manzini, Mcaphozini Area, Swaziland	6	-	-26.435	31.197	Bastos *et al.* (2011)
Melmoth	1	Kwa-Zulu Natal	-28.567	31.317	Bastos *et al.* (2011)
Menlyn, Pretoria	2	Gauteng	-25.791	28.287	This study
Montclair Park, Durban	2	Kwa-Zulu Natal	-29.925	30.965	Bastos *et al.* (2011)
Moreletta Park, Pretoria	2	Gauteng	-25.823	28.295	Bastos *et al.* (2011)
Mountain View, Pretoria	6	Gauteng	-25.701	28.162	This study
Mvuzini, Kwa-Zulu Natal Province	3	Kwa-Zulu Natal	-28.013	30.661	Bastos *et al.* (2011)
Ophuzane	6	Kwa-Zulu Natal	-27.486	30.934	Bastos *et al.* (2011)
OR Thambo International Airport, Johannesburg	10	Gauteng	-26.133	28.230	Bastos *et al.* (2011)
Renosterkop, Limpopo Province	1	Limpopo	-24.916	29.100	Bastos *et al.* (2011)
Richmond	1	Kwa-Zulu Natal	-29.883	30.283	Bastos *et al.* (2011)
Rietfontein, Pretoria	1	Gauteng	-25.705	28.215	This study
Rietondale, Pretoria	1	Gauteng	-25.731	28.219	Bastos *et al.* (2011)
Roodeplaat, Pretoria	13	Gauteng	-25.654	28.358	Bastos *et al.* (2011)
Sekhukhune	2	Limpopo	-24.311	29.770	Bastos *et al.* 2011
Shongweni, Durban	2	Kwa-Zulu Natal	-29.834	30.699	Bastos *et al.* 2011
Sydenham, Durban	2	Kwa-Zulu Natal	-29.832	31.000	Bastos *et al.* (2011)
Tembisa, Johannesburg	43	Gauteng	-26.000	28.214	This study/Bastos *et al.* (2011)
Tholakele	2	Kwa-Zulu Natal	-27.434	30.988	Bastos *et al.* (2011)
Tshilimbani, Limpopo Province	23	Limpopo	-22.766	30.200	Bastos *et al.* (2011)
Umkomaas	5	Kwa-Zulu Natal	-30.217	30.800	Bastos *et al.* (2011)
University of Pretoria (UP) Experimental Farm, Pretoria	33	Gauteng	-25.752	28.251	This study/Bastos *et al.* (2011)
UP Hammanskraal Campus, Hammanskraal	6	Gauteng	-25.404	28.263	This study
UP Main Campus, Pretoria	5	Gauteng	-25.753	28.230	This study
Val de Grace, Pretoria	1	Gauteng	-25.746	28.295	This study

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Verena, Mpumalanga Province	1	Mpumalanga	-25.469	29.036	Bastos *et al.* (2011)
Villeria, Pretoria	1	Gauteng	-25.712	28.232	This study
Warwick Ave, Durban	1	Kwa-Zulu Natal	-29.858	31.010	Bastos *et al.* (2011)

Sampling localities were representative of industrial, sub-urban, formal- and informal residential as well as rural areas. During the 2010-11 sampling, approximately 50 snap traps and 100 Sherman live traps were baited with a peanut butter, fish and oatmeal mixture and placed in and around storage facilities, office buildings and human dwellings. Traps were inspected daily for a trapping period of one week per month. All live-trapped rats were transported to the laboratory at the Department of Zoology and Entomology, University of Pretoria and maintained under the guidelines of the American Society of Mammalogists (ASM; www.mammalogy.org/committees/index.asp; Animal Care and Use Committee 1998) and as approved by the Animal Care and Use Committee of the University of Pretoria (Ethics clearance number EC025-10) while snap-trapped individuals were individually bagged, and transported at 4ºC to the same laboratory. Live animals were euthanised by means of halothane inhalation. All animals sampled in the present study were collected under permit number (CPF6 0032) issued by the Gauteng Department of Nature Conservation and with permission from landowners.

Standard measurements were recorded and oral and rectal swabs were taken and stored in 1:1 PBS/glycerol solution while organ tissue were dissected and preserved at -20º C for subsequent molecular analysis. Where available, ectoparasites were collected and stored in absolute ethanol. Voucher specimens were prepared and deposited in the small mammal reference collection of the Ditsong National Museum of Natural History (formerly Transvaal Museum), Pretoria, South Africa. Genomic DNA was extracted from organ tissue using the Roche High Pure PCR Template Preparation Kit (Roche Diagnostics) according to the manufacturer's protocol and stored at -20º C.

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Morphological identification

Adult individuals of *R. norvegicus* were identified based on their significantly large body size and external morphological features that differ markedly from the other two congeners present in southern Africa (Figure 2). Individuals of *R. norvegicus* typically have small ears in comparison to their heads and short tails compared to its body length whereas the other two congeners have large ears compared to the size of the head and long tails compared to the body length.

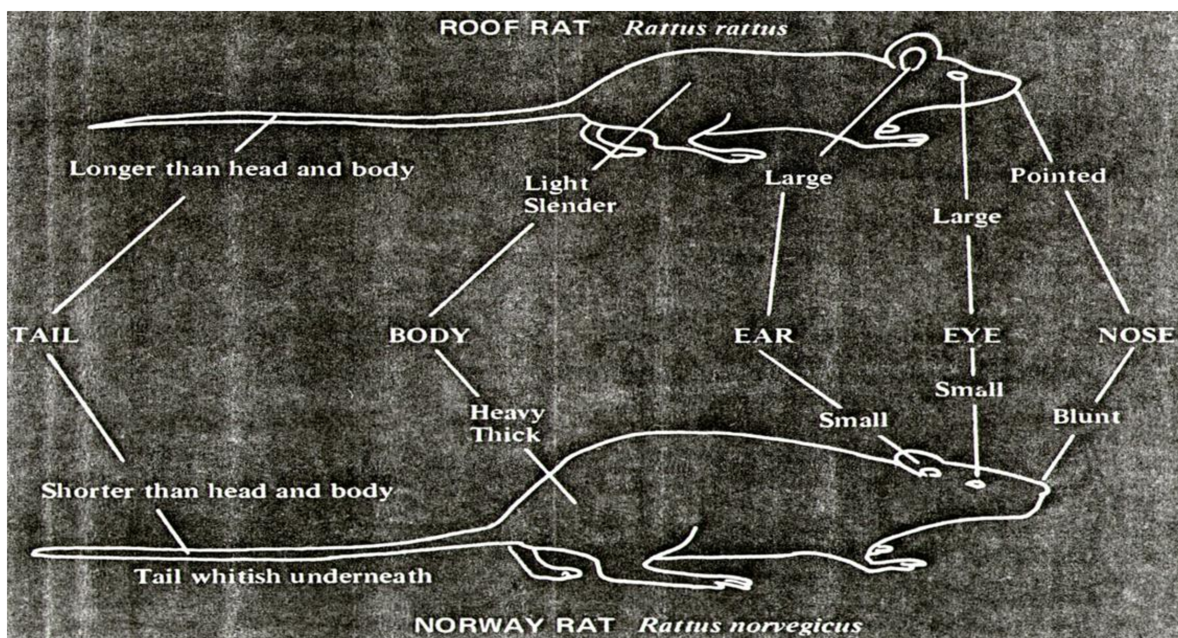

Figure 2 Drawing showing external morphological features of *Rattus rattus* and *R. norvegicus* upon which the species can be differentiated (adopted from Brennan 1980).

Molecular analyses

Polymerase Chain Reaction (PCR)

DNA extracts (either kidney or oral swab) were used to amplify the full length of the mitochondrial cytochrome *b* (cyt *b*) gene using primers L14724-TGAYATGAAAAAYCATCGTTG and H15915- CATTTCAGGTTTACAAGAC that bind in regions flanking the gene and target a 1.2 kbp amplicon and previously described reaction

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conditions (Bastos *et al.* 2011). Briefly, PCRs were performed in a final reaction volume of 50 µl containing 1X buffer (Fermentas), 0.25 µM dNTP's (Fermentas), 0.4 µM of each primer, 1U *Taq* Polymerase (Fermentas) and 100-200 ng DNA template. Thermal cycles were as follows: initial denaturation at 96º C for 20 s; followed by two cycles of denaturation at 96º C for 12 s, annealing at 49º C for 25 s and extension at 72º C for 60 s, five cycles of denaturation at 96º C for 12 s, annealing at 47º C for 20 s and extension at 72º C for 55 s and finally 35 cycles of denaturation at 96º C for 12 s, annealing at 45º C for 15 s and extension at 72º C for 50 s with a final extension at 72º C for 1 minute. This approach was used to molecularly differentiate between the two cryptic species, *R. rattus* and *R. tanezumi* and to confirm the identification of juvenile *R. norvegicus*.

Sequencing and phylogenetic analyses

PCR products were purified using the Roche PCR Product Purification Kit (Roche Diagnostics) and bi-directionally cycle sequenced with each of the amplification primers in separate reactions, using BigDye v. 3.1 terminator cycle-sequencing kit (Perkim-Elmer, Foster City, USA). Samples were run on an ABI 3130 sequencer and the resulting sequence chromatograms were viewed and edited in Mega 5 (Tamura *et al.* 2011) prior to performing a BLAST nucleotide database search (www.ncbi.nlm.nih.gov/blast) to identify the species with the highest sequence similarity. The southern African data set was augmented with 113 reference sequences from diverse sampling localities from around the world that have been reported in published peer-reviewed journals. These were downloaded from the Genbank (www.ncbi.nlm.nih.gov/genbank) database and are summarized in Table 2. As reference sequences consisted of complete (1140 bp) and partial (≥ 550 bp) cyt *b* sequences, two data sets were compiled for analysis and analysed independently and similar to Lack *et al.* (2012) obtained identical results irrespective of when complete sequences or partial sequences were

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used. Therefore, the final dataset and subsequent results are based on the 1140 bp cyt *b* dataset.

Table 2 Global occurrence and haplotype diversity of three mitochondrial DNA (mtDNA) defined *Rattus* species (*n* = sample size) based on the 1140 bp dataset characterising the cyt *b* gene.

Rattus norvegicus					
Country[1]	**Haplotype *n***	**Haplotype**[2]	***n***	**Accession no.**	**Reference**
Cambodia	1	RN08	1	HM217481	Pagès *et al.* (2010)
China	7	RN01	1	GU592971	Lin *et al.* (2012)
		RN19	1	GU592954	Lin *et al.* (2012)
		RN20	1	GU592972	Lin *et al.* (2012)
		RN18	7	HM031681	Lu *et al.* (2012)
		RN14	1	HM031682	Lu *et al.* (2012)
		RN16	1	JQ814256	Conroy *et al.* (2013)
		RN17	1	JQ814260	Conroy *et al.* (2013)
Denmark	1	RN11	1	AJ428514	Nilsson *et al.* (2003)
French Polynesia	2	RN01	1	EF186461	Robins *et al.* (2007)
		RN10	1	EF186462	Robins *et al.* (2007)
Indonesia	1	RN05	1	FJ842279	Bastos *et al.* (2011)
Japan	1	RN12	1	DQ673917	Schlick *et al.* (2006)
South Africa	3	RN01	14	HQ157799	Bastos *et al.* (2011)/This study (*n* = 5)
		*RN02	2	FJ842274	Bastos *et al.* (2011)
		RN12	6	FJ842275	Bastos *et al.* (2011)
Sweden	1	RN01	1	FJ919765	Abhyankar *et al.* (2009)
Thailand	2	RN08	2	HM217370	Pagès *et al.* (2010)
		RN13	1	HM217429	Pagès *et al.* (2010)
USA	2	RN01	1	JQ814283	Conroy *et al.* (2013)
		RN10	1	DQ673916	Schlick *et al.* (2006)
Vietnam	3	RN06	1	FJ842277	Bastos *et al.* (2011)
		RN08	1	AB355902	Bastos *et al.* (2011)
		RN07	1	FJ842278	Bastos *et al.* (2011)
Lab Strains	3	RN01	1	AY172581	Gibbs *et al.* (2004)
		RN09	1	DQ673911	Schlick *et al.* (2006)
		RN12	1	DQ673915	Schlick *et al.* (2006)
		RN15	1	X14848	Gadaleta *et al.* (1989)
Rattus rattus					
Country[1]	**Haplotype *n***	**Haplotype**[2]	***n***	**Accession no.**	**Reference**
Brazil	3	RR32	1	JN675550	Aplin *et al.* (2011)
		RR36	1	JN675551	Aplin *et al.* (2011)
		RR05	1	JN675546	Aplin *et al.* (2011)
Denmark	1	RR06	1	FJ355927	Nilsson *et al.* (2010)
Ethiopia	2	RR15	1	GQ891584	Tollenaere *et al.* (2010)
		RR21	1	GQ891583	Tollenaere *et al.* (2010)
French Polynesia	1	RR06	2	EF186474	Robins *et al.* (2007)

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Grand Comore	3	RR01	3	GQ891591	Tollenaere *et al.* (2010)
		RR09	2	GQ891592	Tollenaere *et al.* (2010)
		RR30	1	GQ891594	Tollenaere *et al.* (2010)
India	4	RR01	2	GQ891572	Tollenaere *et al.* (2010)
		RR35	1	GQ891569	Tollenaere *et al.* (2010)
		RR12	1	GQ891570	Tollenaere *et al.* (2010)
		RR33	2	HM217367	Pagès *et al.* (2010)
Indonesia	1	RR31	1	AB033702	Suzuki *et al.* (2000)
Iran	1	RR28	2	JQ814271	Conroy *et al.* (2013)
Japan	2	RR06	2	AB211042	Chinen *et al.* (2005)
		RR29	2	AB211039	Chinen *et al.* (2005)
Madagascar	6	RR06	1	GQ891599	Tollenaere *et al.* (2010)
		RR15	22	GQ891600	Tollenaere *et al.* (2010)
		RR17	1	GQ891603	Tollenaere *et al.* (2010)
		RR18	1	GQ891605	Tollenaere *et al.* (2010)
		RR20	1	GQ891601	Tollenaere *et al.* (2010)
		RR25	1	JQ814237	Conroy *et al.* (2013)
Mayotte	2	RR37	1	GQ891597	Tollenaere *et al.* (2010)
		RR15	1	GQ891598	Tollenaere *et al.* (2010)
Mozambique	2	RR01	1	GQ891590	Tollenaere *et al.* (2010)
		RR15	2	GQ891588	Tollenaere *et al.* (2010)
New Zealand	1	RR06	3	EU273707	Robins *et al.* (2008)
Oman	6	RR35	1	GQ891576	Tollenaere *et al.* (2010)
		RR13	1	GQ891577	Tollenaere *et al.* (2010)
		RR14	1	GQ891574	Tollenaere *et al.* (2010)
		RR16	2	GQ891578	Tollenaere *et al.* (2010)
		RR23	1	GQ891580	Tollenaere *et al.* (2010)
		RR34	2	HM217366	Pagès *et al.* (2010)
Pakistan	2	RR26	1	JQ814242	Conroy *et al.* (2013)
		RR27	1	JN675602	Aplin *et al.* (2011)
Papua New Guinea	1	RR06	1	EF186472	Robins *et al.* (2007)
Reunion	1	RR19	1	GQ891607	Tollenaere *et al.* (2010)
Samoa	1	RR06	1	EF186475	Robins *et al.* (2007)
South Africa	7	RR01	4	HQ157802	Bastos *et al.* (2011)
		RR05	1	FJ842268	Bastos *et al.* (2011)
		RR06	5	GQ891608	Tollenaere *et al.* (2010)/This study (n = 3)
		RR04	33	FJ842267	Bastos *et al.* (2011)/This study (n = 32)
		RR03	1	HQ157803	Bastos *et al.* (2011)
		*RR10	1	HQ157808	Bastos *et al.* (2011)
		*RR11	1	HQ157809	Bastos *et al.* (2011)
		*RR12	4	-	This study
Tanzania	2	RR01	3	HM217365	Pagès *et al.* (2010)
		RR22	1	GQ891585	Tollenaere *et al.* (2010)
USA	5	RR02	27	JN675541	Aplin *et al.* (2011)
		RR36	22	JQ814189	Conroy *et al.* (2013)
		RR06	1	JQ823431	Lack *et al.* (2012)
		RR24	1	JQ814175	Conroy *et al.* (2013)
		RR03	1	JQ823424	Lack *et al.* (2012)

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Yemen	2	RR07	1	GQ891582	Tollenaere *et al.* (2010)
		RR08	1	GQ891581	Tollenaere *et al.* (2010)
Rattus tanezumi					
Country[1]	**Haplotype *n***	**Haplotype[2]**	***n***	**Accession no.**	**Reference**
China	10	RT03	17	HM031696	Lu *et al.* (2012)
		RT06	1	JN675576	Aplin *et al.* (2011)
		RT07	1	AB096841	Suzuki *et al.* (2003)
		RT09	5	JQ814255	Conroy *et al.* (2013)
		RT12	4	HM031695	Lu *et al.* (2012)
		RT16	2	HM031707	Lu *et al.* (2012)
		RT21	1	EF186440	Robins *et al.* (2007)
		RT28	1	HM031697	Lu *et al.* (2012)
		RT29	3	JQ814205	Conroy *et al.* (2012)
		RT30	1	HM031705	Lu *et al.* (2012)
Indonesia	3	RT06	4	EF186507	Robins *et al.* (2007)
		RT08	1	EF186491	Robins *et al.* (2007)
		RT24	4	EF186494	Robins *et al.* (2007)
Japan	3	RT03	1	AB211040	Chinen *et al.* (2005)
		RT05	1	AB211041	Chinen *et al.* (2005)
		RT20	5	EU273712	Robins *et al.* (2008)
Laos	1	RT27	2	HM217480	Pagès *et al.* (2010)
Papua New Guinea	1	RT03	1	JN675595	Aplin *et al.* (2011)
Phillipines	4	RT08	2	JQ814251	Conroy *et al.* (2013)
		RT18	1	JQ814250	Conroy *et al.* (2013)
		RT19	7	DQ191488	Jansa *et al.* (2006)
		RT26	2	JQ814245	Conroy *et al.* (2013)
Singapore	1	RT25	2	GQ274948	Johansson *et al.* (2010)
South Africa	2	*RT01	90	HQ157807	Bastos *et al.* (2011)/This study (*n* = 20)
		*RT02	1	FJ842265	Bastos *et al.* (2011)
Swaziland	1	RT01	6	FJ842264	Bastos *et al.* (2011)
Thailand	7	RT04	1	HM217398	Pagès *et al.* (2010)
		RT10	1	HM217456	Pagès *et al.* (2010)
		RT11	1	HM217457	Pagès *et al.* (2010)
		RT13	1	HM217430	Pagès *et al.* (2010)
		RT14	1	HM217467	Pagès *et al.* (2010)
		RT15	1	HM217407	Pagès *et al.* (2010)
		RT27	7	HM217410	Pagès *et al.* (2010)
USA	3	RT03	2	JN675596	Aplin *et al.* (2011)
		RT08	2	JQ814225	Conroy *et al.* (2013)
		RT23	1	JQ814235	Conroy *et al.* (2013)
Vietnam	4	RT03	4	AB355899	Truong *et al.* (2009)
		RT06	3	AB355901	Truong *et al.* (2009)
		RT17	1	JQ814210	Conroy *et al.* (2013)
		RT22	1	JQ814267	Conroy *et al.* (2013)
		RT27	1	JQ814269	Conroy *et al.* (2013)

*Unique haplotypes from southern Africa
[1] not politically defined countries
[2] RN denotes *Rattus norvegicus*, RR denotes *R. rattus* and RT denotes *R. tanezumi*

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The model of sequence evolution selected in jModeltest (Posada 2008) under the Aikake Information Criterion (AIC_C) was the SYM (Zharkikh 1994) with Gamma distributed sites (G). Phylogenies were inferred using Maximum Likelihood (ML), Neighbour-Joining (NJ) and Bayesian Inference (BI). Nodal support for ML and NJ was assessed by 10,000 non-parametric bootstrap replications performed in Mega 5 (Tamura *et al.* 2011) while the BI was run for 20,000,000 MCMC generations in MrBayes v. 2.1.3 (Huelsenbeck & Ronquist 2001). Tracer plots were viewed in Tracer v1.5 (Rambau & Drummond 2009) where-after 25 % of trees were discarded as burn-in. Haplotype diversity (Hd) and nucleotide diversity (π) was determined by DNAsp (Rozas *et al.* 2003) for each of the *Rattus* species with a method that excluded base ambiguities and missing data. An unrooted haplotype network was also generated for each species using TCS, a computer program for estimating gene genealogies (Clement *et al.* 2000).

RESULTS

The 107 rats captured in this study were identified to matrilineal (mtDNA lineage) species assignments. Of these, 36 individuals were *R. norvegicus*, 40 individuals were *R. rattus* and 31 individuals were *R. tanezumi* based on morphology or nucleotide sequencing of a mitochondrial gene.

Molecular results

The 55-taxon southern African dataset consisted of partial cyt *b* sequences of 1123 nucleotides in length. Combined with reference sequences, the initial 435-taxon dataset including all three matrilineally assigned *Rattus* species was reduced to 15 taxa from South Africa and 113 taxa from elsewhere to show only unique haplotypes. This final homologous dataset consisted of 1140 bp corresponding to complete cyt *b* gene region. The dataset had 276 variable sites, of which 210 were parsimony informative, and an average nucleotide

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composition of T = 28.2 %; C = 28.7 %; A = 30.5; G = 12.5 %. In addition to the AT bias, there was also a bias in transitions as indicated by the transition: transversion ratio (R) of 4.89.

The southern African samples characterised in this study and augmented with those from Bastos *et al.* (2011), had very closely related haplotypes (π; Table 3) for all species. Eight haplotypes within the *R. rattus* lineage, five within *R. norvegicus* and two within the *R. tanezumi* lineages were recovered. These were similar to findings of Bastos *et al.* (2011) with only one additional haplotype recovered for *R. rattus* despite all the additional samples characterised in this study. The analysis of the southern African samples in a haplotype network revealed that the parent haplotypes of all three *Rattus* species came from both the inland (Gauteng and Limpopo Provinces and Swaziland) and coastal (Kwa-Zulu Natal and Western Cape Provinces) areas in southern Africa (Figure 3). In addition, the most abundant haplotype for the *R. norvegicus* population are represented by RN01, for the *R. rattus* population, this is represented by RR04 and for the *R. tanezumi* population RT01 is the most abundant of the two haplotypes for this species.

Table 3 Genetic diversity indices for the three matrilineally assigned *Rattus* species in southern Africa based on the partial 1123 bp sequence data characterising the cyt *b* gene, where ***n*** denotes the number of sequences, **h** the number of haplotypes, **Hd** is the haplotype diversity and $\boldsymbol{\pi}$ is the nucleotide diversity. Where applicable, **SD** denotes the standard deviation in parentheses.

Species	***n***	**h**	**Hd (SD)**	**π (SD)**
Rattus norvegicus	14	5	0.593 (0.144)	0.0025 (0.001)
Rattus rattus	20	8	0.868 (0.041)	0.0050 (0.001)
Rattus tanezumi	21	2	0.095 (0.084)	0.0001(0.0001)

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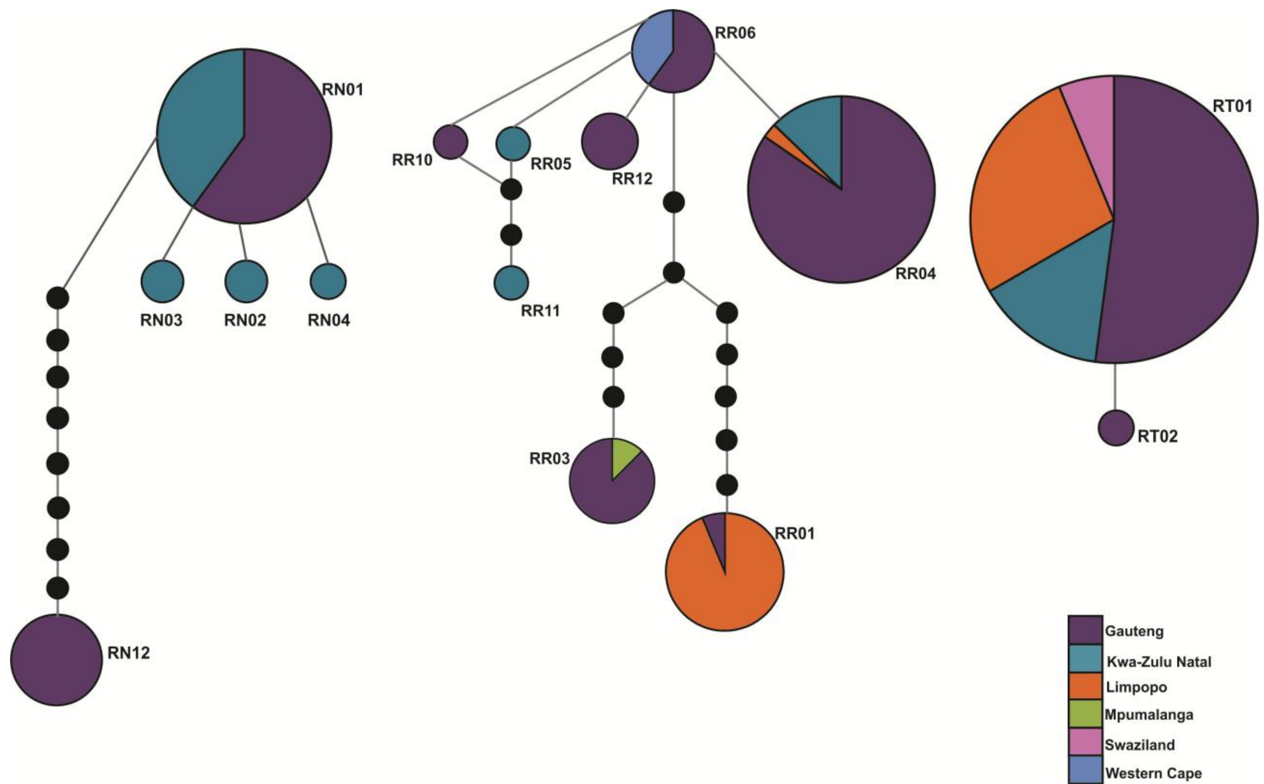

Figure 3 Haplotype networks of three matrilineally assigned *Rattus* species sampled in southern Africa based on the partial 1123 bp sequence data characterising the cyt *b* gene with the *R. norvegicus* (RN) haplotype network displayed on the left, the *R. rattus* (RR) haplotype network shown in the centre and the *R. tanezumi* (RT) haplotype network displayed on the right. The circles represent respective haplotypes and the size of the circle represents the number of individuals per haplotype. The size of the pie in geographically diverse haplotypes represents the number of individuals in proportion to the geographical localities which in turn is represented by the different colours.

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A comprehensive literature search revealed that the three *Rattus* species are found in sympatry in only four of the 28 countries sourced namely, Indonesia, Japan, South Africa and the USA. In addition, an overall of 37 haplotypes for the *R. rattus* lineage, 30 within *R. tanezumi* and 20 within the *R. norvegicus* lineages were recovered (Table 2). Interestingly, each *Rattus* species in South Africa has at least one unique haplotype (RN02, RR04, RR10, RR11, RR12, RT01 & RT02) that is not shared with localities outside of the country.

Haplotype networks revealed the distinct origins of the three invasive *Rattus* species present in southern Africa. All *R. norvegicus* haplotypes originated from Indonesia (Figure 4.1). The most geographically diverse haplotype, RN01 (differing from the ancestral Indonesian haplotype by a single mutation) is found in South Africa and shared among many other countries including China. Similarly, South African haplotype RN02 differs from this common haplotype also by a single mutation, while haplotype RN12 differs by a single mutational step from a Japanese laboratory strain haplotype. The *R. rattus* network analysis revealed RR06 to be the most geographically diverse haplotype (Figure 4.2) and is coincidentally also the most ancestral haplotype. While both South African haplotypes RR05 (which is shared with Brazil) and RR04 is one mutational step removed from this common haplotype, these two haplotypes are not linked. Furthermore, haplotype RR11 is three mutational steps removed from RR05 and haplotype RR12 is two mutational steps removed from the most common and ancestral haplotype as well as from a haplotype from Reunion Island. Haplotypes RR01 and RR03 are distantly removed from the ancestral haplotype RR06. While RR01 is shared with Tanzania and Grand Comore, it is most closely linked to another haplotype from Grand Comore as well as a haplotype from the USA. Haplotype RR03 on the other hand is shared with the USA and most closely linked to a haplotype from Brazil. Network analyses for *R. tanezumi* (Figure 4.3) revealed RT01 to be shared with Swaziland and to be most closely related to South African haplotype RT02. These haplotypes

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are also very distantly linked to the most common and ancestral haplotype, RT03 and are the most closely linked to haplotypes from Indonesia from which they are removed by at least four mutational steps.

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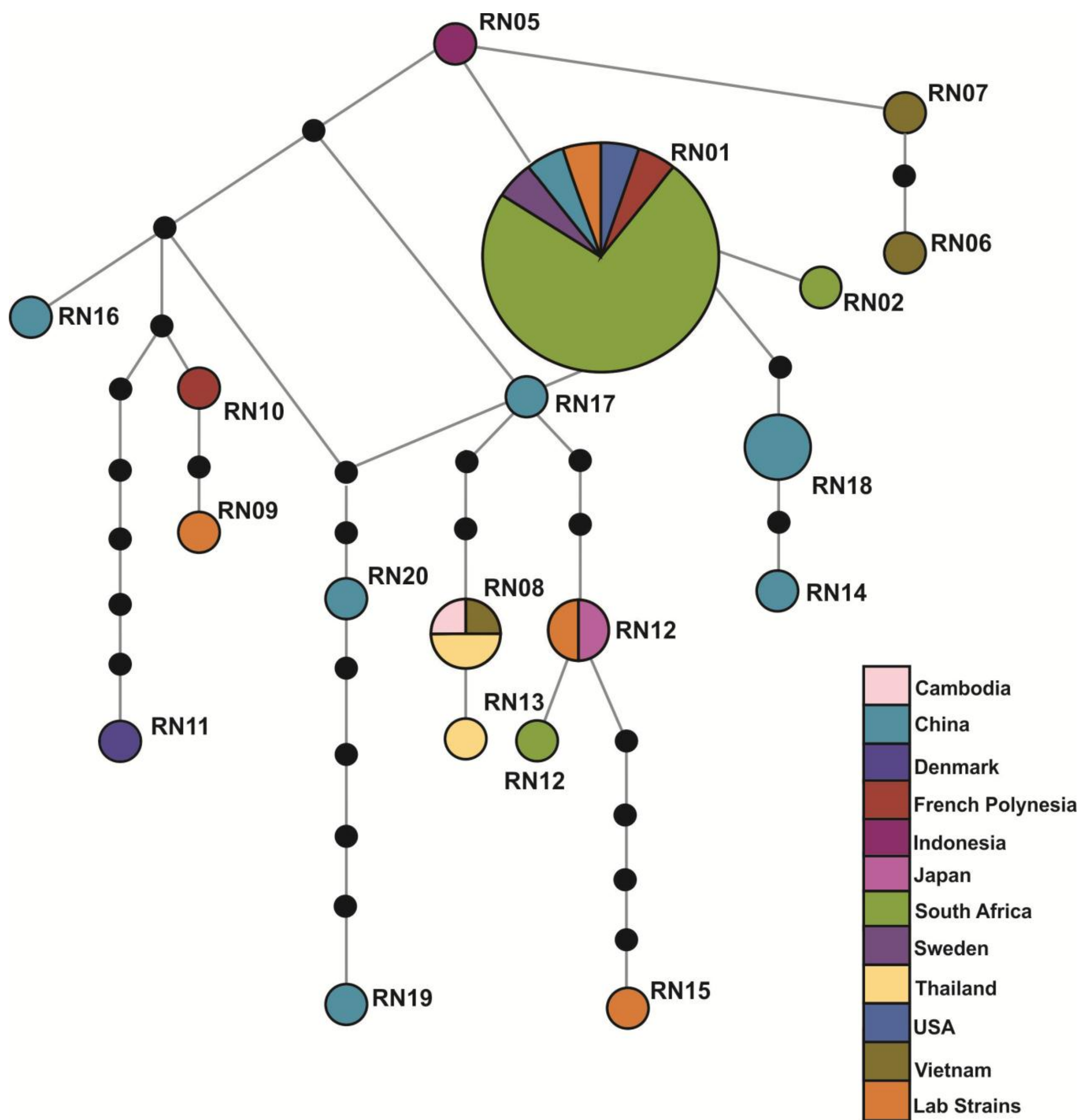

Figure 4.1 Haplotype network of *Rattus norvegicus* based on 1140 bp sequence data characterising the cyt *b* gene where the size of the circle for geographically diverse haplotypes represents the frequency of occurrence in proportion to the geographical locality.

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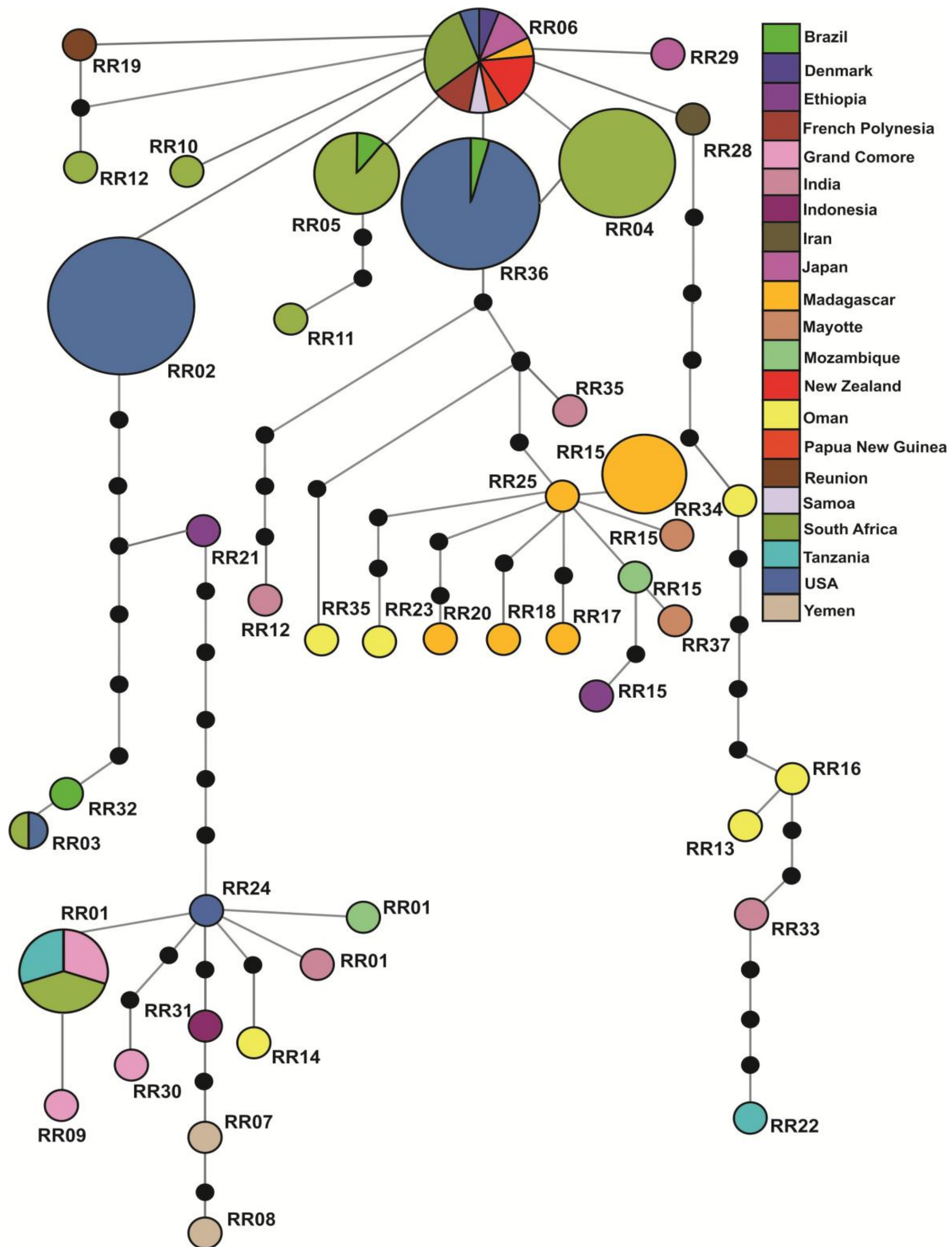

Figure 4.2 Haplotype network of *Rattus rattus* based on 1140 bp sequence data characterising the cyt *b* gene where the size of the circle for geographically diverse haplotypes represents the frequency of occurrence in proportion to the geographical locality.

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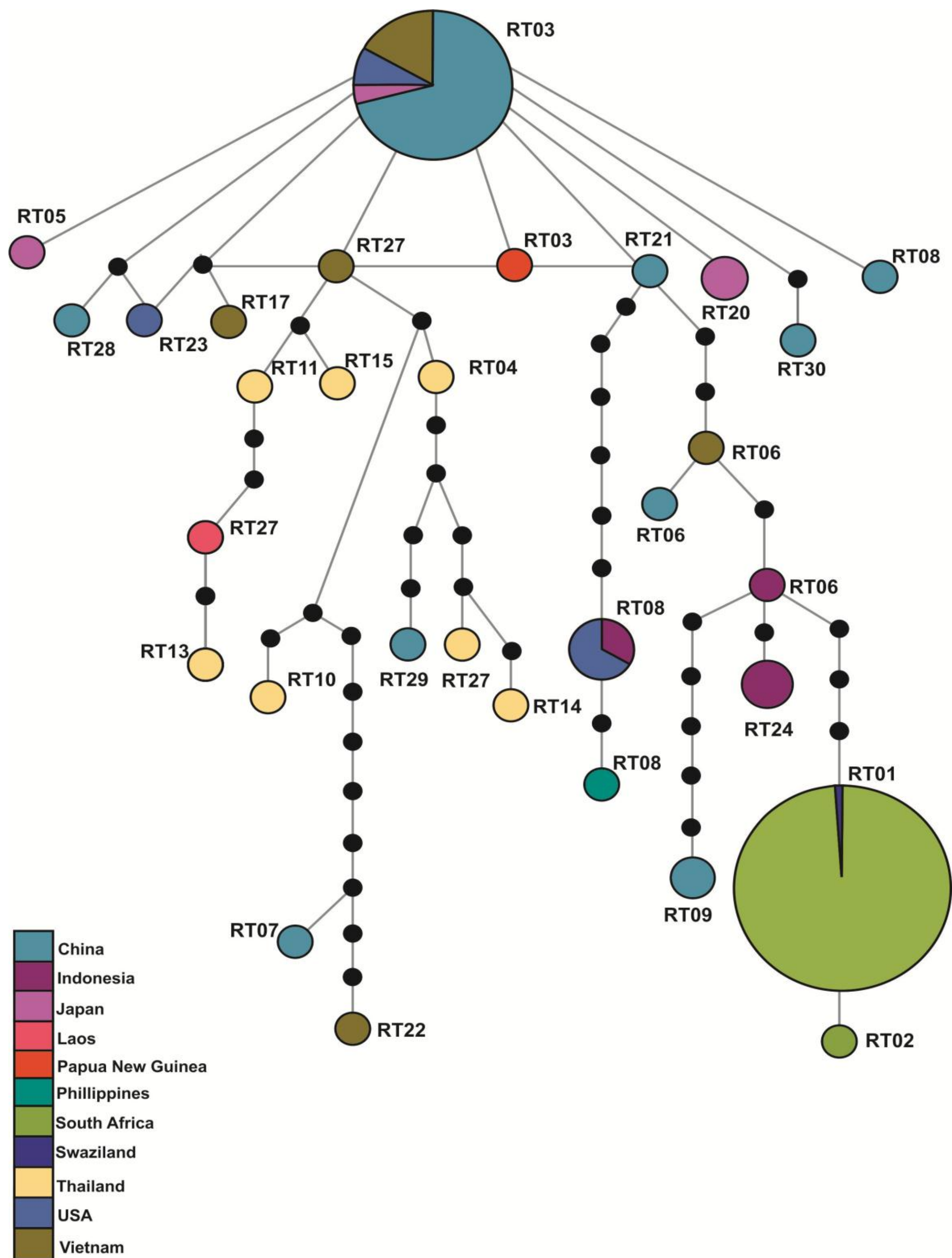

Figure 4.3 Haplotype network of *R. tanezumi* based on 1140 bp sequence data characterising the cyt *b* gene where the size of the circle for geographically diverse haplotypes represents the frequency of occurrence in proportion to the geographical locality.

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The different phylogenetic analyses revealed the same tree topology and therefore only the NJ tree was chosen for illustrative purposes Overall, the major lineages as described in Aplin *et al.* (2011) and Conroy *et al.* (2013) were recovered in addition to the monophyletic *R. norvegicus* clade (Figure 5). The southern African samples: RR01, RR03, RR04, RR05, RR06, RR10, RR11 & RR12 correspond to RrC Lineage I (Figure 5.1) and RT01 & RT02 correspond to RrC Lineage II (Figure 5.2) while two samples from Pakistan make up RrC Lineage III (Figure 5.3) and three samples from the Philippines and one from Singapore make up RrC Lineage IV (Figure 5.4) as defined by Aplin *et al.* (2011). One *R. tanezumi* sample (HM031707) from China did not group with any previously defined lineages and possibly represents a new additional lineage or was a misidentified *R. losea* specimen as it has the closest sequence similarity (99 %) to *R. losea* (HM031710) following a BLAST nucleotide database search of the NCBI (www.ncbi.nlm.nih.gov/blast). Although, RrC Lineages III and IV are both regarded as *R. tanezumi* by Musser & Carlton (2005), these are regarded as *R. rattus* by Aplin *et al.* (2011). Consequently, this study will refer to these lineages only as the *R. rattus* species complex and not assign any taxonomic names to these lineages. High nodal support was recovered for all RrC Lineage I-IV as was the case in Aplin *et al.* (2011) and the *R. norvegicus* phylogeny (Figure 5.5) is also well-supported. The mtDNA *Rattus* phylogenies show distinct clades within all three groups. The southern African *R. tanezumi* samples in the RrC Lineage II form part of the same well-supported clade (Figure 5.2), while in the *R. norvegicus* (Figure 5.5) and RrC Lineage I *R. rattus* (Figure 5.1) phylogenies, these samples form part of two and three distinct (yet not well supported) lineages, respectively.

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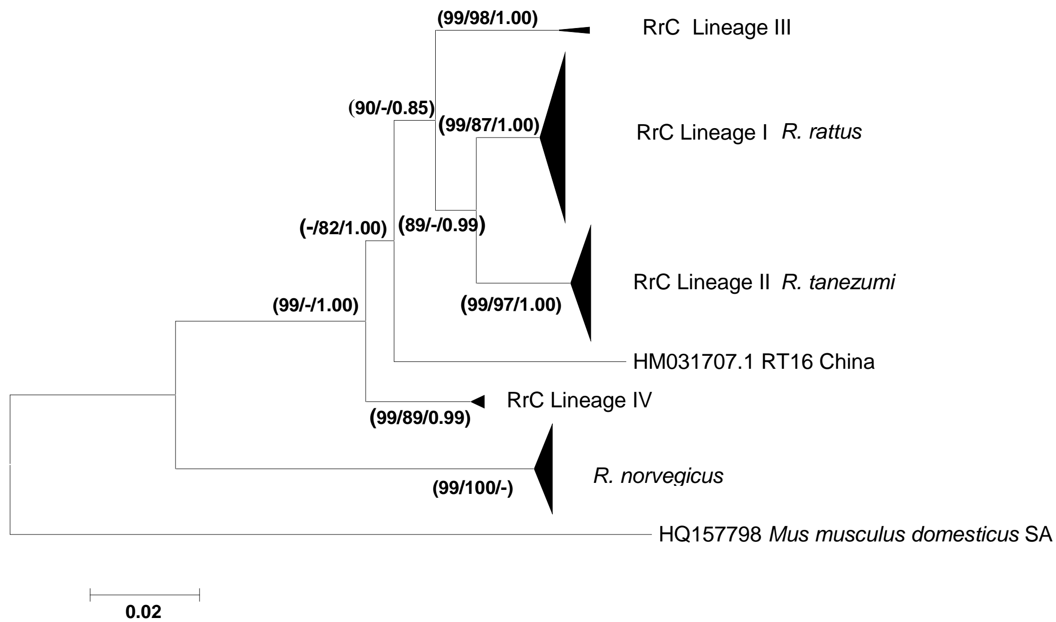

Figure 5 Neighbour-Joining (NJ) tree displaying the phylogeny of mtDNA *Rattus* species based on 1140 bp cyt *b* sequence data. *Rattus rattus* Complex (RrC) lineage names (I-IV) are adopted from Aplin *et al.* (2011). Nodal support (≥ 75 % and ≥ 0.85) is shown in parentheses below or above nodes in the order of NJ, Maximum Likelihood (ML) and Bayesian Inference (BI), respectively.

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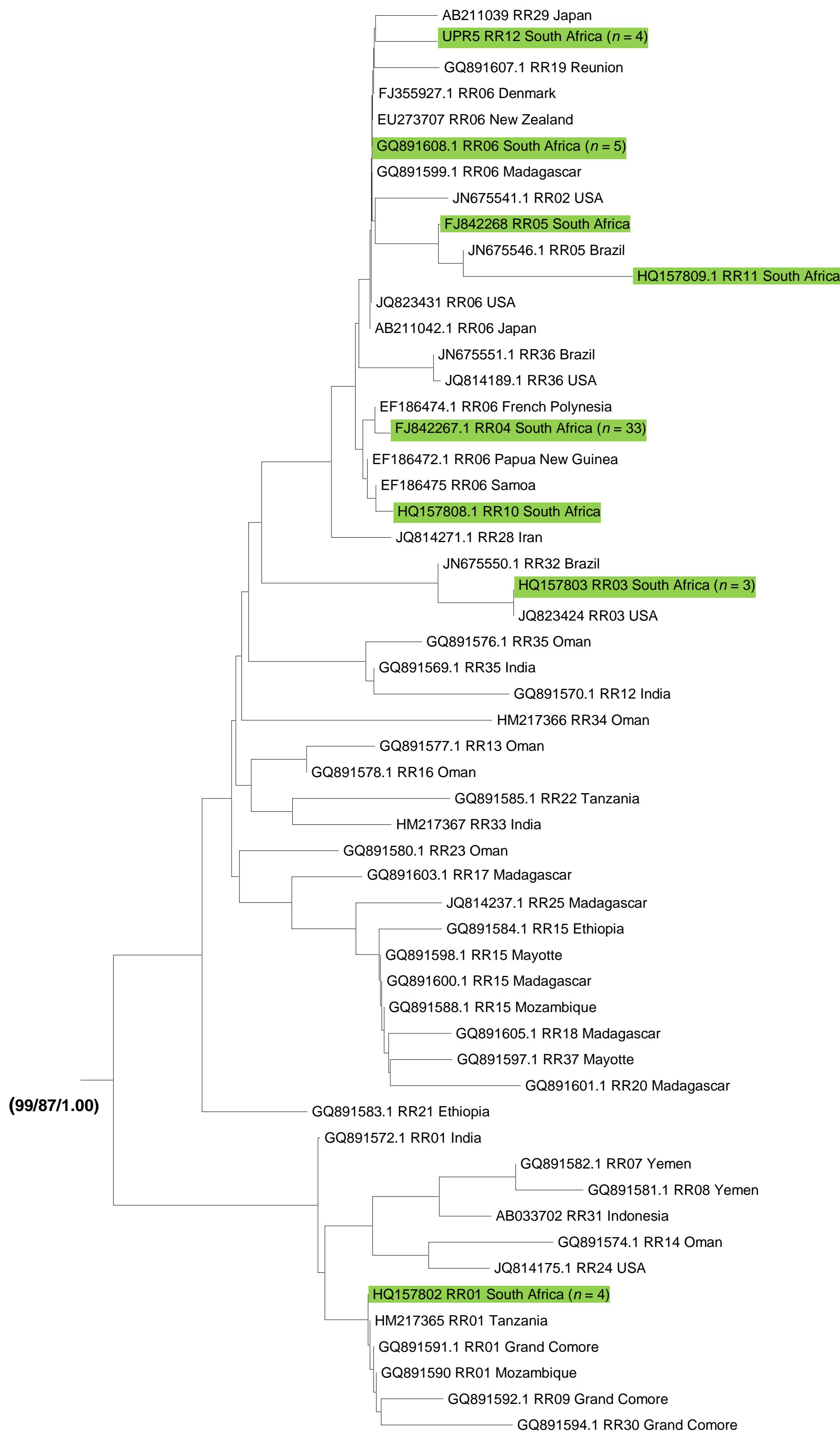

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Figure 5.1 Section of the Neighbour-Joining (NJ) tree displaying the *Rattus rattus* phylogeny (RrC Lineage I; Aplin *et al.* 2011) based on 1140 bp cyt *b* sequence data with the relevant nodal support (≥ 75 % and ≥ 0.90) in parentheses below the node in the order of NJ, Maximum Likelihood (ML) and Bayesian Inference (BI). Taxon name includes Genbank accession number or sample name where available followed by haplotype name and locality. Southern African samples are highlighted in green with the number of identical taxa shown in parentheses.

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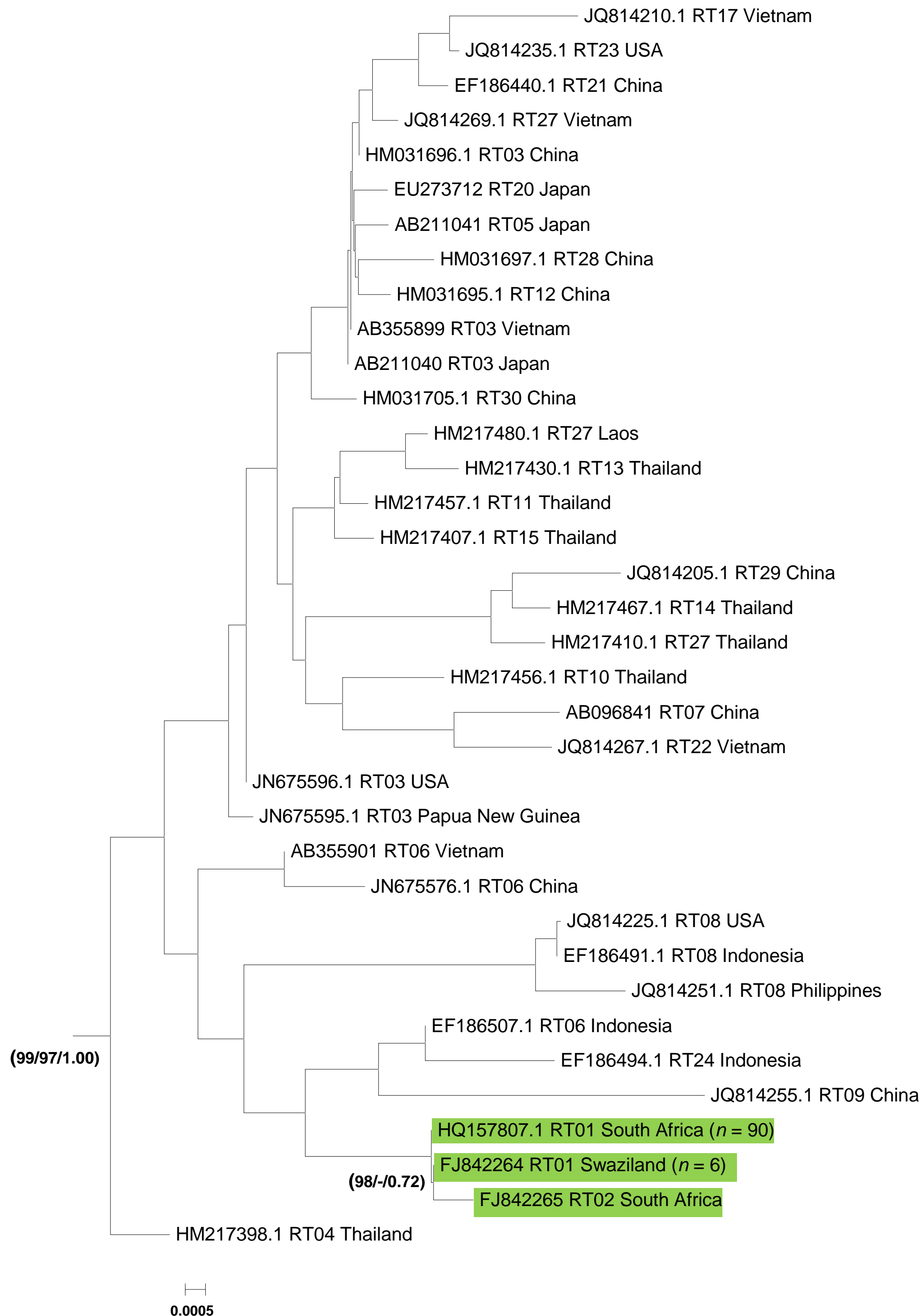

Figure 5.2 Section of the Neighbour-Joining tree displaying the *Rattus tanezumi* phylogeny (RrC Lineage II; Aplin *et al.* 2011) based on 1140 bp cyt *b* sequence data with the relevant nodal support (≥ 75% and ≥ 0.90) in parentheses below the node in the order of NJ, Maximum Likelihood (ML) and

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Bayesian Inference (BI). Taxon name includes Genbank accession number or sample name where available followed by haplotype name and locality. Southern African samples are highlighted in green with the number of identical taxa shown in parentheses.

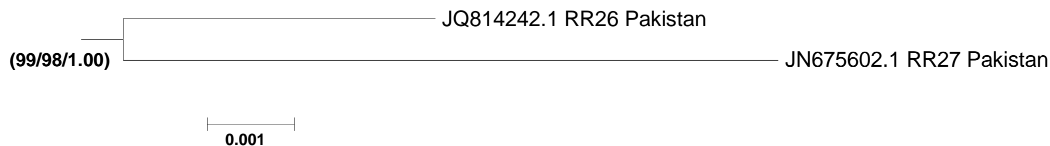

Figure 5.3 Section of the Neighbour-Joining (NJ) tree displaying the *Rattus rattus* Complex (RrC) Lineage III (adopted from Aplin *et al.* 2011) based on 1140 bp cyt *b* sequence data with the relevant nodal support (≥ 75 % and ≥ 0.90) in parentheses below the node in the order of NJ, Maximum Likelihood (ML) and Bayesian Inference (BI). Taxon name includes Genbank accession number followed by haplotype name and locality.

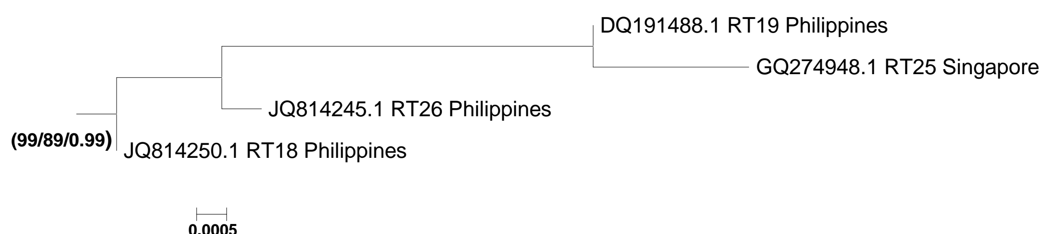

Figure 5.4 Section of the Neighbour-Joining (NJ) tree displaying the *Rattus rattus* Complex (RrC) Lineage IV (adopted from Aplin *et al.* 2011) based on 1140 bp cyt *b* sequence data with the relevant nodal support (≥ 75 % and ≥ 0.90) in parentheses below the node in the order of NJ, Maximum Likelihood (ML) and Bayesian Inference (BI). Taxon name includes Genbank accession number followed by haplotype name and locality.

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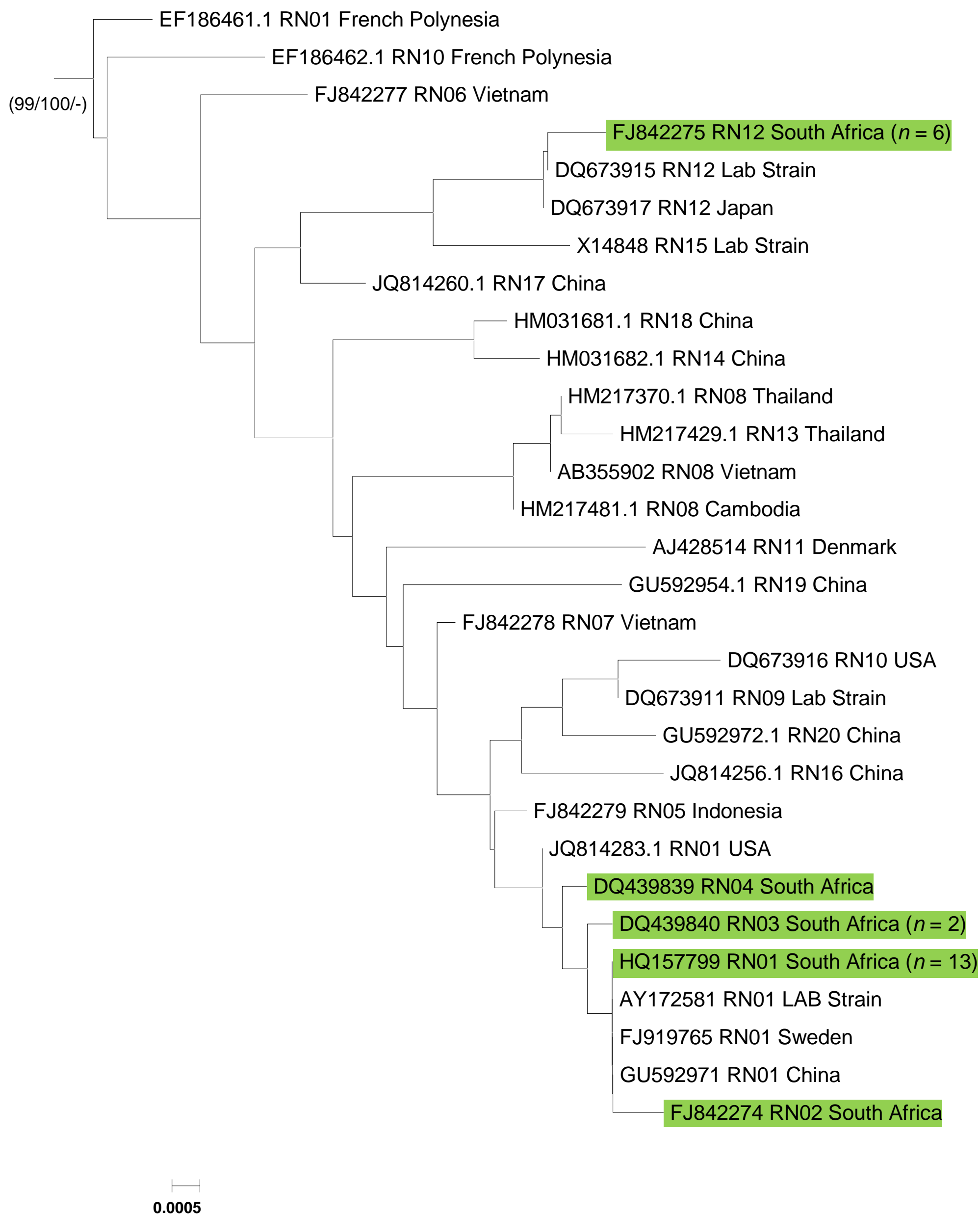

Figure 5.5 Section of Neighbour-Joining (NJ) tree displaying the *Rattus norvegicus* phylogeny based on 1140 bp cyt *b* sequence data with the relevant nodal support (≥ 75 % and ≥ 0.90) in parentheses below the node in the order of NJ, Maximum Likelihood (ML) and Bayesian Inference (BI). Taxon name includes Genbank accession number followed by haplotype name and locality. Southern African samples are highlighted in green with the number of identical taxa shown in parentheses.

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DISCUSSION

All three *Rattus* species known to occur in South Africa were sampled in this study. The genetic characterisation of samples when combined with geographic distribution has the potential to provide some insight into the invasion sites and colonisation routes of these species in the southern African region. Having pooled data from both coastal and inland areas of southern Africa, it was anticipated that the *Rattus* population's initial colonisation sites were coastal localities (having arrived *via* the shipping trade) and subsequently diversified as they spread inland. When looking at the haplotype networks (Figure 3), the parent haplotypes (representing the founding populations) of all three *Rattus* species are represented by a mixture from the inland Gauteng Province and coastal Western Cape or Kwa-Zulu Natal Provinces. Although this is still consistent with a coastal to inland dispersal pattern, the high abundance of the inland samples relative to the coastal samples may be indicative of long distance dispersal probably by means of air or road travel (in addition to shipping) but may also be as a consequence of under sampling of the coastal areas.

The abundance of RN01 and RN12 haplotypes of the *R. norvegicus* mitochondrial DNA (mtDNA) lineage indicates that initial colonisation occurred by these two haplotypes. These two haplotypes are also very distantly related to each other and likely represent two distinct introduction events or possibly correspond to a single invasion by both haplotypes. This finding is supported by Bastos *et al.* (2011) and in the phylogenetic analyses of *R. norvegicus* in the present study where the two haplotypes form part of two separate lineages. Similarly, it is believed that at least three introductions can account for the diversity in the *R. rattus* population represented by the three most abundant haplotypes. This is also supported by the phylogenetic analyses in Bastos *et al.* (2011) and in the present study where each introduction event is represented by a separate lineage. While one haplotype, RR06 is also the parent haplotype from which all other haplotypes are derived from, two haplotypes, RR03 and

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RR01, are so distantly related from this haplotype and to each other that they most likely represent two separate and additional introduction events or one introduction event with three distinct haplotypes. In contrast, *R. tanezumi* populations are believed to have arisen from a single introduction event as the second haplotype (RT02) is derived from the parent haplotype (RT01). Some within-species variation is evident in all three mitochondrial defined *Rattus* species as the majority of haplotypes are closely linked to the parent haplotypes. This is suggestive of high diversification following invasion or multiple invasions of closely related haplotypes from the same geographical area (Lack *et al.* 2012).

Following "global" sourcing of mtDNA data from *Rattus* samples it was found that South Africa is one of only four countries where these three *Rattus* species occur in sympatry. The low number of localities where the three species co-occur is probably due to sampling bias and misidentification. Haplotype diversity for the southern African samples was generally low for all three species when compared to "global" haplotype diversity supporting the findings of Bastos *et al.* (2011), despite the additional samples characterised in the present study. This may be due to inadequate sampling of especially the south and west coasts of South Africa. Lack *et al.* (2013) however, found similarly low genetic diversity in the USA having recovered only 21 haplotypes from 163 *R. rattus* individuals and 11 haplotypes from 184 *R. norvegicus* individuals, respectively. While *R. rattus* is shown to be more genetically diverse than *R. tanezumi*, it must also be considered that the former species was sampled from almost twice as many localities than the latter species. *Rattus tanezumi* probably goes undetected in most parts of the world (Lack *et al.* 2012), where it is confused primarily with *R. rattus* (Plyusnina *et al.* 2009). Interestingly, *R. norvegicus* has markedly lower haplotype diversity than the two congeners and may again be due to the lack of available data on the species (Lack *et al.* 2013). While South Africa has at least one unique haplotype present for each species, this is not surprising as it would seem that whenever there are more than four

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haplotypes present in a country, it is not unusual for at least one of these to be unique and may be explained by the within-species variation for this taxonomic group.

The global haplotype network revealed some insights into the colonisation history of the *Rattus* population of southern Africa. The most abundant haplotypes in the *R. rattus* population is derived from the "shiprat cluster" (Aplin *et al.* 2011) which expanded out of India colonising most of the planet. One haplotype, RR06 is shared with seven other countries and also forms the parent haplotype from which all other haplotypes are derived. Most southern African *R. rattus* haplotypes are unique and form a cluster around this haplotype indicating a rapid expansion of the population following invasion (Lack *et al.* 2012). Other haplotypes which are several mutational steps removed from this parent haplotype likely represent introduction events from the geographical localities with which the haplotype is shared. The within-species variation for the southern African samples seems very low however when compared to the Madagascan haplotypes of *R. rattus* where many more closely related haplotypes cluster around a single parent haplotype (Figure 4.2 in this study; Hingston *et al.* 2005). In contrast, the network analyses of *R. rattus* in southern Africa suggest that only five haplotypes derived from three introductions or from a single introduction with three distinct haplotypes. This in turn indicates that these introductions may have been very recent and not enough time has passed for subsequent diversification to give rise to more haplotypes. However, considering historical trade between the southern African region and Eurasia, initial introduction could not have occurred that recently and possibly interspecific competition with the diverse indigenous rodent fauna (Towns & Broome 2003) of southern Africa may inhibit the diversification and expansion of introduced *Rattus* species.

Similarly, introductions of *R. norvegicus* seem to be rare. Most haplotypes (RN01, RN03 and RN04) are clustered and shared with the most common, geographically diverse and abundant haplotype and this again indicates that the origins *R. norvegicus* are associated with

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the localities with which these haplotypes are shared. Other than *R. rattus*, this species has fewer haplotypes which are very geographically diverse and abundant. As this species was historically associated with a coastal distribution (Apps 2000) and is believed to have only recently invaded the inland areas, the lack of genetic diversity is probably due to the under-sampling of the coastal regions of South Africa. This is consistent with the findings of Lack *et al.* (2013) where nuclear genetic data found higher diversity in coastal *R. norvegicus* populations compared to those of central USA. In addition, the haplotype (RN12) distantly related to the other South African haplotypes shows close links to both a wild caught specimen from Japan and a laboratory strain. Diversification from an escaped laboratory rodent seems more likely rather than an introduction from Japan and subsequent diversification to form a distinct haplotype in South Africa.

The two *R. tanezumi* haplotypes of southern Africa were very far removed from the most geographically diverse and parent haplotype. These haplotypes were unique and their closest links were to haplotypes from Indonesia implying that initial introductions were possibly from the native range. In comparison to the global haplotype diversity recovered for this species, haplotype diversity in southern Africa is markedly low. This, along with the high frequency of one of the southern African haplotypes indicates that this *Rattus* species was recently introduced with only one low frequency haplotype arising from the initial haplotype. Nevertheless, it seems clear that the observed haplotype diversity of all three *Rattus* species in their invaded ranges is likely due to a combination of both introductions from geographically diverse localities and within-species variation leading to subsequent diversification.

Phylogenetic analyses confirmed that all the southern African samples (with the exception of *R. norvegicus*) examined in this study corresponded to the RrC Lineages I and II as defined by Aplin *et al.* (2011) and are commonly identified as *R. rattus* and *R. tanezumi*,

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respectively (Aplin *et al.* 2003; Robins *et al.* 2007; Pagès *et al.* 2010; Bastos *et al.* 2011). In this study, the haplotypes that make up Lineage IV are from the Philippines and Singapore, while Aplin *et al.* (2011) and Conroy *et al.* (2013) described these haplotypes as being concentrated around the lower Mekong River, Cambodia suggesting that this lineage possibly represents the native population. In contrast, haplotypes from Lineage II are more widespread as populations from as far as the USA and southern Africa are included. This, along with divergence estimates (Aplin *et al.* 2011) suggests that Lineage IV haplotypes may be the parent haplotypes of Lineage II despite haplotype network analysis revealing no links between haplotypes from Lineages II and IV (not illustrated). Since rats of Lineage IV are known to commonly occur in natural forest habitats and not so much along human habitation (Aplin *et al.* 2011), it is likely that they represent the wild ancestors to the more commensal *R. tanezumi* rats of Lineage II (Aplin *et al.* 2012). In addition, it is also known that *R. tanezumi* becomes a serious pest in areas outside its native range (Stuart *et al.* 2007).

Conroy *et al.* (2013) found *Rattus* sampled from California, USA to also form part of the RrC I and II Lineages only. However, analyses of microsatellite data found no differentiation between the RrC I and RrC II lineages indicating that introgression of *R. rattus* and *R. tanezumi* occurs in introduced and possibly even in their native ranges (Pagès *et al.* 2013). Furthermore, the paraphyletic origins of the *Rattus rattus* species complex in the mitochondrial defined *Rattus* phylogeny (Figure 5.1) supports this introgression between the two species. However, if the two species interbreed in nature, taxonomic resolution may be further complicated as the two species have been found to be locally sympatric (Bastos *et al.* 2011; this study) with instances of both species being sampled at the same residence.

Phylogenetic analysis provides a powerful tool to monitor the introduction and spread of an invasive species which in turn may provide insights into the best rodent pest management strategies. Given the above data, it is evident that the colonisation and dispersal pattern of all

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three *Rattus* species observed in southern Africa is due to a combination of introductions and subsequent diversification due to some within-species variation. Despite this, genetic diversity for *Rattus* species in southern Africa is low and may be because this global diversity is simply not colonising the region or is yet to be sampled. Nevertheless, biosecurity practices are failing to control rodent invasions. This should be addressed especially in light of introgression occurring in nuclear genomes despite the distinct mitochondrial lineages, which are possibly stimulated through sympatry in the invasive range (Lack *et al.* 2012). In addition, rodent pest management practices may also be impeded through a continuous influx of genotypes of which some may be favourable and will hamper rodent pest control measures.

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Chapter 3

Molecular prevalence and diversity of bacteria in invasive, commensal *Rattus* from South Africa: a broad-range PCR-sequencing approach

ABSTRACT

Rats harbour various pathogens ranging from bacteria, viruses, helminths and protozoan parasites. However, many bacterial zoonotic diseases have a widespread occurrence and can cause severe out-breaks, with important socio-economic implications. Rats are abundant in both urban and rural South Africa, and as a consequence their presence may pose a threat to public health and food security. The present study investigated the potential environmental contamination of bacteria *via* the urinary route by screening kidneys of invasive commensal rodents of the genus *Rattus* using PCR with broad-range, universal PCR primer sets targeting the 16S gene region. The analysis revealed the presence of five genera of two phyla and an overall, sequence-confirmed prevalence of 49 %. The diverse bacterial genera found namely; *Acinetobacter*, *Bartonella*, *Brochothrix*, *Rickettsia* and *Streptococcus*, may be of zoonotic interest and the study highlights the importance of continuous rodent control and disease surveillance among invasive, commensal rodents.

KEYWORDS: 16S, invasive species, PCR, *Rattus*, South Africa, zoonoses

INTRODUCTION

Rattus species play a significant role in the transmission of zoonotic diseases (Gratz 1997). Several hanta- and arena viruses known to cause haemorrhagic fevers (Gratz 1997; Mills & Childs 1998), bacterial pathogens (Ellis *et al.* 1999; Easterbrook *et al.* 2007) as well as helminths and protozoan parasites (Webster & MacDonald 1995; Claveria *et al.* 2005) have been isolated from rats. In addition, rats may harbour several ectoparasites that can act as vectors capable of transmitting pathogens to humans (Carter & Cordes 1980; Azad & Beard

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1998; Yang *et al*. 2009). There are various routes of transmission of infectious agents; either directly through rat bites or scratches, indirectly through environmental contamination by rat urine or faeces, or through ectoparasite vectors.

Rattus species are native to Asia (Musser & Carleton 2005) and are among the most widely introduced vertebrates having successfully established feral populations in both disturbed and pristine habitats that they have gained access to (Amori & Clout 2003). Three matrilineal species of invasive rats occur in South Africa, namely *R. norvegicus*, *R. rattus* and *R. tanezumi*, the latter being only recently genetically-identified in South Africa in 2005 (Bastos *et al.* 2005). These rodents are commensal and exploit human habitation for food and shelter (Atkinson 1985; Taylor *et al*. 2008; Bastos *et al.* 2011).

Many species of indigenous rodents are present in Africa and are actual or potential reservoirs of infectious agents (Gratz 1997). If interspecific exchanges of infections between invasive commensal and indigenous rodents occur, commensal rats may provide a link between indigenous rodents and humans where spill-over of zoonotic diseases are likely to occur. Furthermore, invasive, commensal rats have the potential to spread disease to native rodent populations (Smith & Carpenter 2006) and may cause population declines in susceptible species.

Many bacterial zoonotic diseases have a widespread occurrence and can cause severe outbreaks with important socio-economic implications (Gratz 1997; McMichael 2004; Neerincx *et al.* 2010). Plague is probably the most well documented bacterial disease associated with *Rattus*. The disease is caused by the bacterium, *Yersinia pestis* and it is estimated to have been responsible for around 200 million deaths throughout recorded human history (Perry & Fetherston 1997). In sub-Saharan Africa sporadic outbreaks are common (Neerincx *et al.* 2010) due to various endemic foci (Gratz 1997).

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Invasive, commensal rats are abundant in both urban and rural South Africa (Kirsten & von Maltitz 2005; Taylor *et al.* 2008) and their presence may pose a threat to public health and food security (Ahmad *et al.* 1995; Smith *et al*. 2002; Meerburg *et al.* 2009). Immune-compromised individuals such as people living with HIV-Aids, those suffering from alcoholism or drug addiction as well as the very young and elderly are at greatest risk as many of these rodent-borne diseases develop as a result of opportunistic infections (Comer *et al.* 2001). Furthermore, the detrimental effect invasive, commensal rats have on human nutrition in Africa is significant. Invasive, commensal rats threaten food security, when they contaminate and deplete food stores (Ahmad *et al*. 1995) which may lead to people being nutritionally challenged rendering them more susceptible to disease. Given the zoonotic disease and food security risk that rats pose, it is surprising that very few studies in Africa (Shepherd *et al.* 1982; Gratz 1999; Taylor *et al.* 2008; Mostert 2009; Brettschneider 2010) have assessed the role that rats play as potential carriers of zoonotic diseases as compared to elsewhere in the world (Carter & Cordes 1980; Webster & Macdonald 1995; Ellis *et al.* 1999; Hilton *et al.* 2002; Easterbrook *et al*. 2007).

This study used molecular techniques that are known to provide a fairly low risk, rapid detection and identification of bacteria (Clarridge 2004). The 16S rRNA coding gene in the bacterial genome is most commonly used for taxonomic purposes (Amann *et al.* 1995) as it yields a sequence of considerable length and is highly conserved across all bacterial taxa with enough variable sites to be informative for phylogenetic analyses (Clarridge 2004). Consequently, sequences generated characterising this gene region has a high frequency in nucleotide databases (Clarridge 2004) and therefore allows rapid identification of unknown samples. As such, broad range universal primer sets characterising this gene region has been extensively used for differential diagnosis of pathogens (Amann *et al.* 1995; Marchesi *et al.* 1998; Doern 2000) as it overcomes the limitations associated with culturing techniques.

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The need for more information on infection prevalence and zoonotic potential of invasive, commensal rats in Africa is evident from the literature and especially important in the advent of changes in global climate, land use patterns and human social behaviour which play a major role in emerging and re-emerging diseases (McMichael 2004). To this end, the objective of the present study was to investigate the diversity, prevalence and zoonotic potential of the bacterial community present in *Rattus* kidneys that have the potential to be spread through environmental contamination in South Africa.

MATERIALS AND METHODS

Sample collection

Rats were sampled, processed and identified to matrilineal species assignments as described in Chapter 2. A subset of 54 samples from four provinces (Gauteng, Kwa-Zulu Natal, Limpopo, and Mpumalanga Provinces) in South Africa collected during the 2003-2008 sampling period (Mostert 2009; Bastos *et al.* 2011) were selected based on proportional matrilineally-defined species abundance. This, along with the first 30 samples collected during the 2010-2011 sampling period gives a total of 84 samples used in this study.

Laboratory procedures

Genomic DNA was extracted from kidney tissue using the Roche High Pure PCR Template Preparation Kit (Roche Diagnostics) according to the manufacturer's protocol and stored at -20º C for subsequent molecular analyses. Kidney DNA extracts were screened for overall bacterial prevalence using one of the two universal, broad-range 16S rRNA targeting forward primers *viz* 27F- AGAGTTTGATCCTGGCTCAG or 63F-CAGGCCTAACACATGCAAGTC in combination with the reverse primer, 1522R-AAGGAGGTGATCCAGCCGCA (Hauben *et al*. 1997; Marchesi *et al.* 1998). Each primer combination amplified the full length (1.5kbp) of the 16S rRNA gene region. The PCR

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mixture contained 1X buffer (Fermentas), 0.25 µM dNTP's (Fermentas), 0.4 µM of each primer, 1U *Taq* Polymerase (Fermentas) and 100-200 ng DNA template in a 50 µL reaction volume. Amplification was achieved using a touchdown PCR at which initial denaturation was at 96º C for 10 s, followed by three discrete thermal cycles that consisted of denaturation at 96º C for 12 s, annealing for 20 s and extension at 70º C for 1 min 50 s, with an initial annealing temperature of 63º C, followed by 61º C and finally 32 cycles at 59º C.

Phylogenetic and statistical analyses

All amplified PCR products were purified using the Roche PCR Product Purification Kit (Roche Diagnostics) and cycle sequenced using BigDye v. 3.1 terminator cycle-sequencing kit (Perkim-Elmer, Foster City, USA). Samples were run on an ABI 3130 sequencer and sequence chromatograms were viewed and edited in Mega 5 (Tamura *et al.* 2011) prior to performing a BLAST nucleotide database search (www.ncbi.nm.nih.gov/blast) to identify the genus/species with the highest sequence similarity (> 95 %). The data generated in this study were complemented with reference sequences from the Genbank database (Table 1) that were reported in peer-reviewed journals and/or type strains.

Table 1 List of partial 16S rRNA bacterial sequences generated in this study (indicated in bold) and reference sequences from the Genbank database used for comparative purposes.

Sample	Taxon name	Accession no.	Reference
-	*Acinetobacter* sp.	GQ178044	Ray *et al.* (2009)
-	*Bartonella elizabethae*	NR025889	Daly *et al.* (1993)
-	*Bartonella grahamii*	AB426636	Inoue *et al.* (2009)
-	*Bartonella queenslandensis*	EU111758	Gundi *et al.* (2009)
-	*Bartonella rataustraliani*	EU111753	Gundi *et al.* (2009)
ER2	***Bartonella* sp.**	**-**	**This study**
ER4	***Bartonella* sp.**	**-**	**This study**
-	*Bartonella tribocorum*	NR025278	Heller *et al.* (1998)
ER3	***Brochothrix thermosphacta***	**-**	**This study**
-	*Brochothrix thermosphacta*	AB680248	Unpublished
-	*Brochothrix thermosphacta*_ATCC11509	HQ890942	Nowak *et al.* (2011) (type strain)
-	*Clostridium difficile*_ATCC9689	HM245939	unpublished (type strain)
-	*Clostridium sordellii*	JN048957	Ryzinska-Paier *et al.* 2011

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UPER24	***Clostridium* sp.**	**-**	**This study**
-	*Rickettsia conorii*	NR041934	Zhu *et al.* (2005)
-	*Rickettsia australis*	NR036773	Roux & Raoult (1995)
-	*Rickettsia bellii*	NR036774	Roux & Raoult (1995)
F51	***Rickettsia* sp.**	**-**	**This study**
-	*Rickettsia sp.*	AB185963	Hagimori *et al.* (2006)
-	*Streptococcus agalactiae*	HQ658089	Geng *et al.* (2012)
-	*Streptococcus fryi*	AB588125	Tomida *et al.* (2011)
HK26	***Streptococcus* sp.**	**-**	**This study**
HSYR4	***Streptococcus* sp.**	**-**	**This study**
JZ07	***Streptococcus* sp.**	**-**	**This study**
JZ11	***Streptococcus* sp.**	**-**	**This study**
UPER22	***Streptococcus* sp.**	**-**	**This study**
UPER23	***Streptococcus* sp.**	**-**	**This study**
WK01	***Streptococcus* sp.**	**-**	**This study**
WK02	***Streptococcus* sp.**	**-**	**This study**
-	Uncultured Neiserraceae bacteria	HM108348	Martinson *et al.* (2011)
-	Uncultured *Streptococcus* sp.	AM420197	Bolivar *et al.* (2012)

The K80 model with Gamma distributed sites was selected as best-fit model of sequence evolution using the Akaike Information criterion (AIC_C) in jModeltest (Posada 2008). Phylogenies were inferred using Maximum Likelihood (ML) and Neighbour-Joining (NJ) methods with nodal support assessed by 10, 000 non-parametric bootstrap replications performed in Mega 5 (Tamura *et al.* 2011). Bayesian inference (BI) analysis was performed with MrBayes v.2.1.3. (Huelsenbeck & Ronquist 2001) and run over 10,000,000 MCMC generations where- after 25 % of trees were discarded as burn-in based on tracer plots observed in Tracer v1.5 (Rambaut & Drummond 2009).

Differences in bacterial prevalence between host species were analysed in STATISTICA v10 (StatSoft, Inc., Oklahoma, USA). A one-way analysis of variance (ANOVA) with host species as the fixed factor and a Tukey's *post hoc* test for multiple comparisons between treatments were used.

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RESULTS

A total of 84 kidney samples were screened and revealed an overall prevalence of 62 % PCR amplification with a sequence-confirmed bacterial prevalence of 49 %. Host species showed differences in bacterial prevalence (ANOVA: $F_{2, 81} = 4.73$; $n = 84$; $P = 0.01$) (Figure 1) with *R. rattus* (59 %, $n = 34$) and *R. norvegicus* (60 %, $n = 25$) having similar prevalence and *R. tanezumi* (24 %, $n = 25$) having markedly lower prevalence than the two congeners.

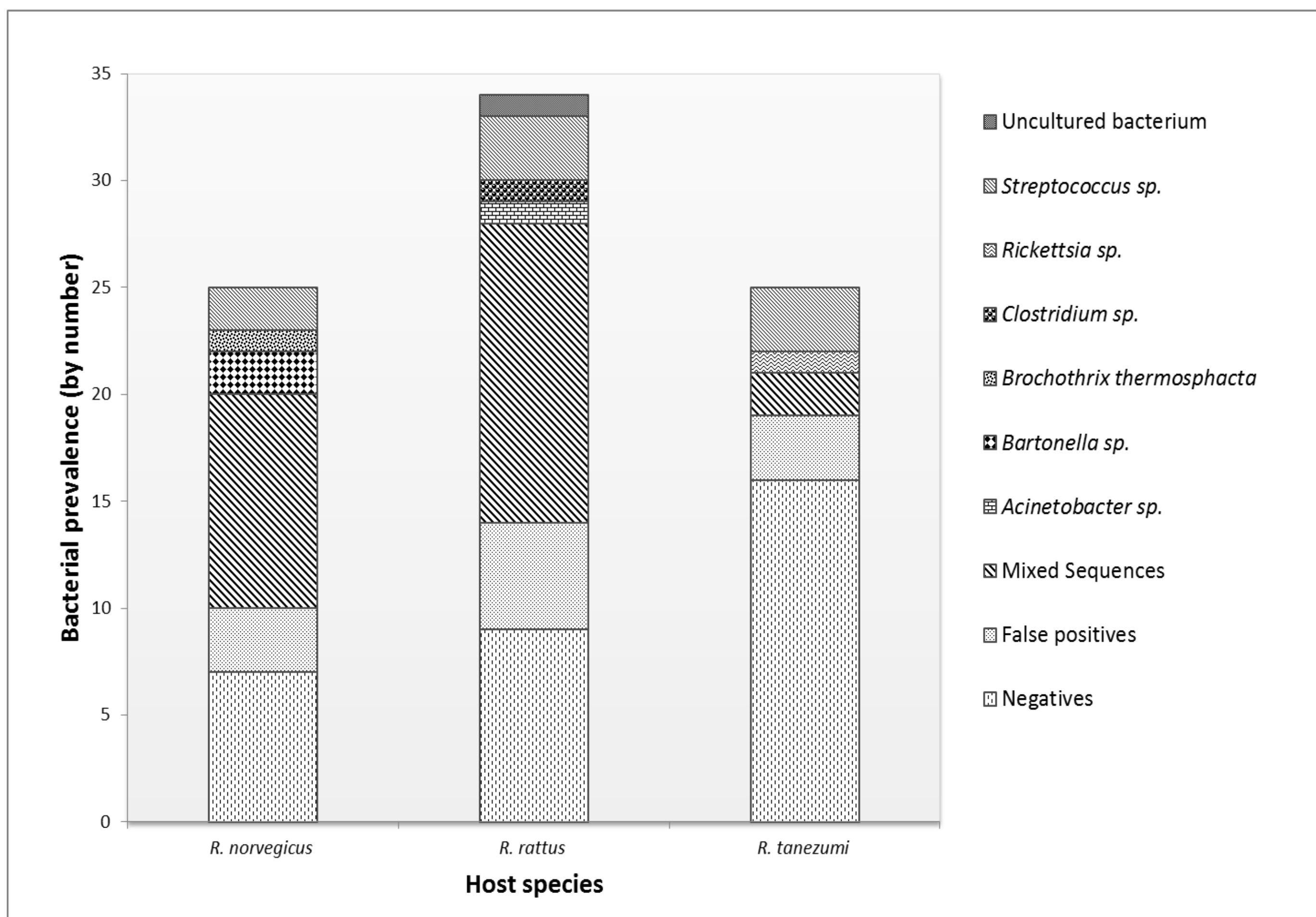

Figure 1 Bacterial prevalence recovered from kidney tissue among the three matrilineally-defined *Rattus* host species in South Africa.

A final homologous dataset 485 nucleotides in length, comprising 28 taxa had 190 variable and 181 parsimony informative sites. The average base pair composition was T = 22.8 %, C = 25.2 %, A = 23.4 %, G = 28.6 %, indicating a slight GC bias (53.8 %). The transition: transversion ratio (R) was 0.88.

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The phylogenetic analyses revealed the same tree topology for the BI and NJ tree yet the ML tree differed from these in grouping the *Rickettsia* and *Bartonella* spp. in one clade. Nevertheless, as two of the three types of phylogenetic analyses revealed the same tree topology, this tree topology was chosen and was represented by the NJ tree (Figure 2). Five bacterial genera from two phyla namely, Proteobacteria and Firmicutes were identified from a 28-taxon data set which included reference sequences. In addition one sample was identified to be 98 % similar to *Acinetobacter* spp. (JQ815602) and another 97 % similar to uncultured Neisseraceae bacteria (HM108348) (not shown in Figure 2; see appendix b).

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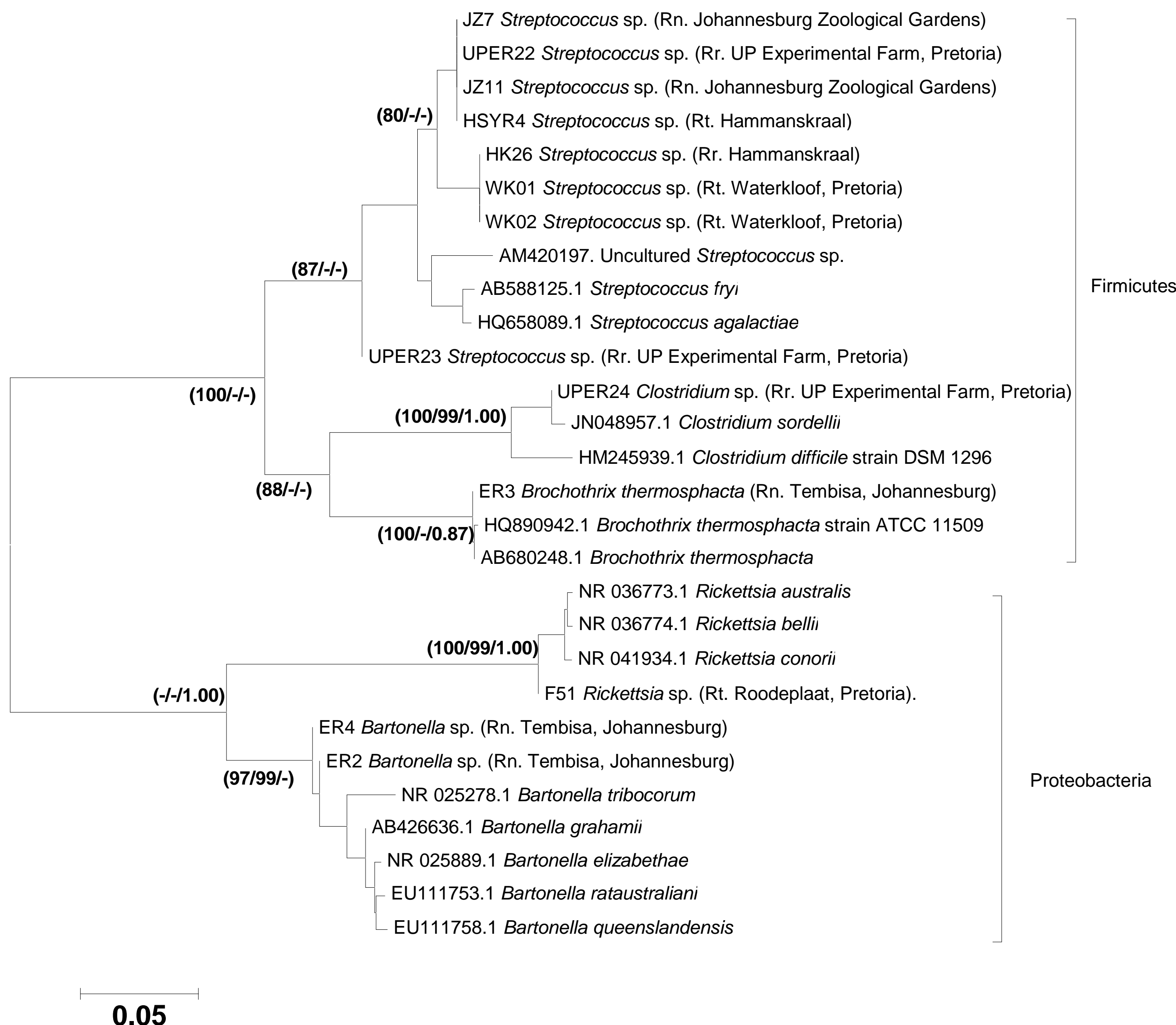

Figure 2 Neighbour joining tree showing the phylogeny of bacteria belonging to the Firmicutes and Proteobacteria phyla based on the 485bp partial 16SrRNA found in *Rattus* host species. Nodal support of ≥ 75 % is displayed below or above the node in the NJ, Maximum Likelihood and Bayesian Inference order. Taxon name includes sample name followed by bacterial species name and in parentheses the *Rattus* host species where Rr = *R. rattus*, Rn = *R. norvegicus* and Rt = *R. tanezumi* and locality. Reference sequences are distinguished by the Genbank accession no. preceding the bacterial species name within the taxon name.

Species of the genus *Streptococcus* (n = 8) were the most prevalent bacteria including three previously undescribed *Streptococcus* species followed by *Bartonella* sp. (n = 2),

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Clostridium sp. (*n* = 1), *Acinetobacter* sp. (*n* = 1), *Rickettsia* sp. (*n* = 1), *Brochothrix thermosphacta* (*n* = 1) and one uncultured bacterium belonging to the Neisseraceae family. Sequencing of multiple amplicons either due to presence of more than one bacterial species or to co-amplification of host and bacterial genomes occurred in 26 samples and was omitted in the phylogenetic analyses. Although host genome amplification by 63F/1522R primer set has not been reported previously, 11 amplicons (1.5kbp) were found to correspond to the host genome and these were not considered when determining bacterial prevalence estimates.

DISCUSSION

All the bacterial genera recovered in this initial assessment have been implicated in adverse effects on human health and food security. *Streptococcus,* a bacterial genus known to cause meningitis, nephritis and systemic infection in humans (Ruoff & Bisno 2010) and disease in rodents (Bell *et al.* 1958; Goodman 2004) was present in more than 50 % of the sequence-confirmed positive samples. In addition, species of the *Bartonella* genus were present which are usually transmitted *via* arthropod vectors and common bacteria in mammalian blood (Ellis *et al.* 1999) known to cause severe endocarditis in immune-compromised individuals (Breitschwerdt & Kordick 2000). Furthermore, *Acinetobacter* sp. and *Clostridium sordellii* are soil and water pathogens and although widely distributed in nature, the former can become problematic in nosocomial environments (Allen & Hartman 2010) while the latter has been linked to toxic shock syndrome in previously healthy individuals (CDC 2010). The presence of a *Rickettsia* sp. in a sample potentially represents a true zoonotic agent, as members of the genus *Rickettsia* are vector-borne and responsible for typhus epidemics, murine typhus and rickettsial pox (Azad & Beard 1998; Raoult 2010). The detection of *Brochothrix thermosphacta* is particularly interesting as this was the only bacterium found in our study to be associated with food spoilage as this organism is principally responsible for the spoilage of meat (Skovgaard 1985). All bacteria identified in the study were extracted

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from kidney samples and have the potential to be spread through rat urine and therefore pose a risk for environmental contamination.

Bacterial prevalence among host species is important from an epidemiological point of view (Mills & Childs 1998). These results suggest uneven infection prevalence among *Rattus* hosts which is not comparable to previous studies as these used fewer *Rattus* host species, different detection methods and focused on different pathogens (Gratz 1999; Taylor *et al*. 2008; Mostert 2009). The low bacterial infection prevalence of *R. tanezumi* as compared to the two congeners cannot be accounted for by the data at hand. *Rattus tanezumi* and *R. rattus* do not share similar infection prevalence and yet the two congeners are cryptic and considered to form a species complex (Aplin *et al.* 2011; Bastos *et al.* 2011). This may be due to regional differences as the two species did not overlap in geographical locality within this particular subset of samples.

It cannot be overlooked that with such a high bacterial prevalence (49 %, $n = 84$), very few bacterial samples could generate sizeable sequence fragments to be used in phylogenetic analyses and thereby be taxonomically identifiable. In addition, the different phylogenetic analyses were not in agreement as the ML analysis grouped the *Bartonella* and *Rickettsia* genera within the same clade whereas the NJ and BI analyses placed them in separate clades. This inconsistency is possible as the particular analyses may have not found sufficient variability in the partial 16S rRNA gene regions of the two genera analysed as the fragment size should ideally be over 1Kbp long (Amann *et al.* 1995). Moreover, these two genera are very closely related and were once erroneously classified within the same bacterial order (Drancourt & Raoult 1994). Nevertheless, this emphasises the need to use multiple and diverse gene regions such as outer membrane protein genes and citrate synthase genes along with various phylogenetic analyses in bacterial taxonomic studies.

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Furthermore, the high incidence (31 %) of non-specific amplification and mixed bacterial infections / bacterial-host co-amplification hampered the identification of some of the bacteria present in the study and has been reported previously (Rantakokko-Jalava 2000). This is an acknowledged drawback of the 16S rRNA broad range bacterial primer approach (Clarridge 2004). Alternative methods include traditional culturing or genus-specific primer sets targeting the 16S rRNA gene region. However, the traditional use of culture methods is not without its own drawbacks as complex biochemical needs renders culturing methods insensitive to target species (Pickup 1991) and the use of genus-specific primer sets would be expensive.

Although no known zoonotic pathogens were identified from the samples, the diverse bacterial genera found have the potential to be of zoonotic interest. In addition, a species implicated in food spoilage were detected. While the confirmation of these bacteria in kidney samples indicates that these organisms are potentially spread *via* the urinary route and are possibly transmitted to humans through environmental contamination, the presence of vector-borne *Rickettsia* and *Bartonella* also implicates *Rattus* as possible reservoir hosts for vector-borne infections. Furthermore, bacterial prevalence seems to differ among host species for unknown reasons and should be further explored as this may be important from a management perspective. The use of the 16S rRNA broad range bacterial primer approach in isolation was not optimal and it is likely the bacterial community found is a great under-representation of the true bacterial diversity present in kidney samples. It is therefore recommended that this genetic approach be used in conjunction with culturing methods to obtain a more complete account of the bacterial community present in the kidneys of these *Rattus* hosts. Nevertheless, the study highlights the importance of continuous rodent control and disease surveillance among commensal rodents especially in informal settlements where conditions are conducive to disease outbreaks.

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Chapter 4

Prevalence and diversity of *Streptobacillus moniliformis* in invasive, commensal *Rattus* from South Africa

ABSTRACT

Streptobacillus moniliformis is one of the causative agents of rat-bite fever, a rare zoonotic infection that is most likely under-reported and misdiagnosed. This bacterial pathogen is considered to be part of the natural flora in the nasopharynx of rats which are potential reservoir hosts, as infected individuals often appear healthy and asymptomatic. A large number of South Africans reside in informal settlements with conditions conducive to rodent infestation, and therefore it is crucial to assess the prevalence of *S. moniliformis* in such settlements where there is also a high prevalence of immune-compromised individuals through the HIV/AIDS pandemic. Consequently, 106 oral swabs and 63 kidney tissue samples were obtained from *Rattus* specimens from urban formal and informal residential areas, industrial areas, office buildings and semi-urban small holdings in Gauteng Province, South Africa. Samples were screened for *S. moniliformis* genome presence by PCR using genus-specific PCR primers and amplified products were purified and sequenced. A prevalence of 54/106 (50.94 %) was obtained in oral swabs while in kidney tissue this was only 4/63 (6.35 %). Overall, *R. tanezumi* had the lowest oral infection prevalence (32.26 %), followed by *R. norvegicus* (55.56 %), with *R. rattus* (61.54 %) having the highest infection prevalence. Sequencing of *S. moniliformis*-positive samples and subsequent phylogenetic analyses revealed two *S. moniliformis* strains specific to the three matrilineal *Rattus* hosts present in South Africa. The high prevalence of *S. moniliformis* coupled with the high abundance of commensal, wild rats is a public health concern. Differences in infection prevalence among *Rattus* host species warrants further investigation and may be important in

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rodent pest management practices. Similarly, the apparent host specificity in *S. moniliformis* strains requires further investigation as it may be important in disease epidemiology.

KEYWORDS: bacteria, informal settlements, phylogeny, public health, rat-bite fever

INTRODUCTION

Streptobacillus moniliformis is a non-motile, non-sporulating, non-encapsulated Gram-negative rod (Gaastra *et al.* 2009). Variation in colony morphology exists and *S. moniliformis* typically forms either the normal bacillary form or the cell wall deficient L-form which is regarded as non-pathogenic (Wullenweber 1995; Elliot 2007). However, the bacterium appears to be less pleiomorphic when observed directly from animal hosts than when in culture (Gaastra *et al.* 2009). With a genome size of about 1.8 Mbp, *S. moniliformis* was previously thought to be part of the Mycoplasmatales (Gaastra *et al.* 2009) but with use of genetic analyses it was later classified within the family Leptotrichiaceae of the phylum Fusobacteria (Nolan *et al.* 2009).

Streptobacillus moniliformis is a bacterial pathogen that is considered to be part of the natural flora in the nasopharynx of rats (Strangeways 1933; Wullenweber 1995) which are potential reservoir hosts, as infected individuals often appear healthy and asymptomatic (Elliot 2007). Infection prevalence for wild rats ranges from 50-100 % and from 10-100 % for laboratory and pet-bred rats, respectively (Elliot 2007). Infection with *S. moniliformis* is possible through direct and indirect contact *via* a rat bite, scratch or through contact with any excreta (e.g. saliva, urine, faeces) (Elliot 2007; Gaastra *et al.* 2009). More importantly, *S. moniliformis* is one of the causative agents of the rare zoonotic infection rat-bite fever. Clinically resembling rat-bite fever, Haverhill fever is also associated with *S. moniliformis* but is distinguished from rat-bite fever by its specific association with the oral ingestion of the bacterium (Gaastra *et al.* 2009). Due to non-specific presentation of rat-bite fever, the

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disease is most likely under-reported (Gaastra *et al.* 2009) and misdiagnosed (Freels & Elliot 2004). In the past, the disease was thought to be restricted to laboratory personnel in frequent contact with rodents but has also long been recognised as a disease affecting children of low socio-economic status (Wullenweber 1995; Hirschorn & Hodge 1999; Freels & Elliot 2004).

In South Africa, the rat population in cities have drawn growing media attention over recent years and is indicative of increasing rodent-human conflict as exemplified by the following prominent South African newspaper headlines: "*Joburg health dept to clean up rat scourge*", *The Citizen*, April 2011; "*Cape Town residents live in fear of rats*", *SAPA,* March 2013; "*Rats wreak havoc in Pretoria*", *Pretoria News*, March 2013; "*All they need in Alexandra township is the Pied Piper of Hamelin*", *The Sowetan*, April 2013). A large number of South Africans reside in informal settlements characterised by overcrowding, poor housing structure, poor living conditions with low lighting and lack of basic furnishing needs, as well as inefficient waste removal services (Wekesa *et al.* 2011). These conditions are conducive to rodent breeding, resulting in rodent populations attaining high abundances which makes the assessment of *S. moniliformis* prevalence critical in such settlements where there is also high incidence of immune-compromised individuals due to the HIV/AIDS pandemic (Richards *et al.* 2007).

Streptobacillus moniliformis has traditionally been detected through tissue culture with the use of human blood and cerebrospinal fluid (CSF) (Graves & Janda 2001; Irvine & Wills 2006; Gaastra *et al.* 2009). This detection method however, delays appropriate treatment, as the bacterium is characterised by slow growth with fastidious growth requirements (Hagelskjaer *et al.* 1998; Irvine & Wills 2006; Gaastra *et al.* 2009). Serological methods have also been employed to detect the bacterium in rodents but have not yet been standardised for detection in humans (Gaastra *et al.* 2009). Consequently, molecular detection by PCR is now

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commonly used as it is rapid, more sensitive and has improved accuracy in identification (van Nood & Peters 2005; Kimura *et al.* 2008; Gaastra *et al.* 2009). Fortunately, rat-bite fever due to *S. moniliformis* infection is relatively easy to treat if accurately diagnosed (Wullenweber 1995; Gaastra *et al.* 2009). When left untreated, however, it may be fatal and mortality rates range from 7-10% (Elliot 2007).

Prior to 2000, information on *S. moniliformis* infection was limited to individual case reports despite the etiological agent having been discovered nearly 100 years earlier (Elliot 2007). Furthermore, the incidence of rat-bite fever on the African continent seems poorly-studied and limited to rat-bite fever caused by *Spirillum minus* (Heisch 1950; Bhatt & Mirza 1992). This study therefore investigated the prevalence and diversity of *S. moniliformis* in commensal, invasive *Rattus* in South Africa, and has implications for rodent pest management practises and the control of rodent-associated zoonotic diseases in South Africa and beyond.

MATERIALS & METHODS

Sample collection

Rats were sampled, processed and identified to matrilineal species as described in Chapter 2. Only the 107 samples collected during the 2010-2011 sampling period in Gauteng Province were used in this study.

Laboratory procedures

Genomic DNA was extracted from kidney tissue (n = 63) and oral swabs (n = 106) using the Roche High Pure PCR Template Preparation Kit (Roche Diagnostics) according to the manufacturer's protocol and stored at -20º C. Oral swabs and kidney extracts were tested for the presence of *S. moniliformis* using a diagnostic primer set S5-ATACTCGGAATAAGATGG and AS2-GCTTAGCTCCTCTTTGTAC amplifying a 269 bp

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fragment of the 16S gene region (Kimura *et al*. 2008). The PCR mixture contained 10x buffer (Biotools B&M Labs), 0.25 µM dNTP's (Fermentas), 0.4 µM of each primer, 1U Taq Polymerase (Biotools B&M Labs) and 100-200 ng DNA template in a 50 µl reaction volume. Amplification was achieved using a touchdown PCR with initial denaturation at 96º C for 10s followed by three discrete thermal cycles each with denaturation at 96º C for 12 s, annealing for 30 s, 25 s and 20 s, respectively and extension at 70º C for 40 s, 35 s and 30 s, respectively and annealing temperatures of 58º C for two cycles, 56.5º C for three cycles and lastly 55º C for 35 cycles with a final elongation at 70º C for 1 minute.

In addition to determining the prevalence *S. moniliformis*, the diversity of *S. moniliformis* in *Rattus* hosts, were also assessed. Oral swab extracts found positive in the first PCR round were then screened with a combination of the diagnostic primers AS2 and S5 of Kimura *et al.* (2008) and the broad range universal primer set, 27F- AGAGTTTGATCCTGGCTCAG and 1522R-AAGGAGGTGATCCAGCCGCA (Hauben *et al*. 1997). The 27F/AS2 primer combination yielded a 1206 bp fragment characterising the16S gene region. Amplification was achieved through another touchdown PCR with the following reaction conditions: initial denaturation at 96º C for 10 s followed by three discrete thermal cycles of denaturation at 96º C for 12 s, annealing at 59º C, 58º C and 57º C for 30 s, 25 s and 20 s, respectively followed by extension at 70º C for 1 minute 40 s, 1 minute 35 s and 1 minute 30 s each and final elongation at 70º C for 1 minute 30 s. The S5/1522R primer combination yielded a fragment of 539 bp through amplification by a touchdown PCR with similar reaction conditions; initial denaturation at 96º C for 10 s with three discrete thermal cycles of 96º C for 12 s, annealing at 56º C for 30 s, 55º C for 25 s and 54º C for 20 s followed by extension at 70º C for 1 minute 40 s, 1 minute 35 s and 1 minute 30s, respectively and final elongation at 70º C for 1 minute 30 s. Each PCR run included a positive and negative control and all positive samples were visualized by 1.5 % agarose gel electrophoresis.

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Phylogenetic and statistical analyses

Amplified PCR products were purified using the Roche PCR Product Purification Kit (Roche Diagnostics) and cycle sequenced using BigDye v. 3.1 terminator cycle-sequencing kit (Perkim-Elmer, Foster City, USA). Samples were run on an ABI 3130 sequencer and sequence chromatograms were viewed and edited in Mega 5 (Tamura *et al.* 2011) prior to performing a BLAST nucleotide database search (www.ncbi.nm.nih.gov/blast) to identify the species with the highest sequence similarity (98-100 %) in Genbank database. The data generated in this study were complemented with reference sequences from the Genbank database that were reported in Kimura *et al.* (2008) (Table 1).

Table 1 Partial 16S rRNA *Streptobacillus moniliformis* strains in Genbank database. Apart from the type strain these were recovered from *Rattus norvegicus* and *R. rattus* rodent host species.

Accession no.	Host	Reference
AB330754	*R. norvegicus*	Kimura *et al.* 2008
AB330757	*R. norvegicus*	Kimura *et al.* 2008
AB330758	*R. rattus*	Kimura *et al.* 2008
AB330760	*R. rattus*	Kimura *et al.* 2008
Z35305.1	-	ATCC 14647 Type Strain (unpublished)

The Tamura-Nei model (TNef) with uniform rates was selected as best-fit model of sequence evolution using the Akaike Information Criterion (AIC_C) in jModeltest (Posada 2008). Phylogenies were inferred using Maximum Likelihood (ML) and Neighbour-Joining (NJ) with nodal support assessed by 10, 000 non-parametric bootstrap replications in Mega 5 (Tamura *et al.* 2011). Bayesian inference (BI) was performed with MrBayes v.2.1.3 (Huelsenbeck & Ronquist 2001) and run over 10,000,000 generations. Tracer plots were viewed in Tracer v.1.5 (Rambau & Drummond 2009) where-after 25 % of trees were discarded as burn-in.

Initial analyses of variance of *S. moniliformis* prevalence between host species were performed with a one-way ANOVA followed by a Tukey's *post hoc* analyses to identify non-

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significant differences between species. Subsequently, a two-way ANOVA between sexes within each host species were performed. The influence of land-use site and sexes on *S. moniliformis* prevalence between host species was also investigated by use of a two-way ANOVA. Unfortunately, these data were only available for *R. rattus* and *R. tanezumi* as differentiation in land-use sites were not available for *R. norvegicus*. Land-use areas were categorised as urban areas when densely populated with buildings with scattered ornamental gardens while semi-urban areas were defined by areas with vast open landscapes of natural vegetation and sparsely populated by buildings. Seasonal data were not available due to the opportunistic nature of sample collection.

To estimate the proportional contribution of each variable to the observed differences in prevalence of each respective host, was undertaken through the partitioning of sum of squares (SSQ; Leamy 1984) of each source of variation obtained from a two-way ANOVA table. Percent SSQ of the four potential sources of variation in the data, namely, sex, land-use site, sex × land-use site interaction, and error (= residual) were obtained by dividing the SSQ associated with each source of variation by the total SSQ. All statistical analyses were performed in STATISTICA v.10 (StatSoft Inc. Oklahoma, USA).

RESULTS

The 107 rats examined in this study comprised 36 *R. norvegicus* (33.64 %), 40 *R. rattus* (37.38 %) and 31 *R. tanezumi* (28.97 %). Of the 106 oral swabs tested, 54 were positive for *S. moniliformis* DNA while only four of the 63 kidney samples tested positive. This corresponds to a molecular prevalence of 50.94 % and 6.35 % in oral swabs and kidney tissue, respectively. Of the four positive kidney tissue samples, two samples were from *R. rattus* (n = 36; 5.56 %), two from *R. norvegicus* (n = 16; 12.5 %) and none from *R. tanezumi* (n = 11; 0%). Infection prevalence in oral swabs among the *Rattus* hosts (Figure 1) were significantly

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different ($F_{(2,103)}$ = 3.30; n = 106; P = 0.04). *Post hoc* analyses revealed that *R. norvegicus* (55.56 %) and *R. rattus* (61.54 %) have similar infection prevalence while *R. tanezumi* (32.26 %) has significantly lower infection prevalence from *R. rattus* but not *R. norvegicus*.

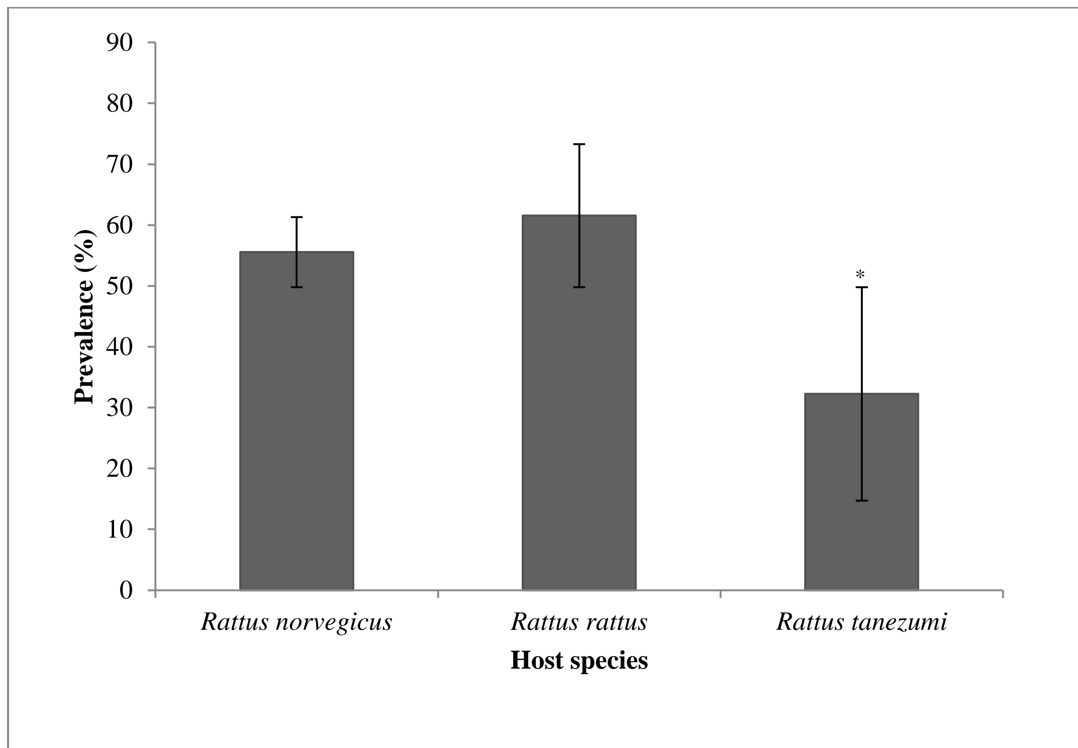

Figure 1 *Streptobacillus moniliformis* oral infection prevalence among *Rattus* host species (* indicates $P < 0.005$).

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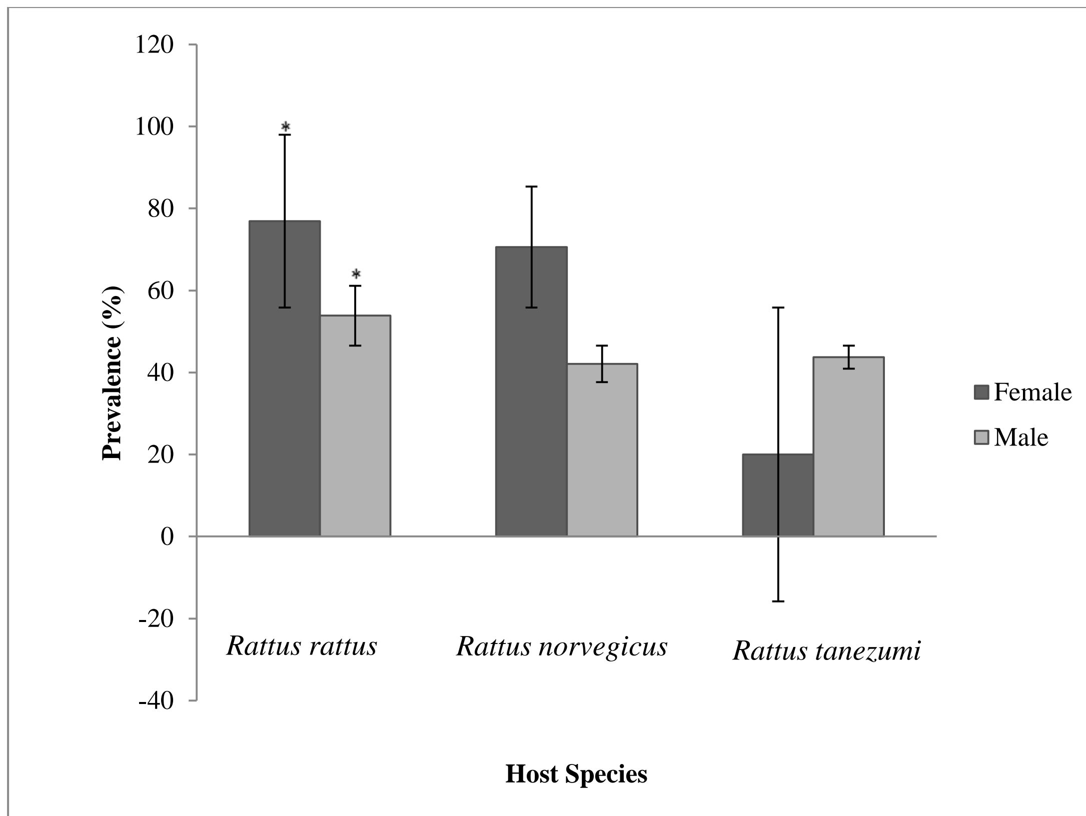

Figure 2 Gender prevalence for *Streptobacillus moniliformis* infection among the three *Rattus* host species

(* indicates $P < 0.005$).

Unlike *R. rattus* and *R. norvegicus* which showed higher infection prevalence in females, *R. tanezumi* showed higher infection prevalence in males (Figure 2) although this was not statistically significant ($F_{(3,100)} = 2.33$; $n = 31$; $P = 0.08$) while *post hoc* analyses revealed that only *R. rattus* have significant differences in infection prevalence between the two sexes. In addition, a comparison of infection prevalence between land-use sites within *R. rattus* and *R. tanezumi* revealed that semi-urban areas showed higher infection prevalence over urban areas and was statistically significant for only *R. rattus* ($F_{(1, 37)} = 5.16$; $n = 40$; $P = 0.003$). A two-way ANOVA showed that both sex and site had a significant effect in *R. rattus* at $P <$ 0.05 (Table 2). This is also reflected in the subsequent assessment of the sources of the variation % SSQs) (Table 2) where *R. rattus* showed relatively high %SSQs for both sex (% SSQ = 10.81 %) and site (% SSQ = 11.25 %) compared to those in *R. tanezumi* (Sex: % SSQ

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= 7.70 %); Site: % SSQ = 8.54 %). Both ANOVA and % SSQ values revealed that the interaction between sex and site had no effect on infection prevalence in the two host species. Both analyses however revealed that the main effect of infection prevalence in *R. rattus* and *R. tanezumi* is due to error (= residual) (*R. rattus*: % SSQ = 76.34 %; *R. tanezumi*: % SSQ = 83.74 %) suggesting that other factors rather than sex, site or the interaction between the two are affecting the infection prevalence in the two host species. Overall, these results support the results of earlier analyses which revealed the relatively low infection prevalence in *R. tanezumi* compared to *R. rattus* and *R. norvegicus*.

Table 2 *F*-value and percentage sum of squares (% SSQ) of four potential sources of variation in *Streptobacillus moniliformis* oral infection prevalence for two host species namely *Rattus rattus* and *R. tanezumi* derived from a two-way analysis of variance (ANOVA) (* indicates $P < 0.005$).

	***F*-value**			**% SSQ**			
Host species	Sex	Site	Sex × Site	Sex	Site	Sex × Site	Error
R. rattus	4.96*	5.16*	0.74	10.81	11.25	1.60	76.34
R. tanezumi	2.48	2.75	0.01	7.70	8.54	0.02	83.74
Mean ($\bar{x}$)				9.26	9.90	0.81	80.04

Sequences generated by the diagnostic/universal primer combination were augmented with reference sequences and yielded a 1224 bp long homologous dataset with 19 taxa. The dataset contained 24 variable sites and 10 parsimony-informative sites. The average base pair composition was T = 22.4 %, C = 18. 7%, A = 29.8 %, G = 29.1 %, indicating an AT bias (52.2 %). The transition: transversion ratio (R) was 1.24. All the analyses revealed the same tree topology and consequently the ML tree (Figure 3) is illustrated with nodal support and posterior probabilities (albeit low) from the NJ and BI analyses, respectively indicated on the tree. *Streptobacillus moniliformis* strains isolated from *R. norvegicus* were genetically different from those isolated from *R. rattus* and *R. tanezumi* (of the *R. rattus* complex), forming a separate clade. However, there is no difference in *S. moniliformis* strains between *R. rattus* and *R. tanezumi* which together, forms one clade.

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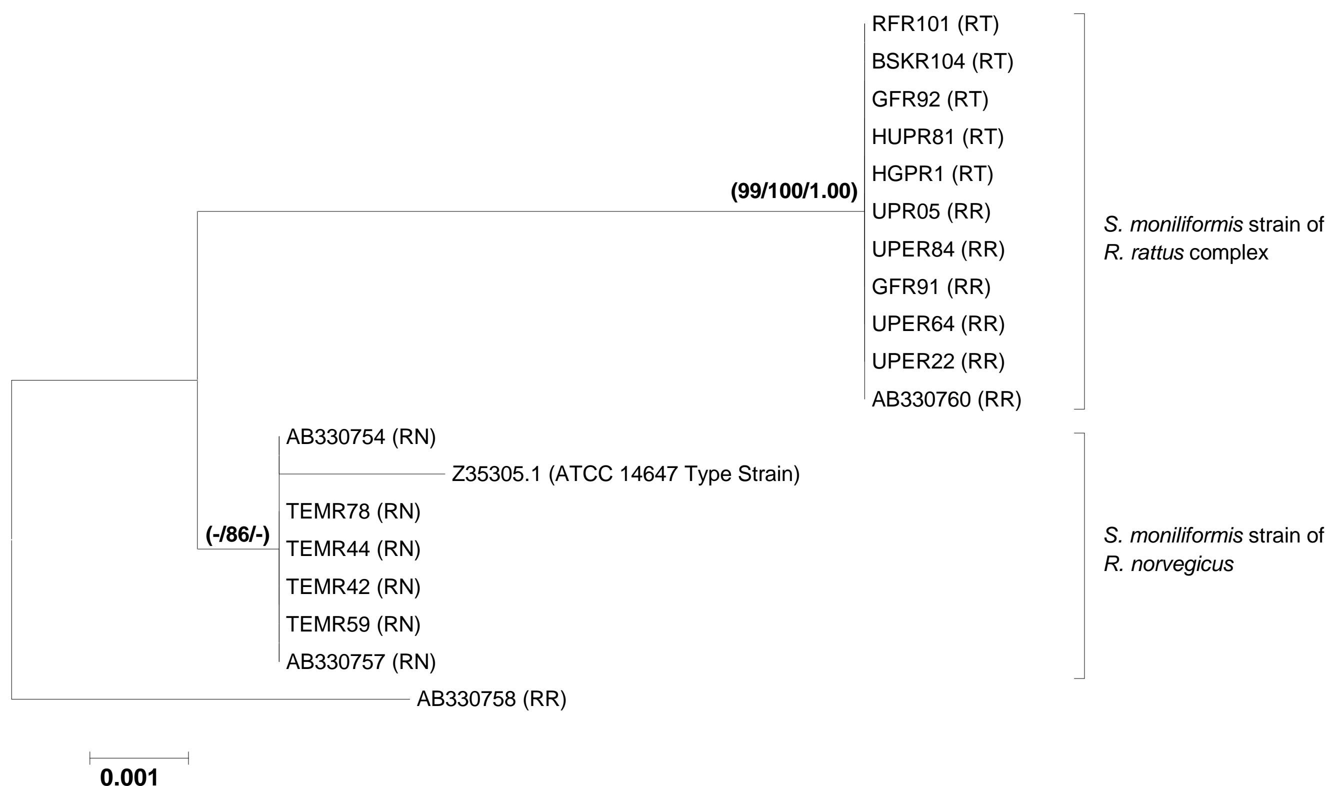

Figure 3 Maximum Likelihood (ML) tree displaying the phylogeny of *Streptobacillus moniliformis* strains isolated from *Rattus rattus* complex and *R. norvegicus* from South Africa. Bootstrap support and posterior probabilities for ML, Neighbour-Joining (NJ) and Bayesian Inference (BI) analyses (in that order) is included in parentheses above the node. Taxon names include either sample codes or Genbank accession numbers where applicable along with *Rattus* host species (abbreviated) from which it was isolated.

DISCUSSION

The low *S. moniliformis* prevalence in *Rattus* kidney tissue suggests that excretion of the bacteria in the urine is of lesser importance. Therefore, rat urine/faeces pose a low risk of transmitting Haverhill fever. In contrast, the results suggest that one out of two wild rats carry *S. moniliformis* in the oral cavity with possible transmission *via* a bite. This is alarming in the South African setting where frequent rat bites occur in both urban and rural areas (Kirsten & von Maltitz 2005 and are increasingly reported in the media such as “*Ma vort, laat kinders*

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vol rotbytmerke " ("*Mother gone, leaving children covered with rat-bite marks*"), the *Beeld*, May 2010; "*Soweto baby bitten to death by rats*", the *Citizen*, June 2011; "*Helen Zille tweets about rat-bite*", *News24*, March 2013 and "*Rats, That's Not Funny - Khayelitsha Child Bitten By Rat*", *GroundUp*, March 2013. These reports indicate that rat-bite incidence is on the increase and places the public at risk of contracting rat-bite fever. The increasing popularity of rats as pets may be an additional source of infection and there are several reports of pet owners and pet shop employees contracting rat-bite fever (Andre *et al.* 2005; van Nood & Peters 2005; Freels & Elliot 2007). Rat-bite fever is also a systemic disease (van Nood & Peters 2005) and susceptible people such as immune-compromised individuals, children, the elderly and those suffering from HIV/AIDS may develop complications including polyarthritis, endocarditis and pneumonia (Hagelskjaer *et al.* 1998; Stehle *et al.* 2003; Thong & Barkham 2003; Chean *et al.* 2012).

Amongst the three *Rattus* species in South Africa, *R. rattus* and *R. norvegicus* were found to have similar infection prevalence. A study by Kimura *et al.* (2008) also assessed *S. moniliformis* prevalence amongst these two *Rattus* host species but unlike this study, found *R. norvegicus* to have a higher infection prevalence compared to *R. rattus*. Of particular interest, is the significantly lower infection prevalence obtained for matrilineally-defined *R. tanezumi* individuals compared to *R. rattus*. *Rattus tanezumi* species was recently discovered in South Africa (Bastos *et al.* 2011) and little is known about its ecology. In addition, phylogenetic analyses revealed the presence of *Rattus* host-specific strains of *S. moniliformis*. Strains isolated from *R. norvegicus* form a distinct clade, while strains isolated from *R. rattus* and *R. tanezumi*, cluster together with no distinct differences in sequences. While these findings would need to be confirmed by sequencing other gene regions, it is possibly related to these latter two host species being considered to be part of a species complex (Aplin *et al.* 2011) with some degree of genome introgression in areas where they occur sympatrically (Lack *et*

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al. 2012). Consequently, because morphologically similar *R. rattus* and *R. tanezumi* (Musser & Carleton 2005; Mostert 2009; Bastos *et al.* 2011) showed no difference in *S. moniliformis* strains, the significantly lower infection prevalence of *R. tanezumi* may reflect possible microhabitat and behavioural differences. An investigation into different land-use areas for the two species however, revealed that although semi-urban areas have higher infection prevalence, the differences in infection prevalence between these two host species is due to unknown factors as revealed from the very high mean % SSQs error (= residual). Similarly, sex differences within each *Rattus* host species could also not account for the differences in infection prevalence as the source of variation was largely due to the error (= residual) component.

This study presents the first detection of *S. moniliformis* in *Rattus* in southern Africa although previous detection in patients may not be discounted. However, rat-bite fever is likely to be underreported and frequently misdiagnosed as the disease is not a reportable disease and shows non-specific presentation (Elliot 2007; Gaastra *et al.* 2009). Nevertheless, the high prevalence of *S. moniliformis* in wild rats along with the high abundance of these rats poses a definitive public health threat. Although, reasons for the observed differences in infection prevalence between *Rattus* host species could not be accounted for by the data at hand, the presence of host-specific *S. moniliformis* strains was confirmed. Therefore, results of this study have important implications in disease epidemiology and rodent pest management practices in South Africa and beyond.

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Chapter 5

Rattus species as reservoir hosts of vector-borne *Rickettsia* in South Africa

ABSTRACT

The genus *Rickettsia* has a number of pathogenic members including the recently discovered *R. felis*. Prevalence of these vector-borne bacteria has been well-documented in arthropods but less so in mammalian hosts. In addition, where prevalence has been reported in mammalian hosts, it has primarily been detected using serological methods, which were favoured in the past. The present study assessed the prevalence of *Rickettsia* in invasive *Rattus* hosts (*R. norvegicus*, *R. rattus* and *R. tanezumi*) from South Africa using a molecular approach. Rickettsial DNA was detected in just one sample of *R. tanezumi* (0.93%) out of 108 rodent samples screened with an *ompB* gene assay. Nucleotide sequencing and phylogenetic analysis of the *ompB* gene identified the bacterial species to be *R. felis*. The species discovered together with the low prevalence suggests that these invasive rodents may only be incidental hosts of *Rickettsia*, and that their associated ectoparasites possibly play a more prominent role in the maintenance and transmission of rickettsioses.

KEYWORDS: arthropods, bacteria, PCR, prevalence, public health, rodents

INTRODUCTION

Rickettsia is a bacterial genus of the phylum Proteobacteria and is closely related to *Bartonella*. The genus contains a number of pathogenic members such as *R. prowasekii* (epidemic typhus), *R. typhi* (murine typhus), *R. akari* (rickettsial pox) and various spotted fever group members such as *R. rickettsii*, *R. conorii* and *R. africae*. Furthermore, new members are continuously being discovered of which some are pathogenic. Members of this bacterial genus are therefore often regarded as agents of emerging zoonoses (Parola *et al.*

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2005; Perez-Arellano *et al.* 2005). As different *Rickettsia* are transmitted by an assortment of hosts such as fleas, ticks, mites and lice, the diversity of *Rickettsia* within a geographical location is determined in part by regional arthropod host distribution and diversity (Kelly *et al.* 2002; Schex *et al.* 2011). For this reason, few to several *Rickettsia* spp. may be endemic to a particular area (see Table 1 for present or potentially present species of *Rickettsia* in South Africa) and each may cause different rickettsioses. Based on phylogenetic analyses and serology, two groups are characterised within the genus, namely the spotted fever group (SFG) and the typhus group (TG).

The prevalence and diversity of *Rickettsia* in *Rattus* and their associated ectoparasites has rarely been assessed (Christou *et al.* 2010; Abramowicz *et al*. 2011). In contrast, several studies have investigated *Rickettsia* presence in ectoparasites in general, while a few studies have investigated those associated with indigenous rodents in southern Africa (Gear 1954) and elsewhere (Christou *et al.* 2010; Schorn *et al*. 2011). Consequently, the reported prevalence varies greatly with respect to rodent host, arthropod vector, *Rickettsia* spp. and methods used for detection (see Table 2).

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Table 1 *Rickettsia* spp. present or potentially present in South Africa as the species were either isolated from human cases who reside or travelled to South Africa* or are associated with arthropod vectors in the country# or associated with cosmopolitan mammalian hosts$ where SFG = spotted fever group and TG = typhus group.

***Rickettsia* spp.**	**Disease/Group**	**Vector**	**Host**	**Reference**
*R. aeschlimannii**#	SFG	Ticks: *Hyalomma marginatum, H. m. rufipes, Rhipicephalus appendiculatus*	Cattle	Pretorius & Birtles 2002; Parola 2006; Fernandez-Soto *et al.* 2009
*R. africae**#	African tick bite fever/SFG	Ticks: *Amblyomma variegatum*, *A. hebraeum*	Cattle, ungulates	La Scola & Raoult 1997; Kelly *et al*. 2002; Jensenius *et al*. 2003; Pretorius & Birtles 2004; Parola *et al*. 2005
R. akari$	Rickettsial pox/SFG	House mouse mites: *Liponyssoides sanguineus*	*Mus musculus*, rats	Azad & Beard 1998; Comer *et al*. 2001; Kelly *et al*. 2002; Zavala-Castro *et al*. 2009
*R. conorii**#$	Mediterranean spotted fever /SFG	Ticks: *Rhipicephalus sanguineus*, *R. pumillo, Haemaphysalis* spp.	Rodents, Dogs	La Scola & Raoult 1997; Azad & Beard 1998; Kelly *et al.* 2002; Parola *et al.* 2005
R. felis#$	Flea-borne spotted fever/SFG	Fleas: *Ctenocephilides felis*, *Xenopsylla cheopsis, Leptopsylla segnis* Ticks: *Haemaphysalis flava*, *Rhipicephalus sanguineus*, *Ixodes ovatus*, and *Carios capensis*	*Rattus norvegicus*, opossums	La Scola & Raoult 1997; Azad & Beard 1998; Comer *et al.* 2001; Christou *et al.* 2010; Abramowicsz *et al.* 2011; Parola 2011
*R. mongolotimonae**	SFG	Ticks: *Hyalomma asiaticum*, *H. truncatum*	Cattle	Pretorius & Birtles 2004
R. typhi$	Murine typhus/TG	Fleas: *Xenopsylla cheopsis* and others	Rats, opossums, other rodents	La Scola & Raoult 1997; Azad & Beard 1998; Comer *et al.* 2001; Civen & Ngo 2008; Abramowicsz *et al.* 2011; Ko *et al.* 2011

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Table 2 The reported prevalence (%) of *Rickettsia* spp. in rodents and arthropods as detected by PCR and/or serology that have caused or have the potential to cause human disease in South Africa.

***Rickettsia* spp.**	**Arthropods**	**Rodents (PCR/Serology)**	**Region**	**Reference**
R. aeschlimannii	1.9-19.6	-	Europe, South America, Africa	Parola 2006; Fernandez-Soto *et al.* 2009; Tomassone *et al.* 2010; Mutai *et al.* 2013
R. africae	1-70	-	Africa, Europe	Jensenius *et al.* 2003; Angelakis *et al.* 2012
R. akari	n/a	-/23	eastern Europe, North America	Eremeeva *et al.* 1995; Bennett *et al.* 2007
R. conorii	0	-/9.4-39.1	Asia, northern Africa, southern Africa	Gear 1954; Khaldi *et al.* 2012; Kuo *et al.* 2012
R. felis	0.9-100	0-100/-	North America, Europe, Asia, northern Africa	Tay *et al*. 2002; Christou *et al.* 2010; Pluta *et al.* 2010; Abramowicsz *et al.* 2011; Schex *et al.* 2011; Khaldi *et al.* 2012; Kuo *et al.* 2012
R. mongolotimonae	0.96	n/a	Africa	Mutai *et al*. 2013
R. typhi	4-50	75/0.1-21	Europe, Asia, North America	Christou *et al.* 2010; Abramowicsz *et al.* 2011, Kuo *et al.* 2012

Rickettsia africae is considered to be endemic to sub-Saharan Africa and is responsible for African tick-bite fever (Jensenius *et al.* 2003; Parola *et al*. 2005) which is transmitted by ticks of the genus *Amblyomma* that are most commonly found on ungulates (Jensenius *et al.* 2003; Pretorius *et al.* 2004). African tick bite fever has long been recognised but was confused with Mediterranean spotted fever that is caused by *R. conorii* (also present in Africa) until the etiological agent was successfully isolated (Jensenius *et al.* 2003). In contrast, *R. felis* (a flea-borne rickettsia) was not recognized as a human pathogen until 1994 (Perez-Arellano *et al.* 2005) and has since been implicated in disease across Europe, the Americas and recently in sub-Saharan Africa where it has a high prevalence (Parola 2011). In addition to *R. africae* and *R. conorii,* two more pathogenic rickettsia were isolated in patients in South Africa (Pretorius & Birtles 2002; Pretorius & Birtles 2004) namely, *R. mongolotimonae* and *R.*

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aeschlimannii. The former was first isolated in 1991 and confirmed to be pathogenic in 1996 while the latter was first detected in 1997 and its pathogenicity confirmed in 2002 (Parola 2006). Other rickettsia such as *R. typhi*, responsible for murine typhus and *R. akari*, the etiological agent of rickettsial pox, have a worldwide distribution and are particularly associated with ectoparasites of invasive, commensal rodents such as *R. rattus R. norvegicus* and *Mus musculus* (Azad & Beard 1998; Kelly *et al*. 2002; Bennett *et al.* 2007).

The duration of bacteraemia in a rodent host was experimentally assessed by Arango-Jaramillo *et al.* (1984), and found that rickettsia could not be detected from rodent organs (kidney, blood and brain) 4-5 weeks post-inoculation by culturing methods, yet using serological methods, the bacteraemia could be detected 60 weeks post-infection. This is clearly illustrated in Figure 1 (adopted from Richards 2012) where PCR analysis was sensitive enough to detect infection prior to the patient becoming symptomatic, with either fever or rash. Antibodies, because they are long-lived are detectable long after symptoms have subsided.

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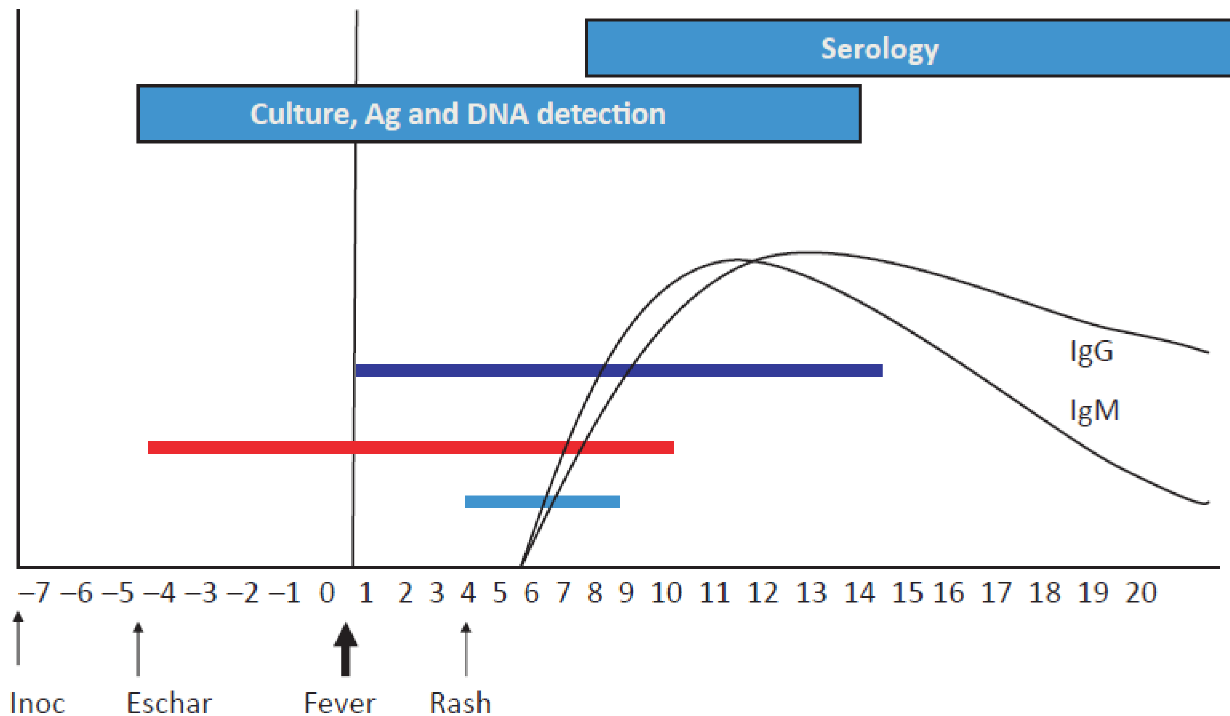

Figure 1 Bacteraemia in patients following *Rickettsia* spp. infection (adopted from Richards 2012). Red bar indicates duration of visible eschar at bite site, dark blue bar indicates the onset of fever and the duration of other symptoms associated with rickettsial infection, while the light blue bar indicates the appearance and duration of skin rash. By the 3rd and at most the 19th day post-inoculation, *Rickettsia* will be detectable by culture, antigens and PCR, yet only after the 14th day post-inoculation will *Rickettsia* be detectable by serology (antibodies).

Currently, there are no clear guidelines on the optimal organ(s) to use when screening for *Rickettsia* in vertebrate reservoir hosts. Abramowicz *et al.* (2011) represents one of a few studies which tested tissue tropism for *Rickettsia* (by PCR) and found the kidney, heart and brain to be most efficient, while blood tested negative. However, most studies use blood rather than organs to detect rickettsia but this is probably as a consequence of the traditional use of serology (Davis 1948; Arango-Jaramillo *et al.* 1984; Dupont *et al.* 1995). Recently, the use of PCR has become more widespread as it is relatively inexpensive, more specific and

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sensitive and does not require the same level of specialised facilities and expertise as in the case of serology (La Scola & Raoult 1997; Richards 2012). In light of this, the present study attempts to investigate the prevalence and diversity of *Rickettsia* spp. in *Rattus* hosts in South Africa by molecular screening of kidney samples for evidence of bacterial genome presence.

MATERIALS AND METHODS

Sample collection

Samples were collected, processed and *Rattus* specimens identified as described in Chapter 2. Samples collected in the 2003-2004 period which tested positive for *Rickettsia* with universal 16S primer sets (Chapter 3) were included in this study along with all the samples collected during 2010 and 2011.

Laboratory procedures

Genomic DNA was extracted from kidney tissue using the Roche High Pure PCR Template Preparation Kit (Roche Diagnostics) according to the manufacturer's protocol and stored at -20º C. Kidney extracts were tested for the presence of *Rickettsia* using two genus-specific primer sets. The primer set RpCS.877p-GGGGGCCTGCTCACGGCGG and RpCS.1258n-ATTGCAAAAAGTACAGTGAACA characterising a fragment of the citrate synthase (*gltA*) gene region (Regnery *et al*. 1991) as well as M59-CCGCAGGGTTGGTAACTGC and 807-CCTTTTAGATTACCGCCTAA (Roux & Raoult 2000) which characterises a fragment of the *ompB* gene region (which codes for the outer membrane protein) were used in this study. These primer sets yielded an amplicon of 381 bp and 833 bp, respectively. The PCR mixture contained 1X buffer (Fermentas), 5% Glycerol, 0.25 µM dNTP's (Fermentas), 0.4 µM of each primer, 1U *Taq* Polymerase (Fermentas) and 100-200 ng DNA template in a 50 µl reaction volume. Percentage GC content of both primer sets were high and necessitated the addition of glycerol to the PCR mixture to facilitate reactivity of the primers in the reaction.

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Bacterial genome presence was initially confirmed by amplification of the *gltA* target. This was achieved using a touchdown PCR in which initial denaturation was at 96º C for 10 s, followed by three thermal cycles of denaturation at 96º C for 12 s, annealing for 30 s and extension at 70º C for 35 s with an initial annealing at 51º C for two cycles, 49º C for 8 cycles and lastly 48º C for 32 cycles. Final extension was at 70º C for 1 minute.

In order to identify the *Rickettsia* to species level, the *gltA*-positive samples were screened with the second set of primers targeting the *ompB* gene region using the following thermal cycling reaction conditions: initial denaturation at 96º C for 10 s, followed by three discrete thermal cycles that consisted of denaturation at 96º C for 12 s, annealing for 20 s and extension at 70º C for 1 min 40 s, with an initial annealing at 58º C for two cycles, followed by eight cycles at 56º C and finally 32 cycles at 54º C. Each PCR run included a positive control which was identified in chapter 3 using universal 16S primers, and a negative control. Amplicons were visualized and identified by 1.5 % agarose gel electrophoresis against a size standard.

Phylogenetic analyses

All amplified PCR products were purified using the Roche PCR Product Purification Kit (Roche Diagnostics) and cycle sequenced using BigDye v. 3.1 terminator cycle-sequencing kit (Perkim-Elmer, Foster City, USA). Samples were run on an ABI 3130 genetic analyser and sequence chromatograms were viewed and edited in Mega 5 (Tamura *et al.* 2011) prior to performing a BLAST nucleotide database search (www.ncbi.nm.nih.gov/blast) to identify species with the highest sequence similarity (> 95%) in the Genbank database. The data generated in this study were complemented with reference sequences from this database (Table 3) that were reported in published peer-reviewed journals, where available. These

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reference sequences included all *Rickettsia* species present or potentially present in South Africa.

Table 3 Partial sequences characterising the *ompB* gene obtained from Genbank as reference sequences in the *ompB Rickettsia* phylogeny.

***Rickettsia* species**	**Vector/Host**	**Accession no.**	**Reference**
R. aeschlimannii	tick	GQ180863	Tomassone *et al.* 2010
R. africae	tick	AF123706	Roux & Raoult 2000
R. akari	human	AF123707	Roux & Raoult 2000
R. conorii	tick	AF123726	Roux & Raoult 2000
R. felis	rat	-	This study
R. felis	louse	GQ385243	Behar *et al.* 2010
R. felis	louse	GQ329879	Behar *et al*. 2010
R. felis	louse	GQ329875	Behar *et al.* 2010
R. felis	flea	AF210695	unpublished
R. felis	tick	DQ102711	Reeves *et al*. 2006
R. felis	flea	AY394854	Stevenson *et al.* 2005
R. felis	flea	AF182279	Moron *et al.* 2000
R. mongolotimonae	human	DQ423364	De Sousa *et al*. 2006
R. typhi	human	L04661	Roux & Raoult 2000

The three-parameter model (TPM3uf) with unequal base frequencies and rate variation among sites (G) was selected as the best-fit model of sequence evolution using the Akaike Information Criterion (AIC_C) in jModeltest (Posada 2008). Phylogenies were inferred using Maximum Likelihood (ML) and Neighbour-Joining (NJ) methods with nodal support assessed by 10, 000 non-parametric bootstrap replications in Mega 5 (Tamura *et al.* 2011). Bayesian Inference (BI) was performed with MrBayes v.2.1.3 (Huelsenbeck & Ronquist 2001) and run over 10,000,000 generations. Tracer plots were viewed in Tracer v.1.5 (Rambaut & Drummond 2009) where-after 25% of trees were discarded as burn-in.

RESULTS

A total of 108 kidney samples were included in this study of which the host composition was as follows: 36 *R. norvegicus* (33.33 %), 40 *R. rattus* (37.04 %) and 32 *R. tanezumi* (29. 63 %)

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individuals. The genus-specific primer set characterising the *gltA* gene region did not yield any *Rickettsia*-positive samples but instead resulted in non-specific amplification of incorrect target size. The species-specific primer set characterising the *ompB* gene region yielded only one *Rickettsia*-positive in a *R. tanezumi* specimen, along with several non-specific amplicons of incorrect target size in the other samples. Sequencing of the *ompB*-positive sample yielded a fragment 741 nucleotides in length which had 99 % sequence identity to a number of *Rickettsia felis* Genbank entries identified in ectoparasites (see Table 3 and Figure 2 for accession numbers). The final homologous dataset consisted of 14 taxa, 744 nucleotides in length, 107 parsimony-informative and 539 variable sites. The average base pair composition was T = 32 %, C = 17 %, A = 31.3%, G = 19.7 %, indicating an AT bias (63.3 %). The transition: transversion ratio (R) of 0.74 indicated a slight transversional bias.

All phylogenetic analyses revealed the same tree topology and consequently the NJ tree (Figure 2) is illustrated with nodal support and posterior probabilities from ML and BI, respectively transferred to the relevant nodes. The phylogeny shows that the *Rickettsia felis* strain recovered from the kidney tissue of an *R. tanezumi* specimen clustered with strains recovered from a range of ectoparasites including a tick species, *Carios capensis* (DQ102711) and a louse species *Liposcelis bostrychophila* (GQ385243, GQ329879, GQ329875) that are considered to be uncommon hosts of this particular *Rickettsia* species (Reeves *et al.* 2006; Behar *et al.* 2010).

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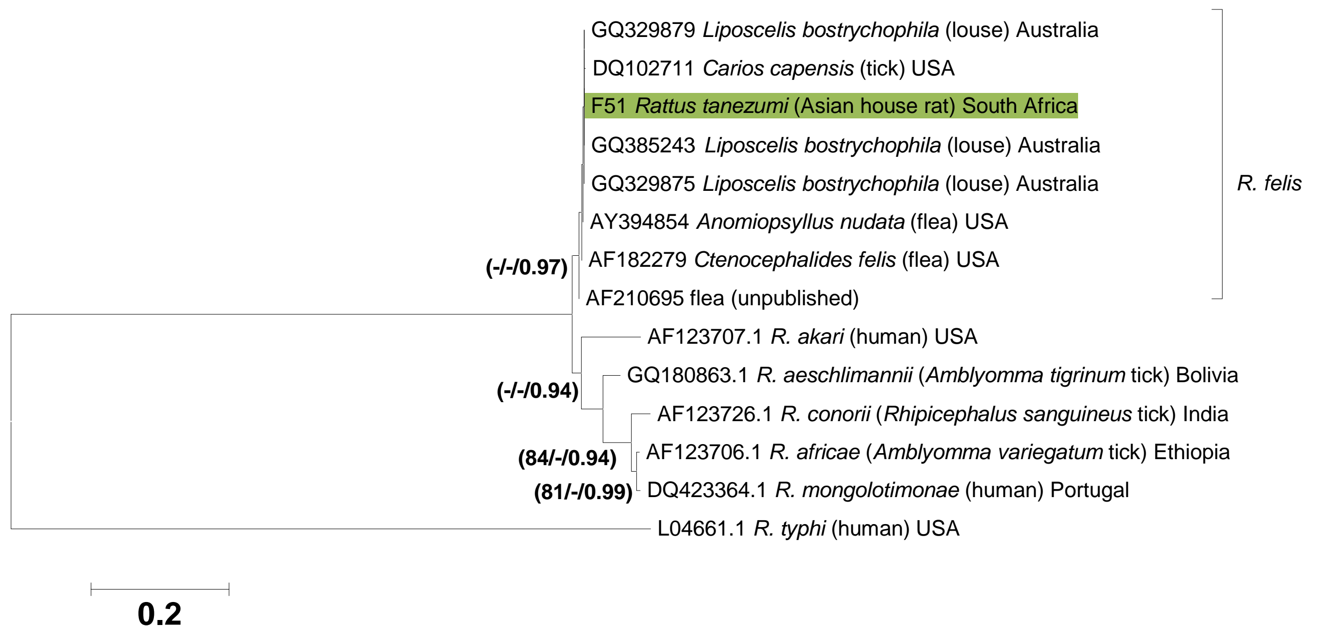

Figure 2 Neighbour-Joining (NJ) tree displaying *ompB* gene relationships of *Rickettsia* species present or potentially present in South Africa. Where available the taxon name is preceded by the Genbank accession number followed by either the host or vector from which it was recovered. Bootstrap values and posterior probabilities, greater than 75 % and 0.90 respectively, from the NJ, Maximum Likelihood (ML) and Bayesian Inference (BI) analyses are indicated in this order next to the relevant node.

DISCUSSION

The prevalence of *Rickettsia* in *Rattus* species from South Africa was found to be very low with only one of the 108 (0.9 %) *Rattus* samples tested, being positive. The method of detection along with the particular host species investigated may possibly account for this low prevalence, as tissue type has been shown to influence prevalence. A previous study by Abramowics *et al.* (2011) show that although the kidney, spleen, brain and heart tested positive for *R. felis,* the bacterium was most frequently isolated from the ear, whereas the liver, lung and blood tested negative. In addition, the detection by PCR in isolation may not

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be sufficient as isolates have been recovered from tissue by cultures that were negative by PCR (Angelakis *et al.* 2012). Furthermore, serology generally recovers higher *Rickettsia* prevalence (Ibrahim *et al.* 1999; Kuo *et al.* 2012) which is consistent with antibody response remaining detectable long after disease symptoms have diminished (Richards 2012).

An actual low prevalence of *Rickettsia* in the rodents used in this study may not be unusual. Other studies in which wild rodents were tested for the presence of *Rickettsia* by PCR were found to be as low as 0-7.6 % (Tay *et al.* 2002; Pluta *et al.* 2010; Shex *et al.* 2011). In one study where 100 % prevalence for *R. felis* and *R. typhi* were found, it should be mentioned that a sample size of only four animals was used (Abramowicz *et al.* 2011). This suggests that rodents and other mammals may be incidental hosts while their associated ectoparasites are the maintenance and reservoir hosts for *Rickettsia* (Parola *et al.* 2005, Perez-Osorio *et al.* 2008).

After 1994 recognition of *R. felis* as a zoonotic agent (Parola *et al.* 2005; Perez-Osorio *et al.* 2008), it became apparent that this rickettsial species is widespread. It has caused illness in humans from at least nine countries (USA, Mexico, Brazil, Germany, Thailand, South Korea, Tunisia, Laos and Spain) and occurs on five continents including Africa (Perez-Osorio *et al.* 2008). Since then, more human cases from Africa emerged with a reported 4.4 % and 3.7 % prevalence of *R. felis* in patients from Senegal and Kenya, respectively (Parola 2011). With regards to the vectors, *R. felis*-infected fleas have been detected in Ethiopia, Gabon, Ivory Coast and Morocco (Parola 2011) while Khaldi *et al.* (2012) recently found high infection prevalence (95 %) in fleas from hedgehogs in Algeria. Consequently, flea-borne spotted fever is predicted to be endemic in Africa and the relatively rare incidence of human infections is indicative of undetected and under-reported rickettsial disease (Kernif *et al.* 2012; Khaldi *et al.* 2012). Furthermore, *R. felis* has shown cross-reactivity with *R. typhi* infections during serological tests (Parola 2011) and was placed in the SFG group of

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Rickettsia distinct from *R. typhi* based on genetic analyses (Moron *et al.* 2000). The need for specialized laboratory testing complicates *R. felis* detection especially in Africa where such facilities are limited, resulting in a severe under-estimation of *R. felis*-associated rickettsial disease (Parola 2011; Khaldi *et al.* 2012).

The precise role of arthropod and mammalian hosts in rickettsial epidemiology remains unclear and warrants further investigation. Ideally, the associated ectoparasites of the rodent hosts should also be screened to refine the role of vector and reservoir host in rickettsial epidemiology. However, as encountered in the course of this study, rickettsial detection is dependent on various factors including duration of infectivity, tissue type and method of detection that needs to be considered for future studies. Nevertheless, *R. felis* infection is probably more common than previously thought and must be considered in urban areas as the arthropod hosts frequently infest domestic cats and dogs kept as pets, as well as urban pests such as rats (Kernif *et al.* 2012). Although, most flea-borne spotted fever infections present with fever and a maculo-papular rash (Brouqui *et al.* 2007; Kernif *et al*. 2012), particularly in Africa, these symptoms may be masked by co-infection with malaria amongst others (Parola 2006; Mutai *et al.* 2013). Fortunately, treatment with doxycycline is very effective against most rickettsiosis, and is readily available and relatively inexpensive (Kernif *et al.* 2012; Mutai *et al.* 2013). At present, mortality rates are unknown (Brouqui *et al.* 2007), but may be considerable in Africa, which is plagued by pandemics such as HIV/AIDS and malaria, as the disease is associated with complications involving meningitis and pneumonia (Parola 2011).

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Chapter 6

GENERAL DISCUSSION

In Chapter 2 the origin and diversity of the three matrilineal *Rattus* species known to occur in South Africa were investigated. Subsequent chapters (Chapter 3-5) investigated their role as potential zoonotic disease reservoirs in primarily urban environments with particular interest in their potential to transmit and spread zoonotic disease through direct contact as well as indirect contact through the urinary route.

Previous studies (Taylor *et al.* 2008; Aplin *et al.* 2011) reported the difficulty of species assignments to members of the *R. rattus* species complex. This is because *R. tanezumi* and *R. rattus* are cryptic (Taylor *et al.* 2008; Aplin *et al.* 2011; Bastos *et al.* 2011) and genome introgression in invaded areas where these species occur in sympatry have recently been reported (Lack *et al.* 2012; Conroy *et al.* 2013). Consequently, taxonomic resolution of the genus with regards to certain taxa remains elusive and the present study identified the invasive, commensal *Rattus* species occurring in southern Africa by means of sequencing of the mitochondrial gene (cytochrome *b*) and therefore assigned only matrilineal species names to *R. rattus*, *R. tanezumi* and *R. norvegicus*, respectively.

Genetic diversity of *Rattus* in southern Africa has previously been explored (Bastos *et al.* 2011), and despite the additional samples used in the present study, there was no significant increase in genetic diversity. In comparison with global genetic diversity of the three *Rattus* species, it is apparent that genetic diversity in the southern African *Rattus* population is relatively low. Phylogenetic analyses revealed that apart from *R. tanezumi*, *R. rattus* and *R. norvegicus* populations colonised southern Africa *via* multiple introduction events. Subsequently, a few unique haplotypes were derived from geographically diverse parent

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haplotypes due to some within-species diversity associated with this taxonomic group. The low genetic diversity arising from multiple introductions or a single introduction with several distinct haplotypes suggest that these *Rattus* species may have been recently introduced into the region. This however, seems unlikely especially with the possibility of multiple introductory pathways that are present, which in addition to introduction through shipping (historically and presently), long distance dispersal by road or air travel may also be prevalent. Consequently, diversification and expansion following initial invasion is possibly impeded through interspecific competition with indigenous rodents (Towns & Broome 2003). In addition, genetic diversity may also remain low if colonisation occurs through repeated introductions from the same geographical areas or closely related populations.

In contrast to its congeners, *R. tanezumi* is characterised by a single introduction event evident from the well-supported clade to which both haplotypes belong. In addition, network analyses revealed that most individuals belong to one highly abundant haplotype linked most closely to Indonesia while the other haplotype of lower frequency is derived from the former. This scenario indicates a recent introduction possibly from its native range. Furthermore, evidence in the present study suggests that *R. norvegicus* was likely also introduced from Indonesia with some escaped laboratory rats accounting for the *R. norvegicus* population in southern Africa, while initial colonisers of *R. rattus* came from several geographical areas.

Nevertheless, genetic diversity observed in *Rattus* from southern Africa is probably due to a combination of introductions and subsequent diversification. The null hypothesis that there is no genetic diversity within members of invasive, commensal *Rattus* in South Africa is therefore rejected and the second alternative hypothesis is accepted of low genetic diversity among the invasive, commensal *Rattus* in South Africa. Introductions of invasive species must be curbed not only because of the accompanying diseases introduced with them but also because the continuous influx of genetic diversity may influence rodent control efforts. This

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may also compromise the integrity of the species by the stimulation of introgression through sympatry in the invaded range (Conroy *et al*. 2013).

Rodent-associated bacterial diseases are widespread and may cause severe outbreaks with serious socio-economic and health implications (Gratz 1997; Meerburg *et al.* 2009). The present study determined that invasive, commensal rats have the potential to spread and transmit potentially zoonotic bacteria through the environment *via* their urine, and confirmed the role of *Rattus* in the indirect transmission of a vector-borne zoonotic disease. Although bacterial prevalence in kidney samples was high when assessed with universal 16S bacterial primers, true bacterial diversity and abundance was masked by co-amplification of host genome and mixed infections and is a known drawback of the 16S approach (Clarridge 2004). It is therefore suggested that culture techniques in combination with molecular techniques be used for future studies on *Rattus* associated with zoonotic disease. Nevertheless, the diverse combination of bacteria detected in these rats, may not only cause opportunistic infection in immune-compromised individuals but also contribute to food spoilage. The presence of invasive, commensal rats therefore poses both a public health and food security threat. The null hypothesis that there is no diversity, prevalence or zoonotic potential of bacteria that are potentially transmitted through environmental contamination by invasive, commensal *Rattus* in South Africa is therefore rejected and the alternative hypothesis is accepted.

Streptobacillus moniliformis is the etiological agent of rat-bite fever and considered to be part of the normal flora in the nasopharynx of rats (Gaastra *et al.* 2009). Clinically resembling rat-bite fever, Haverhill fever is also associated with this bacterium but is characterised by oral ingestion of infective material (Gaastra *et al.* 2009). As expected, the bacterium was highly prevalent in oral cavities, but less so in the kidneys of sampled *Rattus* species in the present study. Direct transmission of rat-bite fever through rat bites and/or scratches is

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therefore highly probable especially in light of frequent contact between rats and humans in South African informal settlements (Kirsten & von Maltitz 2005). The observed low prevalence of *S. moniliformis* in the kidneys of rats, however, indicates a lower transmission potential of Haverhill fever. Differences in oral infection prevalence were observed among *R. tanezumi* and *R. rattus* and it was proposed that this possibly reflect microhabitat and behavioural differences.

Genetic sequencing of *S. moniliformis* revealed genetic diversity in strains from *Rattus* hosts as observed by Kimura *et al.* (2008). Not only was *S. moniliformis* detected in *R. tanezumi* for the first time during the present study, strains isolated from this species showed were not genetically distinct from *S. moniliformis* strains detected from matrilineally defined *R. rattus* specimens. This supports the notion of genome introgression developing in the two matrilineally distinct *R. rattus* and *R. tanezumi* proposed by others (Aplin *et al.* 2011; Lack *et al.* 2012; Conroy *et al.* 2013). Consequently, since the two species are infected by the same *S. moniliformis* strain, yet show infection prevalence differences, it was proposed that this is possibly due to microhabitat and behavioural differences. It was however found, that although this difference in infection prevalence is potentially associated to land use sites, the source of variation in infection prevalence between the species is largely unknown. The study presents the first detection of *S. moniliformis* in southern Africa although previous detection in patients may not be discounted as the disease is not reportable and has non-specific presentation. The null hypothesis that there is no diversity and prevalence of zoonotic, *S. moniliformis* and no host specificity of invasive, commensal *Rattus* in South Africa is rejected and the alternative hypothesis is therefore accepted.

The role of rodents in vector-borne zoonoses is often complex as they may be either incidental or maintenance hosts in the epidemiology of the disease. Flea-borne *Rickettsia felis* and associated with spotted fever, was detected in a *Rattus* specimen from South Africa in the

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present study. However, *R. felis* had low prevalence among the *Rattus* hosts, which may indicate that the sampled *Rattus* species play an insignificant role in the maintenance of this rickettsial disease. It is possible that *Rattus*-associated ectoparasites may instead be more important in the epidemiology of this rickettsial disease as it is highly prevalent in arthropods elsewhere in Africa (Khaldi *et al.* 2011; Kernif *et al.* 2012) and the disease is maintained by vertical transmission in the arthropod host (Parola 2011). The null hypothesis that there is no prevalence and diversity of potentially zoonotic, *Rickettsia* in invasive, commensal *Rattus* species in South Africa is rejected and the alternative hypothesis that there is prevalence of zoonotic *Rickettsia* is accepted yet no diversity of *Rickettsia* in invasive, commensal *Rattus* species in South Africa.

Not only have *Rattus* species been historically introduced through the shipping trade, currently, they are still continuously being introduced, with new species even entering the continent through additional modern air and road transportation systems. Undoubtedly, diseases accompany these introductions and the present study has successfully determined that invasive, commensal *Rattus* in urban settings of South Africa are carriers of either actual or potential zoonotic diseases and may transmit these either directly through bites and/or scratches or indirectly through environmental contamination *via* the urinary route or potentially through their ectoparasites. In urban South Africa, and especially informal settlements, conditions are conducive to disease outbreaks and emphasises the need for continuous disease surveillance and rodent control efforts in not only urban settings but also trade ports such as airports and harbours. Certainly, effective disease surveillance rely on proper identification of the carriers or hosts so that when disease outbreaks occur in either the human population or indigenous rodents, control methods may be directed to the correct target species.

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Although some insights into the colonisation of *Rattus* in South Africa were provided in the present study, any inferences are limited by the lack of extensive, geographically representative sampling of southern Africa. Future sampling of especially the under-represented south and west coast of South Africa and also neighbouring countries, Namibia, Botswana, Zimbabwe, Mozambique and Lesotho that form part of the southern African sub-region is needed. In addition, since the potential of *Rattus* as carriers of actual or potential bacterial zoonoses has been demonstrated in South Africa, there is a need to also assess other possible zoonotic infections such as those from helminthic, protozoan and viral origin to determine the extent of the role of *Rattus* in human disease prevalence. Also, more knowledge is needed regarding *Rattus* ecology in the invaded range. Little is known of the *R. rattus* species complex and *R. norvegicus* regarding their demographic characteristics, their home ranges, niche partitioning and other ecological factors many of which may aid detection of cryptic species and facilitate the development of improved control methods.

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APPENDIX A

Code	Locality	Species	Sex	Weight	Ecto-parasites?	Month	2010-2011	Comments	Organs	Foetuses
HGP-R1	Hammanskraal	Rattus tanezumi	male	40.98	No	Aug	2010		H-L-K-GIT-MS-LG-RO	
HGP-R2	Hammanskraal	Rattus tanezumi	male	109.93	Yes	Aug	2010		H-L-K-GIT-MS-LG-RO	
HGP-R3	Hammanskraal	Rattus tanezumi	male	135.52	Yes	Sept	2010		H-L-K-GIT-MS-LG-RO	
HSY-R4	Hammanskraal	Rattus tanezumi	female	127.82	No	Sept	2010	pregnant	H-L-K-GIT-MS-LG-RO	*6
UP-R5	Hatfield	Rattus rattus	female	145.9	No	Nov	2010		H-L-K-GIT-MS-LG-RO	
UPE-R6	Hatfield	Rattus rattus	male	190.37	Yes	Dec	2010		K-GIT-MS-RO	
UPE-R7	Hatfield	Rattus rattus	female	38.43	Yes	Dec	2010		K-GIT-MS-RO	
UPE-R8	Hatfield	Rattus rattus	male	33.19	Yes	Dec	2010		K-GIT-MS-RO	
UPE-R9	Hatfield	Rattus rattus	male	35.81	Yes	Dec	2010		K-GIT-MS-RO	
UPE-R10	Hatfield	Rattus rattus	male	46.43	Yes	Dec	2010		K-GIT-MS-RO	
UPE-R11	Hatfield	Rattus rattus	male	95.83	Yes	Dec	2010		K-GIT-MS-RO	
UPE-R12	Hatfield	Rattus rattus	male	31.26	Yes	Dec	2010		K-L-GIT-RO	
UPE-R13	Hatfield	Rattus rattus	female	166.32	Yes	Dec	2010		K-GIT-MS-RO	
UPE-R14	Hatfield	Rattus rattus	male	33.86	Yes	Dec	2010		K-GIT-MS-RO	
UPE-R15	Hatfield	Rattus rattus	male	43.54	Yes	Dec	2010		K-GIT-MS-RO	
UPE-R16	Hatfield	Rattus rattus	male	195.93	Yes	Dec	2010		K-GIT-MS-RO	
UPE-R17	Hatfield	Rattus rattus	male	37.05	Yes	Dec	2010		K-GIT-MS-RO	
HSY-R18	Hammanskraal	Rattus tanezumi	male	30.54	Yes	Dec	2010		H-L-K-GIT-MS-LG-RO	
UPE-R19	Hatfield	Rattus rattus	female	19.55	Yes	Dec	2010		K-GIT-MS-RO	
UPE-R20	Hatfield	Rattus rattus	male	34.63	Yes	Dec	2010		K-GIT-MS-RO	
UPE-R21	Hatfield	Rattus rattus	male	39.6	Yes	Dec	2010		K-GIT-MS-RO	
UPE-R22	Hatfield	Rattus rattus	male	186.23	Yes	Dec	2010		K-GIT-MS-RO	
UPE-R23	Hatfield	Rattus rattus	male	193.54	No	Dec	2010		K-GIT-MS-RO	
UPE-R24	Hatfield	Rattus rattus	female	179.82	Yes	Dec	2010		K-GIT-MS-RO	
UPE-R25	Hatfield	Rattus rattus	female	213.75	Yes	Dec	2010		K-GIT-MS-RO	
UPE-R26	Hatfield	Rattus rattus	male	193.88	No	Dec	2010		K-GIT-MS-RO	
TEM-R27	Tembisa	Rattus norvegicus	male	82.52	Yes	Dec	2010		H-L-K-GIT-MS-LG-RO	
TEM-R28	Tembisa	Rattus norvegicus	male	70.27	Yes	Dec	2010		H-L-K-GIT-MS-LG-RO	
TEM-R29	Tembisa	Rattus norvegicus	male	48.51	No	Dec	2010		H-L-K-GIT-MS-LG-RO-	
TEM-R30	Tembisa	Rattus norvegicus	male	44.74	Yes	Dec	2010		H-L-K-GIT-MS-LG-RO-	
TEM-R31	Tembisa	Rattus norvegicus	male	45.26	Yes	Dec	2010		H-L-K-GIT-MS-LG-RO-	
HUP-R32	Hammanskraal	Rattus tanezumi	male	139.14	Yes	Jan	2011		H-L-K-GIT-MS-LG-RO-RS	
VL-R33	Villeria	R.tanezumi	male	72.16	Yes	Jan	2011		H-L-K-GIT-MS-LG-RO-RS	
HUP-R34	Hammanskraal	Rattus tanezumi	female	94.5	Yes	Jan	2011		H-L-K-GIT-MS-LG-RO-RS	
HUP-R35	Hammanskraal	Rattus tanezumi	female	61.8	Yes	Jan	2011	live caught	H-L-K-GIT-MS-LG-RO-RS	
UP-R36	Hatfield	R. rattus	male	20.29	Yes	Feb	2011	live caught	H-L-K-GIT-MS-LG-RO-RS	
UP-R37	Hatfield	R. rattus	male	18.78	Yes	Feb	2011	live caught	H-L-K-GIT-MS-LG-RO-RS	
UP-R38	Hatfield	R. rattus	male	21.06	Yes	Feb	2011	live caught	H-L-K-GIT-MS-LG-RO-RS	
TR-R39	Centurion	R. tanezumi	female	99.49	No	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
TEM-R40	Tembisa	Rattus norvegicus	female	363.61	No	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
TEM-R41	Tembisa	Rattus norvegicus	female	356.71	No	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
TEM-R42	Tembisa	Rattus norvegicus	female	236.45	Yes	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
TEM-R43	Tembisa	Rattus norvegicus	male	182.7	Yes	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
TEM-R44	Tembisa	Rattus norvegicus	female	84.22	Yes	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
TEM-R45	Tembisa	Rattus norvegicus	female	287.94	Yes	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
TEM-R46	Tembisa	Rattus norvegicus	male	160.73	No	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
TEM-R47	Tembisa	Rattus norvegicus	female	225.73	Yes	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
TEM-R48	Tembisa	Rattus norvegicus	female	55.9	Yes	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
TEM-R49	Tembisa	Rattus norvegicus	female	154.17	Yes	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
TEM-R50	Tembisa	Rattus norvegicus	male	104.93	Yes	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
TEM-R51	Tembisa	Rattus norvegicus	female	78.02	Yes	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
TEM-R52	Tembisa	Rattus norvegicus	female	55.27	Yes	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
TEM-R53	Tembisa	Rattus norvegicus	female	124.44	Yes	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
TEM-R54	Tembisa	Rattus norvegicus	female	105.13	No	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
TEM-R55	Tembisa	Rattus norvegicus	male	61.87	Yes	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
HUP-R56	Hammanskraal	Rattus tanezumi	female	91.41	Yes	Feb	2011	/poisoned?	H-L-K-GIT-MS-LG-RO-RS	
HSY-R57	Hammanskraal	Rattus tanezumi	female	60.75	No	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
HSY-R58	Hammanskraal	Rattus tanezumi	female	63.9	Yes	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
TEM-R59	Tembisa	Rattus norvegicus	female	272.05	Yes	Feb	2011		H-L-K-GIT-MS-LG-RO-RS	
UP-R60	Hatfield	R. rattus	female	115.41	No	Mar	2011		H-L-K-GIT-MS-LG-RO-RS	
UPE-R61	Hatfield	R. rattus	male	78.73	Yes	Mar	2011		H-L-K-GIT-MS-LG-RO-RS	
UPE-R62	Hatfield	R. rattus	male	138.52	Yes	Mar	2011		H-L-K-GIT-MS-LG-RO-RS	
UPE-R63	Hatfield	R. rattus	male	127.5	Yes	Mar	2011		H-L-K-GIT-MS-LG-RO-RS	
UPE-R64	Hatfield	R. rattus	male	125.46	Yes	Mar	2011		H-L-K-GIT-MS-LG-RO-RS	
UPE-R65	Hatfield	R. rattus	male	100.6	Yes	Mar	2011		H-L-K-GIT-MS-LG-RO-RS	
UPE-R66	Hatfield	R. rattus	male	94.47	Yes	Mar	2011		H-L-K-GIT-MS-LG-RO-RS	
UPE-R67	Hatfield	R. rattus	female	93.16	Yes	Mar	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
UPE-R68	Hatfield	R. rattus	male	57.9	Yes	Mar	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
UPE-R69	Hatfield	R. rattus	female	103.37	Yes	Mar	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
UPE-R70	Hatfield	R. rattus	male	53.33	Yes	Mar	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
TEM-R71	Tembisa	Rattus norvegicus	female	148.8	Yes	Apr	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
TEM-R72	Tembisa	Rattus norvegicus	male	145.63	Yes	Apr	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
TEM-R73	Tembisa	Rattus norvegicus	female	74.94	Yes	Apr	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
TEM-R74	Tembisa	Rattus norvegicus	male	89.98	Yes	Apr	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
TEM-R75	Tembisa	Rattus norvegicus	male	94.94	No	Apr	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
TEM-R76	Tembisa	Rattus norvegicus	male	79.61	No	Apr	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
TEM-R77	Tembisa	Rattus norvegicus	male	89.73	Yes	Apr	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
TEM-R78	Tembisa	Rattus norvegicus	female	158.22	Yes	Apr	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
TEM-R79	Tembisa	Rattus norvegicus	male	136.67	Yes	Apr	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
TEM-R80	Tembisa	Rattus norvegicus	male	97	Yes	Apr	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
HUP-R81	Hammanskraal	Rattus tanezumi	male	80.17	Yes	Apr	2011	live caught	H-L-K-GIT-MS[1]-LG-RO-RS[1]	
HUP-R82	Hammanskraal	Rattus tanezumi	male	63.83	Yes	Apr	2011	live caught	H-L-K-GIT-MS[1]-LG-RO-RS[1]	
HF-R83	Hatfield	R. tanezumi	male	11.9	Yes	Apr	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
UPE-R84	Hatfield	R. rattus	male	124.73	Yes	May	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
MV-R85	Mountain View	R. tanezumi	male	178.78	No	Feb	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
UPE-R86	Hatfield	R. rattus	female	84.32	Yes	May	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
DS-R87	Diepsloot	R. norvegicus	male	74.85	Yes	Jun	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
DS-R88	Diepsloot	R. norvegicus	female	228.31	Yes	Jun	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
DS-R89	Diepsloot	R. norvegicus	male	371.54	Yes	Jun	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
DS-R90	Diepsloot	R. norvegicus	male	542.83	No	Jun	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
GF-R91	Garsfontein	Rattus rattus	female	78.04	No	Jul	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
GF-R92	Garsfontein	R. tanezumi	male	110.42	Yes	Jul	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
MV-R93	Mountain View	R. tanezumi	male	48.25	Yes	Aug	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
MV-R94	Mountain View	R. tanezumi	female	133.55	No	Aug	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
MV-R95	Mountain View	R. tanezumi	male	107.6	Yes	Jul	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
MV-R96	Mountain View	R. tanezumi	female	118.87	Yes	Jul	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
MV-R97	Mountain View	R. tanezumi	female	82.16	Yes	Jul	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
VDG-R98	Val de Grace	R. tanezumi	female	72.45	Yes	Nov	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
ML-R99	Menlyn	R. rattus	female	70.68	Yes	Dec	2011	live caught	H-L-K-GIT-MS[1]-LG-RO-RS[1]	
ML-R100	Menlyn	R. rattus	female	68.87	Yes	Dec	2011	live caught	H-L-K-GIT-MS[1]-LG-RO-RS[1]	
RF-R101	Rietfontein	R. tanezumi	male	186.52	Yes	Dec	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
BSK-R102	Boschkop	R. tanezumi	female	227.81	Yes	Nov	2011	pregnant	H-L-K-GIT-MS[1]-LG-RO-RS[1]	*8
BSK-R103	Boschkop	R. tanezumi	male	194.87	Yes	Nov	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
BSK-R104	Boschkop	R. tanezumi	female	240.5	Yes	Nov	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
BSK-R105	Boschkop	R. tanezumi	male	227.82	Yes	Nov	2011		H-L-K-GIT-MS[1]-LG-RO-RS[1]	
BSK-R106	Boschkop	R. tanezumi	female	206.25	No	Nov	2011	pregnant	H-L-K-GIT-MS[1]-LG-RO-RS[1]	*3
BSK-R107	Boschkop	R. tanezumi	female	241.62	Yes	Nov	2011	pregnant	H-L-K-GIT-MS[1]-LG-RO-RS[1]	*10

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APPENDIX B

Blue- sequences of bad/low yield/mix quality

Green- good quality sequences of sizable length Red- negative samples as detected by both primer setsOrange- host amplification

Primer: 27F/1522R

Sample	PCR result	Seq ID fw	Quality	Size(bp)	Result	Seq ID rv	Quality	Alignment	Size(bp)	Result
ER3	bpos	ZCN51	good	553	Brochothrix thermosphacta 100%	ZCN57	good	used	495	Brochotrix thermosphacta 99%
ER4	bpos	ZCN52	medium	495	Bartonella tribocorum 100%	ZCL15	good	used	431	Bartonella elizabethae. 99%*
ER5	bpos	ZCN53	medium	343	Streptococcus sp 92%	ZCH27/ZCN58	bad/mix		298	Streptococcus suis 97%
F39	bpos	ZBA17	very bad	-	-	ZCH28/ZCN59	medium low yield/low yield mix		653	Uncultured Neisseriaceae bacterium 98%*
F51	bpos	ZBA12	medium	369	Rickettsia sp. 99%	ZCL17	good	used	377	Rickettsia sp 99%
HK26	bpos	ZCN54	medium	491	Uncult bacteria 91%*	ZCL16	good	used	535	Streptococccus suis 96%
HK30	bpos	ZBA10	mix low yield	610	Zebrafish???	ZCH31	very low yield		243	Human?
HSYR4	bpos	ZCL23	medium	427	Uncult bacteria/Streptococcus sp. 93%*	ZCH32	good	used	569	Uncultured organism/ Streptococcus 97%*
JZ11	bpos	ZBO92/ZCL24	very bad very low yield/mix	205	Uncultured bacterium/Streptococcus sp 75%*	ZBQ40	very good	used	519	Uncultured organism/Streptococcus (97%)
JZ15	bpos	ZBA05	bad mix	-	-	ZCH34/ZCl18	salts/bad mix		-	-
JZ7	bpos	ZCL25	mix	425	Uncultured bacterium/Streptococcus sp 91%*	ZCL19	medium		414	Streptococcus cristatus 96%*
MP02	bpos	ZBA11	medium mix to bad	271	Uncult bacteria	ZCH35/ZCN60	bad/medium	not used	310	Uncultured Neisseriaceae bacterium99%*
OT16	bpos	ZCN55	medium	453	Uncultured Staphylococcus(96%*)	ZCH36/ZCN71	bad/bad		-	-
UPER12	fpos	ZBO90	bad low yield	110	Xanthosomas arboricola	ZBQ41	low yield bad		-	-
UPER13	fpos	ZBO91	bad low yield	200	Microbacterium	ZBQ42/ZCH38	pellet/bad low yield		209bp	Bartonella??
UPER14	fpos	ZBM35	very bad mix	740	Firmicutes bacterium 80%*	ZCH39/ZCN61	bad/bad mix		303bp	Uncultured bacterium100%
UPER22	bpos	ZBM36	medium	379	Streptococcus suis 92%*	ZCH40	good	used	660	Streptococcus sp 97%
UPER23	bpos	ZBM37	good	501	Streptococcus suis 92%*	ZCH41	good	used	399	Streptococcus sp. 97%
UPER24	bpos	ZBM38	medium	709	Clostridium sordelii (99%)	ZBO93	good low yield	used	580	Clostridium sordelii (99%)
UPER8	bpos	ZBM34	medium mix	448	Lactobacillus murinus 85%*	ZCH37	bad		587	Human?
WK01	bpos	ZCN56	good	556	Uncult bact 92%*	ZCL20	good	used	507	Streptococcus agalactiae 97%
WK02	bpos	ZBA14	medium	406	Uncult bacterium 90%	ZCL21	good	used	743	Streptococcus agalactiae 97%
WK06	bpos	ZBA15	bad mix low yield	110	Human???	ZCL22	very low yield bad mix		-	-
ER1	fpos									
ER2	neg									
F40	fpos									
F43	neg									
F45	neg									
F49	neg									
F53	neg									
F55	neg									
F57	neg									
HK04	neg									
HK06	neg									
HK07	neg									
HK09	neg									
HK10	neg									
HK12	neg									
HK14	neg									
HK22	neg									
HK24	fpos									
HK25	fpos									
HK27	fpos									
HK28	fpos									
JZ1	neg									
JZ3	neg									
JZ5	fpos									
JZ9	neg									
JZ13	neg									
KZN1	neg									
MP01	neg									
OT2	neg									
OT4	fpos									
OT6	neg									
OT8	neg									
OT10	neg									
OT12	neg									
OT14	neg									
PP01	neg									
WK04	neg									
WK07	neg									
HGP-R1	fpos									
HGP-R2	neg									
HGP-R3	neg									
UP-R5	fpos									
UPE-R6	neg									
UPE-R7	fpos									
UPE-R9	fpos									
UPE-R10	neg									
UPE-R11	neg									
UPE-R15	neg									
UPE-R16	neg									
UPE-R17	neg									
HSY-R18	neg									
UPE-R19	neg									
UPE-R20	fpos									
UPE-R21	neg									
UPE-R25	neg									
UPE-R26	neg									
TEM-R27	fpos									
TEM-R28	neg									
TEM-R29	neg									
TEM-R30	neg									
TEM-R31	neg									

Primer: 63F/1522r

Sample	PCR result	Seq ID fw	Quality	Size (bp)	Result	Seq ID rv	Quality	Alighnment	Size (bp)	Result
ER3	neg									
ER4	fpos	ZCI26	medium mix	204	Bartonella sp. 85%*	ZCI76	Bad mix		460	Uncult bacteria
ER5	pos	ZCI27	medium	691	Mus musculus 83%*					
F39	neg									
F51	neg									
HK26	neg									
HK30	pos	ZCI28	medium	657	Human??					
HSYR4	pos	ZCI29	bad	424	Mus musculus/human???					
JZ11	pos	ZCI30	medium	595	mus musculus					
JZ15	pos	ZCI31	bad	-						
JZ7	fpos	ZCI32	mix	730	Mus musculus					
MP02	fpos	ZCI33	bad	207	mus musculus/Bacillus sp.	ZCI77	Bad			not used
OT16	fpos	ZCI34	vey bad mix	-						
UPER12	pos	ZCI35	bad	576	Human??					
UPER13	pos	ZCI36	medium	588	Mus musculus					
UPER14	pos	ZCI37	medium mix	559	Uncultured bacterium					
UPER22	fpos	ZCI38	medium short	300	Mus musculus					
UPER23	pos	ZCI39	medium	705	Mus musculus					
UPER24	pos	ZCI40	bad mix	-	Mus musculus					
UPER8	pos	ZCI41	medium	626	Human??					
WK01	neg									
WK02	neg									
WK06	neg									
ER1	fpos	ZCI42	bad/mix	-	-					
ER2	pos	ZCI43	good	682	Bartonella sp. 99%	ZCI78	Good		508	Bartonella elizabethae (99%*)
F40	neg									
F43	neg									
F45	neg									
F49	neg									
F53	neg									
F55	neg									
F57	neg									
HK04	neg									
HK06	neg									
HK07	neg									
HK09	neg									
HK10	neg									
HK12	neg									
HK14	neg									
HK22	neg									
HK24	pos	ZCI44	medium	480	Acinetobacter baumanii 93%	ZCI79/ZCN72	Medium/b	not used	278	Uncultured Acinetobacter 98%*
HK25	neg									
HK27	pos	ZCI45	good	301	Mus musculus					
HK28	pos	ZCI46	medium mix	468	Mus musculus					
JZ1	neg									
JZ3	neg									
JZ5	neg									
JZ9	pos	ZCI47	mix	-						
JZ13	pos	ZCI48	mix	-						
KZN1	neg									
MP01	neg									
OT2	pos	ZCI49	bad/mix	-						
OT4	pos	ZCI50	bad/mix	574	Mus musculus					
OT6	neg									
OT8	neg									
OT10	neg									
OT12	pos	ZCI51	medium	664	Bartonella sp. 98%	ZCI80/ZCN73	medium mix/bad		367	Bartonella sp (98%)*
OT14	neg									
PP01	neg									
WK04	neg									
WK07	neg									
HGP-R1	pos	ZCI52	medium	561	Mus musculus					
HGP-R2	pos	ZCI53	medium	541	Brasssica??? Cabbage?					
HGP-R3	pos	ZCI54	bad/mix	-						
UP-R5	neg									
UPE-R6	neg									
UPE-R7	pos	ZCI55	medium/mix	561	Mus musculus					
UPE-R9	pos	ZCI56	medium	734	Mus muculus					
UPE-R10	pos	ZCI57	short mix	270	Pseudomonas 96%*					
UPE-R11	pos	ZCI58	medium mix	459	Mus musculus					
UPE-R15	pos	ZCI59	mix	-						
UPE-R16	pos	ZCI60	medium/mix	637	Mus musculus					
UPE-R17	pos	ZCI61	bad mix	570	Mus musculus					
HSY-R18	pos	ZCI62	medium	688	Mus musculus					
UPE-R19	pos	ZCI63	bad mix	562	Mus musculus					
UPE-R20	pos	ZCI64	medium	729	Mus musculus					
UPE-R21	pos	ZCI65	medium mix	488	Mus musculus					
UPE-R25	pos	ZCI66	medium	410	Mus musculus					
UPE-R26	pos	ZCI67	bad/mix	522	Mus musculus					
TEM-R27	pos	ZCI68	medium/mix	563	Mus musculus					
TEM-R28	pos	ZCI69	medium	516	Mus musculus					
TEM-R29	pos	ZCI70	medium	767	Mus musculus					
TEM-R30	pos	ZCI71	medium	424	Mus musculus					
TEM-R31	pos	ZCI72	bad	-						

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